The Synthesis of Novel O-Alkyl Analogues of the Energy-Repartitioning β-Agonist Clenbuterol and Their Physiological and Immunological Characterisations

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A thesis submitted for the degree of Doctor of Philosophy in the Department of Chemistry, Faculty of Business and Technology, University of Western Sydney (Macarthur)
SYDNEY, AUSTRALIA

September 1995
PLEASE NOTE

The greatest amount of care has been taken while scanning this thesis,

and the best possible result has been obtained.
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STATEMENT OF ORIGINALITY

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material to which to a substantial extent has been accepted for the award of any other degree or diploma of a university or other institute of higher learning, except where due acknowledgement is made in the text of the thesis.

Signed .................................................. Timothy Brown ..................................................

Date .................................................. 29. 6. 95 ..................................................
ACKNOWLEDGEMENTS

A multi-disciplinary project such as this would not have been possible without the help and support of a number of people. I would like to thank both the CSIRO Division of Animal Production and the University of Western Sydney (Macarthur) for provision of my scholarship, Dr. Ron Hoskinson (CSIRO) for his leadership of the Clenbuterol project, of which this work forms part, and his support and helpful comments on thesis drafts, and the Beef and Cattle Cooperative Research Centre for funding of the Clenbuterol project. I would like to especially acknowledge the assistance and encouragement of my supervisors, Dr. Robin Rigby (CSIRO) and Dr. Leonid Tarasoff (UWS, Macarthur), for their careful guidance over the last four years, and their comments and critique during the drafting of this thesis.

I am very grateful for the support and help of my colleagues at CSIRO during my time at Prospect: Dr. Van Huynh, Dr. Mark Jones (also for critique of thesis drafts), Dr. Swee Foong Ng, Mr. Justin Finnerty, Mr. Peter Donnolley and Mr. Ron Newman, and especially the excellent technical assistance from Ms. Sandra Walton (nee Cuthbert), Mr. Stephen Harmer, Ms. Donna McLean and the Library and Small Animal Colony staff at CSIRO.

A number of people have given generously of their time in helpful discussion, for which I am most grateful: Professor Bob Raison (University of Technology, Sydney), Ms. Lynn Boscatto (Garvan Medical Research Institute) and Dr. Mary Campbell (UWS). I would also like to thank Dr. Campbell, as well as Dr. David Adelson (CSIRO) and Dr. Janice Aldrich-Wright (UWS) for their critical and invaluable comments on the thesis drafts. The insulin and glucose assays (Chapter 4) were kindly performed by Ms. Vicki Theos (Garvan Medical Research Institute), the NMR spectra by Dr. Jim Hook (University of NSW), and the mass spectra by Dr. Joe Brophy and Mr. Ray Lidgard (University of NSW), for which I am grateful.

I particularly wish to acknowledge the tremendous contribution of Dr. Swee Foong Ng, for her encouragement and chiding as I sought to come to grips with new scientific disciplines, and critique of thesis drafts; a friend and mentor who provided support and loyalty beyond measure.

Finally I wish to acknowledge the love and support of my wonderful wife Wendy-Jane, who has stood by me and lightened my load, my Mum and Dad who believe in me, and many dear friends. “Trust in the LORD with all your heart and do not rely on your own insight. In all your ways acknowledge him, and he will make straight your paths” (Proverbs 3:5,6)
PUBLICATIONS ARISING FROM THIS THESIS

1. T.J. Barden, R.D.G. Rigby, L. Tarasoff, 
   “Synthesis of O-alkyl analogues of the beta-2 agonist clenbuterol”, Proceedings of the 
   RACI 14th National Conference (Division of Organic Chemistry) , 1994, P57.

2. T.J. Barden, S.F. Ng, S.J. Cuthbert, J.J. Finnerty, R.D.G. Rigby, 
   “Repartitioning effects of some O-alkyl analogues of clenbuterol” Proc. of Nutrition 
   Society of Australia , 1994, 18, 124.

   of the Energy-repartitioning β₂-Adrenoceptor Agonist Clenbuterol” (in preparation)

   Analogues of the β₂-Adrenoceptor Agonist Clenbuterol in Rodents” (in preparation)
**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>%CR</td>
<td>Percentage Cross Reactivity</td>
</tr>
<tr>
<td>%SB</td>
<td>Percentage Specific Binding</td>
</tr>
<tr>
<td>βAA</td>
<td>β-Adrenergic Agonist</td>
</tr>
<tr>
<td>βARK</td>
<td>β-Adrenergic Receptor Kinase</td>
</tr>
<tr>
<td>[3H]CB</td>
<td>[3H]Clenbuterol</td>
</tr>
<tr>
<td>[125]I CYP</td>
<td>[125]I Iodocyanopindolol</td>
</tr>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>Ab1</td>
<td>Idiotypic Antibody</td>
</tr>
<tr>
<td>Ab2</td>
<td>Anti-Idiotypic Antibody</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic</td>
</tr>
<tr>
<td>ADR</td>
<td>Adrenaline</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AQIS</td>
<td>Australian Quarantine and Inspection Service</td>
</tr>
<tr>
<td>AR</td>
<td>Adrenergic Receptor or Adrenoceptor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BW</td>
<td>Bodyweight</td>
</tr>
<tr>
<td>CB</td>
<td>Clenbuterol</td>
</tr>
<tr>
<td>CIM</td>
<td>Cimaterol</td>
</tr>
<tr>
<td>CSIRO DAP</td>
<td>Commonwealth Scientific and Industrial Research Organisation Division of Animal Production</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>Da</td>
<td>daltons</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>DCHA</td>
<td>3,5-Dichloro-4-hydroxyacetophenone</td>
</tr>
<tr>
<td>DEPT</td>
<td>Distortionless Enhancement by Polarisation Transfer</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modification of Eagle’s medium</td>
</tr>
<tr>
<td>DMF</td>
<td>N,N-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DMT</td>
<td>3,4-Dimethoxytyramine</td>
</tr>
<tr>
<td>EAS</td>
<td>ELISA Assay Solution</td>
</tr>
<tr>
<td>ECDI</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ED-BSA</td>
<td>Ethylenediamine-modified BSA</td>
</tr>
<tr>
<td>EDL</td>
<td>Extensor Digitorum Longus</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Et</td>
<td>Ethyl</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
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FCS
Foetal Calf Serum

FSH
Follicle-Stimulating Hormone

Gastroc.
Gastrocnemius

GDP
Guanosine Diphosphate

Gel
Gelatin

GTP
Guanosine Triphosphate

HAT
Hypoxanthine-Aminopterin-Thymidine culture medium supplement

HD
Hydrophobic Domain

HEPES
$N$-2-Hydroxyethylpiperazine-$N'$-2-ethanesulfonic acid

HT
Hypoxanthine-Thymidine culture medium supplement

ISC
Iscove’s modification of Dulbecco’s medium

ISF
Incomplete Serum-Free culture medium

ISO
Isoprenaline

LH
Luteinizing Hormone

Me
Methyl

NEFA
Non-esterified fatty acids

NHS
$N$-Hydroxysuccinimide

NMR
Nuclear Magnetic Resonance

NOR
Noradrenaline

NSB
Non-Specific Binding

NTP
Normal Temperature and Pressure

OA
Ovalbumin

PBS
Phosphate-buffered Saline

Plant.
Plantaris

PNA
Phosphate-Sodium-Azide buffer

Quads
Quadriceps

RAC
Ractopamine

RIA
Radioimmunoassay

RT
Room Temperature

SALB
Salbutamol

SALM
Salmeterol

SF
Serum-Free

Sol.
Soleus

SPG
Soleus/Plantaris/Gastrocnemius muscle bundle

T4
Thyroxine

THF
Tetrahydrofuran

TICA
Trichloroisocyanuric acid
ABSTRACT

It was proposed that some O-alkyl analogues of the β-adrenergic agonist clenbuterol would be effective structural and functional congeners of clenbuterol which may then be used for the production of clenbuterol-specific idiotypic antibodies. These antibodies could possibly then be used to generate anti-idiotypic antibodies that mimic the energy-repartitioning effects of clenbuterol. Therefore, the aim of this work was to synthesise and characterise these compounds, evaluate their physiological effects, characterise the specificity of antibodies produced in response to protein conjugates of two of the novel compounds, and then use this data to determine the utility of these compounds for the generation of anti-idiotypic antibodies which mimic clenbuterol.

The target compounds were synthesised in five steps from 3,5-dichloro-4-hydroxyacetophenone in overall yields of 5-28%. A synthetic scheme similar to that which has led to clenbuterol was used to form the phenylethanolamine backbone, with modifications to include the O-alkyl moiety via a modified Williamson ether synthesis, and elimination of a synthetic chlorination step. Overall, 15 new compounds were synthesised, which were characterised and their structure confirmed from proton and carbon-13 NMR, IR and mass spectral data. The two haptenic analogues were then conjugated to carrier proteins using carbodiimide-based chemistries.

Female Wistar rats were treated with either saline, clenbuterol or one of the four target compounds for 22 days. The clenbuterol treatment group showed decreased fat and increased protein in the carcass, significantly greater 22-day weight gain, increased weights and decreased β₂-adrenergic receptor density of hindlimb skeletal muscles. Some target compound treatment groups showed changes in weight gain, skeletal muscle weights and receptor density (smaller than those observed in the clenbuterol treatment group), but none showed any significant change from saline in carcass composition. These results suggested that the para-amino group of clenbuterol was important for its energy-repartitioning effects.

Polyclonal antibodies to the analogue-protein conjugates were produced in sheep, and titres of anti-clenbuterol antibodies were comparable to those produced by the reported clenbuterol-protein conjugate, although higher titres persisted longer with the analogue-protein conjugates. Competitive binding in a radioimmunoassay indicated that these antibodies were similarly specific for both clenbuterol and the O-alkyl analogues. Attempts to produce monoclonal antibodies to the analogue-protein conjugates were unsuccessful.

In conclusion, the results indicated that the O-alkyl analogues, although structurally similar, were ineffective functional mimics of clenbuterol. Therefore, the anti-clenbuterol antibodies produced from the novel O-alkyl analogues would appear to be unsuitable for production of anti-idiotypic antibodies that mimic the energy-repartitioning effects of clenbuterol since the antibodies were unable to distinguish between the compound which demonstrated energy-repartitioning effects (clenbuterol) and those that did not (O-alkyl clenbuterol analogues).
ERRATUM

Piv Para 5 L1: change analogues to analogues
P5 Para 2 L3: "...pharmacologically, based on a series of adrenergic agonists..." should read "...pharmacologically, based on the relative specificities of a series of adrenergic agonists..."
P5 Para 3 L5: change "...subtypes are not..." to "...subtypes is not..."
P5 Para 5 L1: change ionotropic to inotropic
P6 Para 2 L4: change activity to activity
P10 Para 4 L11: add "...target tissue and ligand receptor affinity."
P15 Figure 1.5: remove allotypes from diagram
P15 Para 3 L6: change occured to occurred
P18 Table 1.2 Footnote L4: change immunosuppressive to immunosuppressive
P19 Para 3 L10: change "...Ab2β which functionally mimic hapten..." to "...Ab2β which functionally mimic some aspects of the hapten..."
P20: add final paragraph "Therefore, it would appear that potential candidates for Ab2β which are true internal images of the original hapten must not only structurally mimic the hapten (e.g. competitively binding to a cellular receptor or Ab1 in the presence of hapten in vitro) but must also elicit similar biological activity in vivo."
P25 Para 1 L2: add "Thus,..." to beginning of sentence
P30 last para L1,2: sentence should read "CB has been synthesised from 4-aminoacetophenone in four synthetic steps (chlorination,..., ketone reduction; refer Scheme 1.1)
P31 Para 1 L5: change difficultly to difficulty
P35 Para 3 L3: "...numbers consistent..." should read "...numbers being consistent..."
P37 Para 2 L4: change consistent to consistent
P37 Para 2 L7: change prefered to preferred
P40 Para 2 L10 & P45 Para 1 L5: "...data was consistent..." should read "...data were consistent..."
P41 Para 3 L5: "...peak may have..." should read "...peak, which may have..."
P46 Figure 2.4 legend: should read "Newman projection of target compounds demonstrates the non-equivalence of the amino protons due to the chirality of the hydroxyl-bearing carbon"
P53 Para 2 L8: "...hapten density..." should read "...hapten density (number of hapten molecules attached to the carrier protein)..."
P53 Para 4 L3: "...as able..." should read "...as being able..."
P65 Para 1 L6: change neutral to neutrality.
P77 Para 2 L2,3: change hr to h
P78 Para 2 L1: change lyopholisation to lyophilisation
P79: remove section 3.2.3.5
P85 Table 3.5: remove insulin results from table and "insulin" from table title
Figure 3.5: Scatchard plots from receptor saturation studies of Quads muscle group from the Saline treatment group (LHS) and the CB treatment group (RHS).

Table 3.8 legend: remove "soleus/plantaris/gastrocnemius (SPG) and..."

Para 2 L7: change "which has given..." to "which have given..."

Para 3 L4-7: replace last sentence with "However, despite a significant increase in heart weight observed for the VUF 8303 treatment group, it was unclear from the present data that this effect was due to a greater β1 activity of this compound, or that such activity was responsible for the lack of CB-like effects observed in this treatment group.

Para 5 L6: change "...rats23 found no change..." to "...rats23 induced no change..."

Para 5 L2: remove ", and the important metabolic hormone, insulin...(refer section 3.3.5)"

Para 1 L3: change "...accretion) were not..." to "...accretion was not..."

Para 2 L14 & Para 3 L1: change "...physiological..." to "...growth-promoting..."

Para 1 L8: change "...2 000 rpm..." to "...2 000 rpm (80 g)...

Para 1 L2: change "...2 400 rpm..." to "...2 400 rpm (110 g)..."

Para 3 L6: change 8 000 rpm to 800 rpm (11 g)

Para 4 L8: change 6 000 rpm to 600 rpm (6 g)

Para 1 L6: change "...centrifuged..." to "...centrifuged (desktop centrifuge)"

Para 5 L7: change "...and reaction..." to "...and the reaction..."

Table 4.5: omit SEMs

Figure 4.5 legend: add "(sheep #624 - C2/ECDI/BSA conjugate, sheep #439 - C2/DCC/BSA conjugate, sheep #451 - CB/Diazo/BSA conjugate)"

Figure 4.6 legend: add "(sheep #608 - C6/ECDI/BSA conjugate, sheep #552 - C6/DCC/BSA conjugate, sheep #451 - CB/Diazo/BSA conjugate)"

Figure 4.7: add "(sheep #547 - C2/ECDI/BSA conjugate, sheep #469 - C2/DCC/BSA conjugate, sheep #608 - C6/ECDI/BSA conjugate, sheep #552 - C6/DCC/BSA conjugate, sheep #437 - CB/Diazo/BSA conjugate)"

Para 1: change "...Figures 4.8A-4.9E shows..." to "...Figures 4.8A-4.8E show...". Also change "...curves these ligands..." to "...curves for these ligands..."

Para 4.8 legend: change "...Figure 4.8..." to "...Figure 4.8A-E"

Para 1 L2: change (4.2.2.4.1) to (4.2.2.5.1)

Para 4.12: change Log Dilution to Log10 Dilution

Para 1 L1: change "Specificity of pAbs..." to "Affinity of pAbs..."

Para 2 L10: change "...Rank Orders of Affinity..." to "...Rank Order of Affinities..."

Para 3 L10: change moieties to moieties

Para 2 L12-15: replace last sentence with "The Ab2β paratope includes that structural feature of the antibody responsible for immunological mimicry, and is influenced by the paratope of the Ab1 used to generate the Ab2β. Therefore, screening anti-CB Ab1 to determine that portion of the CB molecule recognised by these anti-CB Ab1 was important, since it gives
an indication of what structural features of CB may or may not be mimicked by Ab2β generated from these anti-CB Ab1.”

P148 Para 3 L2: change suggets to suggest

P149 Para 2 L8: change “...no data was reported...” to “...no data were reported...”

P149 Para 2 L14: change “...binding site is easily...” to “...binding site would appear to be reasonably accessible ...”

P171 L3: change Propanolol to Propranolol

P171 L21: change Silenius to Silenus
ERRATUM - DISCUSSION

P21 Para 2 and P23: since the structural features important for energy-repartitioning effects, as opposed to β2-AR binding and activation, and the mechanism(s) of action of these βAAs still require further clarification, the author wished to take up these points in the discussion sections of the appropriate chapter as well as the general concluding discussion. In this way, the introduction was designed to draw attention to work which had clearly elucidated important structural features, and use the present data to comment on those matters less well understood. In this way repetition in the introduction may be avoided. The biological consequences of the chosen conjugation site were not known at the commencement of this work; the present work contributes some conclusions on this subject. Indeed, the report in the literature of the reduction in carcass fat following treatment with the des-amino CB analogue suggested that the conjugation through this position may be feasible.

P54 Para 3: no attempts were made to accurately determine the hapten density of the conjugates made, since the analogues did not readily lend themselves to such determinations. Enquiries are currently being made to determine if such analysis, using high resolution mass spectrometry/electrospray ionisation techniques, could be carried out.

P73 Para 4 L7 & P74 Para 2 L7: centrifugations were done with a desktop centrifuge. The precise speed of centrifugation in g was not known, but was not important for this experiment.

P79 Para 4 L4: g values for centrifugations are unavailable since assay was conducted by other workers in another laboratory

P81 Para 4 L2: post hoc determinations refers to tests for significance between groups carried out after the actual analysis of variance.

P77, P82 & P83 Tables 3.1 & 3.2, P90 Paras 3 & 4, P91 Para 4 (NA):

Comments regarding statistical analysis

Comments regarding the statistical analysis of whole body data from the rodent study focus on the apparent difference in starting weights. These differences were noted at the commencement of the study, and found to be statistically insignificant by ANOVA, although the author agrees with the examiner that on inspection the starting weights appear slightly different. Therefore, following supervisory advice (CSIRO DAP), it was felt that there should be no alteration to the random assignment of drug treatments to groups of rats. Further, at the completion of the study, an ANOVA of the data (one-way, repeated measures) showed significant difference
between the final bodyweights. Therefore, the suggestion was made by the statistician (CSIRO DAP) that the data also be analysed by analysis of covariance (ANCOVA), using the final bodyweight as the covariate, and that when the between cage (or between box) variation was significant in this analysis, then the adjusted means should be used to determine significant differences. In this way, the adjustment of the data according to bodyweight can be statistically justified. This kind of ANCOVA is a more severe analysis of the data since it markedly reduces the power of the significance tests, and since there were significant differences between the final bodyweights, was possibly more demanding than an ANCOVA using initial bodyweight as a covariate.

Examination of the data in this way revealed that between box variation was only significant for the soleus and EDL muscles, and the liver and kidneys. From these adjusted means, both the soleus and EDL muscles from any of the treatment groups were not significantly different from saline. Such a result would modify the conclusions made with respect to the anabolic effects of CB, although it should be noted that the weight of the soleus was still \( \approx 14\% \) greater in CB-treated rats than saline, \( \approx 7\% \) for the MeO group, and the VUF 8303 group mean was actually less than the saline group. Overall, the \( O \)-alkyl CB analogues still tended to have much less of an effect on skeletal muscle than CB. In the case of the liver, CB had a liver weight significantly lower than the saline treatment group, whereas the original analysis showed it to be significantly larger. From the adjusted kidney data, C2 and C6 Hapten groups were significantly lower than saline (previously insignificant), whilst CB was not significantly different to saline (previously significant). When the final bodyweight was adjusted for initial bodyweight, CB was still significantly different from saline whereas VUF 8303 was not.

Importantly, the between cage variation was not statistically significant when final bodyweight was used as a covariate in an ANCOVA of the percentages of Fat, Protein and Water in the carcass. Therefore, both the author and supervisor (CSIRO DAP) in this study believed the use of unadjusted means for these measurements was justified. The changes in % Fat and Protein in the carcasses of CB-treated animals has given rise to the description of CB as an energy-repartitioning agent, and these changes were observed in the present study with CB but not for any of the analogues. Therefore, the conclusion that the analogues would be of little use in the generation for the generation of CB-like anti-idiotypes which mimic this desired energy-repartitioning effects remains unaltered.

Since only a small minority of the tissues and growth parameters measured were found to need adjustment, it was decided after consultation with the supervisor of this study (CSIRO DAP) that unadjusted means would be used throughout for consistency. Indeed, the majority of similar studies reported in the literature also report unadjusted data. It should also be noted that advice from the statistician (CSIRO DAP) regarding the experimental protocol, suggested that
for a true randomized block design one rat from each of the six treatment group should be housed in each box (total 6 rats per box). However, the cages available for housing of the animals could only fit three and still meet animal ethics requirements. Further, it was felt that there may be some bias in such a setup since bigger animals (due to the treatment) may compete more successfully for food. Hence the protocol, as detailed in the thesis was used.

P85 Table 3.5 (PW), P92 Para 2 (PW & NA) & P79 Para 3 (NA): the main aim of the rodent study (Chapter 3) was primarily to compare the effects of CB and the analogues on skeletal and non-skeletal tissue weights and carcass characteristics using a saline group as control, but since there was also experimental opportunity to take blood samples, such samples were envisaged to be of possible interest with respect to metabolic substrates and hormones. However, there have not been consistently reported effects of clenbuterol on these substances. Therefore, in view of the comments by 2 examiners, in addition to the levels of insulin observed below the sensitivity of the assay, and the fact the assay was not carried out by the author, it would be sensible to remove these results from the thesis. Importantly, this does not change any of the conclusions of the thesis.

P87 Figures 3.3 & 3.4: Non-Specific Binding in the β2-AR Assay was determined using 5 µM propranolol (as described in section 3.2.3.6.3) and this data has been incorporated, indirectly, into Figures 3.3 and 3.4 since the specific binding (SB) was calculated by subtraction of the non-specific binding (NSB) from the total binding (TB). In the case of the actual receptor preparations, NSB values were routinely less than 10% of the TB values, and at the higher radiolabel concentrations around 3-5%. NSB values from the assay of supernatant, however, were much closer to the TB values since these supernatants contained proportionally larger amounts of non-receptor proteins (refer P87 Para 1). The graph below is representative of those obtained from other receptor preparations with respect to the relative magnitudes of TB and NSB values for both the receptor preparation and the supernatant.
Figure Saturation curves (total binding and non-specific binding) from assay of a $\beta_2$-AR preparation from SPG muscle and the corresponding supernatant (saline treatment group)

P89 Figure 3.5: Saturation curves from both muscle types (SPG and Quads) and all treatment groups tended to suggest that all sites had been saturated (plateau in curve at higher radiolabel concentrations as above). Importantly, a range of radiolabel concentrations was used in all saturation studies of the receptors isolated from each treatment group (refer section 3.2.3.6.3). After consultation with supervisors, it was decided not to include saturation graphs and Scatchard plots for all data so as not to “pad out” the thesis. Graphical limitations precluded both Scatchard plots being given on a single graph.

The suggestion (PW) to conduct competitive displacement assays using the analogues on a single receptor preparation was considered and preliminary assays were carried out. However, the data was difficult to fit to either a one or a two site model of receptor binding, and hand-drawn Scatchard plots appeared very shallow (suggestive of lower affinity for the receptor than CB) and data points which tended to be non-linear. Further work was not carried out since the receptor binding characteristics, although of interest, were not considered essential to the overall anti-idiotypic project, in light of the lack of physiological effects of the analogues; hence the purchase of further quantities of expensive radiolabel could not be justified.

P89 Table 3.8: This table shows the results of an ANOVA where the clenbuterol treatment group was excluded from the analysis (as explained in the text). Therefore data from this group have not been included in this table, but is given in Table 3.7. Further, Table 3.8 only shows data for the quads muscles, because this muscle from the clenbuterol treatment group showed rather large intra-group variation in $K_D$ and $B_{\text{max}}$; hence the additional ANOVA was only carried out for this muscle group.

Physical Characterisation Comments (PW): On the advice of supervisors, characterising spectra and HPLC chromatograms were not included since all data from these spectra were presented in a format consistent with publication in a chemistry journal, and in the interests of not “padding out” the thesis. However, the author acknowledges that inclusion of representative spectra to represent this data pictorially would have been of some value to the non-chemist.

P91 Para 3 L4-7: it is quite reasonable to suggest that VUF 8303 may have increased $\beta_1$ activity and decreased $\beta_2$ activity, since heart weight was increased. The author was reluctant to draw too definite conclusions on the strength of the present data and agrees the sentence is confusing, and has therefore been rewritten.
P92 Para 3: Previous work in this field has found that treatment of an animal with CB leads to anabolic activity (increased muscle mass) as well as a reduction in receptor number. The author does not wish to suggest that a close relationship between the two exists, merely that these appear to be coincidental effects. It is true to say that the intensity of stimulation of the relevant signal transduction pathways is the most important determinant of anabolic activity, and not receptor down-regulation, and also that down-regulation is a protective mechanism to prevent overstimulation. It is therefore of interest that CB has been proposed as a β3-AR agonist, a receptor which is apparently resistant to down-regulation and is responsible for non-shivering thermogenesis in brown adipose tissue. Also, not all βAAs which stimulate the β2-AR are anabolic (e.g. salbutamol); the mechanism(s) of action of CB require(s) further elucidation.

P92 Para 4, P93 Para 1: the author entirely agrees with the examiner's comments with regard to the differences in K_D of β2-ARs from the CB-treated group. The long biological half-life of CB could quite conceivably result in isolation of receptors from this treatment group which still contain CB, leading to errors in the estimation of K_D as suggested by the examiner.

P102 Para 2 L18(?) and P132 Para 1 L1: the examiner is correct to point out that displacement of radiolabelled CB by unlabelled CB is more accurately described in terms of reversible binding and affinity, rather than specificity. However, since the accurate determination of affinities (K_a values) was not attempted, but rather a comparison and ranking of the competing ligands according to cross reactivity, the author was reluctant to make to frequent use of the word affinity and hence preferred to talk in terms of (relative) specificities.

P109 Para 3 L1: molecular weight of PEG appears in Appendix A.

P133 Para 1 L7-9: inspection of Figure 4.11 (Scatchard referred to on P133 Para 1 L7-9) possibly suggests a two site model of binding, consistent with the heterogeneous population of antibodies found in a polyclonal anti-sera. However, the steeper portion of the curve is defined by only 3 (possibly 4) points and the author preferred a more conservative interpretation of the present data.

P147 Para 3 L10-11: the author does not wish to suggest that changes in receptor density are important in describing the magnitude of the biological response, and agrees that the magnitude of the signal transduction response is more important with respect to anabolic activity. However, reductions in receptor density have been reported to accompany anabolic activity (refer previous comments).

There would also have been some value in further characterising the analogues, using a cell type that responds to β2 agonists, and looking at events associated with signal transduction.
such as increased cyclic AMP and receptor phosphorylation. Indeed, such an assay was in the final stages of validation at the CSIRO at the conclusion of the author’s studies. However, time did not permit a full study of the analogues in this way. Further, although such characterisation would be of interest, increased cyclic AMP and β2-AR phosphorylation could be elicited by β-agonists which demonstrate little or no energy-repartitioning effects.

Therefore, the final test of the utility of the O-alkyl CB analogues was to assess their physiological effects in vivo, since these are the desired effects required by the CB anti-idiotypic project. Since the precise mechanism(s) of action of CB have not been clearly elucidated, in vitro tests or assays which predict in vivo energy-repartitioning activity are as yet unavailable. However, the in vitro tests suggested by the examiner could provide data useful in the further elucidation of the mechanism of action of BAAs with energy-repartitioning effects, such as CB. Such work was considered beyond the scope of the present thesis.
1.1 Aims of Project

Investigations (CSIRO DAP laboratories) into the notion that the energy-repartitioning effects of clenbuterol may be mimicked immunologically by the action of an anti-idiotypic antibody bearing the "internal image" of this β-adrenergic agonist growth promotant, have provided the opportunity to examine some novel O-alkyl clenbuterol analogues. It was proposed that these O-alkyl analogues of clenbuterol would be effective structural and functional congeners of clenbuterol, and thus may be useful in the generation of anti-idiotypic mimics of clenbuterol. Therefore, the aim of this project was to determine the utility of these O-alkyl clenbuterol analogues for the generation of anti-idiotypic antibodies which mimic the energy-repartitioning effects of clenbuterol by characterising these analogues in the following ways:

a) chemically, through the development and demonstration of their synthesis, spectroscopic characterisation to confirm structure (including any new intermediates), and the use of them in the formation of hapten-protein conjugates;

b) physiologically, through investigation, in vivo, of the effect of chemical structure modifications on various growth parameters;

c) immunologically, through production of anti-clenbuterol antibodies using the novel analogue-protein conjugates, and an assessment of their specificity.
1.2 Clenbuterol - an Energy-Repartitioning β-Adrenergic Agonist

1.2.1 Physiological Effects

Clenbuterol [4-amino-α-(tert-butylamino)methyl-3,5-dichlorobenzyl alcohol] is a synthetic therapeutic compound, originally developed as a potent bronchodilator for the treatment of equine asthma, as detailed in the 1970 patent. The phenylethanolamine structural moiety (Figure 1.1, dotted circle) is common to both clenbuterol (CB) and the endogenous adrenergic hormones, adrenaline (ADR) and noradrenaline (NOR) and the synthetic analogue isoprenaline (ISO).

![Diagram of Clenbuterol, Adrenaline, Noradrenaline and Isoprenaline](image)

**Figure 1.1** Structures of the phenylethanolamines (dotted circle): Clenbuterol, Adrenaline, Noradrenaline and Isoprenaline

CB has been shown to have various physiological effects, typically:

a) increased skeletal muscle protein accretion (≈10-30%)
b) decreased fat (white adipose tissue) deposition (≈10-30%)
c) skeletal muscle hypertrophy
d) increased weight gain
e) increased nitrogen retention

These effects have been observed in a number of livestock and laboratory species (summarised in Table 1.1). Accurate comparisons between studies have been difficult, even for those of the same species due to variations in the dosages used, the method of administration and duration of the treatment as well as the growth phase of the animals. In addition, the intake of exogenous protein in the diet had not been standardised across the various studies as restrictions on the intake of exogenous protein can lessen the effects of CB. However, the consistently observed changes in carcass composition have demonstrated the anabolic properties of this compound, and led to the subsequent description of it as an "energy-repartitioning" agent.
Table 1.1 A summary of reported studies on physiological effects of clenbuterol

<table>
<thead>
<tr>
<th>Species</th>
<th>Physiological Effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>↑ Protein ▼ Fat ▲ Muscle ▲ Weight ▲ Weight Gain ↑ N- Retention</td>
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<tr>
<td>Rodents</td>
<td>x ✓</td>
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<td>Sheep</td>
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<tr>
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<tr>
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<td>Rodents</td>
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<td>Sheep</td>
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<tr>
<td>Rodents</td>
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<td>✓ ✓ NR</td>
</tr>
</tbody>
</table>

✓ = statistically significant effect  
❌ = no statistically significant effect  
NR = not reported
Cimaterol (CIM), ractopamine (RAC) and L-644,969 contain structural similarities to CB (Figure 1.2). These compounds are also reported to have physiological effects similar to CB and together they are referred to as β-Adrenergic Agonist (βAA) growth promotants (for reviews see\textsuperscript{4,5,35-40}).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{chemical_structures.png}
\caption{Chemical structures of some β-adrenergic agonist growth promotants}
\end{figure}

Other observed physiological effects of βAAs growth promotants have included:

e) increased metabolic rate\textsuperscript{11,20,41}
f) increased heat production and body temperature\textsuperscript{20}
g) loss of appetite at high dietary doses\textsuperscript{41}
h) increased heart rate\textsuperscript{2,4,41-43}
i) decreased blood pressure\textsuperscript{41}
j) increased energy expenditure\textsuperscript{3,11,20,22}
k) increased oxygen consumption\textsuperscript{2}
l) reversal of muscle atrophy\textsuperscript{6,44-50}

These physiological effects of βAAs have also been found to be attenuated over time.\textsuperscript{25,37,38,51}

The increased muscle mass caused by βAAs has been thought to be truly hypertrophic, through a demonstrated increase in muscle fibre cross-sectional area rather than fibre number\textsuperscript{52-55} and increased skeletal muscle protein and RNA, but not DNA,\textsuperscript{6,15,22,53,56} which suggested increased protein synthesis. CB has also been shown to increase satellite cell proliferation in some studies,\textsuperscript{57} but not others,\textsuperscript{56} while the CB-induced reversal of muscle atrophy has been associated with satellite cell activation.\textsuperscript{58} However, the specificity of this action for either Type I (slow-twitch, oxidative) or Type II (fast-twitch, mixed glycolytic/oxidative) muscle fibres has remained unclear.\textsuperscript{5,37,52}

The endocrine effects of βAAs have been less well characterised and differences between acute and chronic effects have been observed.\textsuperscript{42,59} Reports of reduced plasma insulin levels,\textsuperscript{53,59} increased growth hormone levels,\textsuperscript{8} increased non-esterified fatty acid (NEFA) levels,\textsuperscript{59-61} increased T4 levels\textsuperscript{53,61} and decreased insulin-like growth factor-1 levels\textsuperscript{53} have contrasted with suggestions that the effects of βAAs were not mediated by insulin,\textsuperscript{25,27} gonadal or adrenal
hormones, or growth hormone. Others have reported no effects of βAAs on cortisol, prolactin, or growth hormone. However, the latter study suggested alteration of the rhythm of growth hormone secretion without change in the overall mean concentrations. Modulation of glucocorticoid action by CB has also been suggested.

1.2.2 Adrenergic Receptors and Signal Transduction

The adrenergic receptors (adrenoceptors; AR) are cellular receptors recognised by the endogenous adrenergic hormones, ADR and NOR (Figure 1.1), both of which are catecholamines. ARs were originally classified pharmacologically, based on a series of adrenergic agonists and their stimulation of physiological effects in various tissues. This study postulated the existence of a heterogeneous population of AR: α for smooth muscle contraction and β for smooth muscle relaxation. However, significant exceptions were also observed: stimulation of the α-receptors in the intestine which resulted in relaxation, and stimulation of β-receptors in the heart which induced contraction.

Subsequent studies further subdivided the AR types, based on the relative potency of a series of sympathomimetic amines, into β₁- and β₂-AR and α₁- and α₂-AR subtypes. Further advances in synthetic chemistry since then have identified ligands with increased selectivity and resulted in the elucidation of further subtypes: α₁A, α₁B, α₁C and α₁D ARs, α₂A, α₂B, α₂C and α₂D ARs and β₃. However, the function of some of these receptor subtypes are not yet fully understood.

The pharmacological distinction between β-AR subtypes is reflected in the order of affinity of the adrenergic agonists ISO, ADR and NOR:

- β₁: ISO > NOR ≥ ADR
- β₂: ISO > ADR > NOR
- β₃: NOR > ISO > ADR

Increased heart rate (positive ionotropic effect), increased cardiac output, lipolysis and intestinal smooth muscle relaxation have been identified as β₁ effects, whilst relaxation of bronchial and tracheal smooth muscle, arteriole and vasodilation, increased heart rate due to decrease in peripheral resistance, muscle tremor, uterine relaxation and hepatic and skeletal muscle glycogenolysis have been regarded as β₂ effects. The greater affinity of NOR (a neurotransmitter) for β₁ than β₂ has led to the conclusion that β₁-ARs pertain to the sympathetic nervous system, while β₂-ARs are hormonal receptors, although it was noted that there has not been widespread agreement with this classification.
Early suggestions of an atypical β-AR that was neither a β₁ or β₂-AR\textsuperscript{85} which mediated lipolysis in rat adipocytes,\textsuperscript{86} culminated in identification of this receptor as the third β-AR.\textsuperscript{77,78} The β₃-AR has been thought to mediate lipolysis in white adipose tissue,\textsuperscript{84} and non-shivering thermogenesis in the richly-innervated brown adipose tissue through the regulated uncoupling of oxidative phosphorylation by a mitochondrial uncoupling protein (for review see\textsuperscript{87}). In this way, energy released from fatty acid oxidation in brown adipose tissue is released as heat rather than for ATP synthesis. Large numbers of mitochondria give brown adipose tissue the darker appearance (compared with white adipose) and it is thought to be under sympathetic nervous control.\textsuperscript{88} Indeed, NOR has been shown to have a greater affinity than ISO or ADR for β₃-ARs which suggested these receptors were part of the sympathetic nervous system.\textsuperscript{89} However, the precise physiological function of this receptor has remained unclear.\textsuperscript{90}

ISO is regarded as a full agonist at the β₂-AR, and is arbitrarily assigned an intrinsic activity of 1.0 (maximum functional response to agonist), while an antagonist, which elicits no functional response upon binding to the receptor, has an intrinsic activity of zero.\textsuperscript{82,91} CB is a partial agonist,\textsuperscript{92} since it has an intrinsic activity only half that of ISO despite the similar phenylethanolamine structure. Partial agonists are thought to have properties of both an agonist and an antagonist.\textsuperscript{93} CB is a partial agonist in peripheral tissues,\textsuperscript{92,94-96} consistent with its bronchodilatory effects, and has a long duration of action. However increased intracellular concentration of cyclic AMP, an effect usually associated with β-agonists, is only weakly stimulated by CB at β₂-ARs in the central nervous system,\textsuperscript{97} and CB is thought to be less selective for central nervous system β₂-ARs.\textsuperscript{98} The increased heart rate (β₁ effect) observed with CB treatment\textsuperscript{2,4,41-43} indicates that β₂-AR selectivity does not equate to exclusive action at this receptor subtype.\textsuperscript{99}

The β₂-AR has been sequenced, cloned and extensively studied.\textsuperscript{100} The receptor protein, which contains 413 amino acid residues (molecular mass 64 kDa), is thought to be embedded in the cell membrane, and seven helical, hydrophobic domains (HD) are thought to span the cell membrane. These transmembrane domains are presumed to form a three-dimensional “bundle” perpendicular to the membrane, similar to that of the photoreceptor rhodopsin, based on hydrophilicity analysis and comparison (for reviews see\textsuperscript{101-105}). The N-terminus is thought to be on the extracellular side with two sites for glycosylation, whilst the C-terminus is intracellular.\textsuperscript{106,107} The β₂-AR is also stereo specific, recognizing R(-)- isomers with greater affinity than S(+) - isomers of phenylethanolamines containing a chiral hydroxyl-bearing carbon\textsuperscript{108} (for review see\textsuperscript{109,110}).

Studies employing site directed mutagenesis of the β₂-AR have identified amino acid residues important for agonist binding\textsuperscript{111-114} using ISO (Figure 1.3). These residues have been
identified as Ser 204 and Ser 207 (both in the fifth HD, probably H-bonded to the aromatic hydroxyl groups), Asp 113 (in the third HD, probably acting as the counter ion to the amino group, which is cationic at physiological pH), Ser 165 (fourth HD, possibly hydrogen-bonded to the ethanolic hydroxyl group), and Phe 289, Phe 290 and Tyr 326 (sixth HD, possibly interacting with the aromatic ring). Figure 1.3 is a schematic diagram of this arrangement, (looking at the receptor bundle from above) and does not reflect precise positions of the HDs.

![Schematic diagram of the binding of isoprenaline to the β2-AR](image)

**Figure 1.3** Schematic diagram of the binding of isoprenaline to the β2-AR (adapted from\textsuperscript{101,105,112})

Asp 79 has also been found to be critical for agonistic effects, but not for antagonist binding,\textsuperscript{115} while Asn 312 has been identified as important for antagonist binding.\textsuperscript{116} Some extracellular cysteine residues have been identified as necessary for ligand binding, probably by formation of disulfide linkages which stabilise the receptor bundle.\textsuperscript{117,118} Covalent incorporation of the reactive ligand, \(p\)-(bromoacetamido)benzyl-1-[\textsuperscript{125}I]iodocarazolol, in the region of residues 83-96 has suggested that these residues form a region critical for ligand binding.\textsuperscript{119} Asp 79 and Asp 113 have also been identified in the analogous position of the β1-AR.

Investigations of chimeric β1/β2-ARs (synthetic hybrids of portions of the two receptors) have suggested that HD 4 was important for agonist selectivity, whilst HDs 6 and 7 were important for antagonist selectivity.\textsuperscript{114,120} Studies with chimeric \(\alpha_2/\beta_2\)-ARs suggested that HD 7 was also an important determinant of ligand binding specificity.\textsuperscript{121} Agonist binding has been characterised by large decreases in enthalpy and thermodynamically unfavourable decreases in entropy, whilst antagonist binding has given rise to small enthalpy decreases and large entropy increases.\textsuperscript{122,123} The β2-AR ligand binding site has been estimated to be \(\approx 11\text{Å}\) from the extracellular surface of the receptor, approximately one third the depth of the hydrophobic core of the receptor bundle.\textsuperscript{124}
However, despite these elegant experiments, there has still been some suggestion from more recent chimeric studies that the ligand binding site of the \( \beta_2 \)-AR is defined by the ligand itself,\(^{125}\) a hypothesis supported from similar investigations into the dopaminergic \( D_2 \) receptor.\(^{126}\) Although important structural elements required for \( \beta_2 \)-AR ligand binding have been identified (using ISO and its analogues), the precise nature of the binding of individual ligands has still not been completely elucidated. The evidence to date has suggested that the ligand binding pocket of the \( \beta_2 \)-AR is buried within the hydrophobic core of the receptor, and it also appeared that most, if not all, of the HDs are involved in ligand-receptor binding, either allosterically or directly. The binding site for CB has not been determined. However, CB contains structural elements in common with ISO (both phenylethanolamines; refer Figure 1.1), which implies it may bind similarly as ISO to the receptor.

Structure-activity relationships of \( \beta \)-AR agonists, highlighted in an excellent review of over 150 compounds\(^{127}\) (and references therein), have indicated that:

a) the majority of \( \beta \)-agonists are phenylethanolamines whilst almost all \( \beta \)-antagonists are phenoxypropanolamines (Figure 1.4). However, this rule is not absolute and some exceptions have been shown. A variety of ring substituents (\( X, Y \) and \( Z \)) have been reported including hydroxy, amino, cyano, chloro and hydroxymethyl.

b) branching of the \( N \)-alkyl substituent adjacent to the nitrogen has increased the \( \beta \)-selectivity of the compound, and compounds with bulkier \( N \)-alkyl substituents (\( e.g. \) tert-butyl) have tended to have greater \( \beta_2 \)-selectivity. Aryl-substituted \( N \)-alkyl groups (with maintenance of branching adjacent to the nitrogen, \( e.g. \) RAC, Figure 1.2) have been found to enhance \( \beta \)-agonist affinity.

c) a 3,4-dihydroxy ring substitution (catechol, \( e.g. \) ISO, Figure 1.1), rather than a 3,5-dihydroxy substitution (resorcinol), has improved the potency of \( \beta \)-agonists. Replacement of the meta-hydroxyl group in catechol analogues with a hydrogen-bonding group has yielded potent \( \beta \)-agonists. By contrast, similar changes to the para-hydroxyl group have reduced the activity of the compound.
Removal of either of the ring hydroxyl groups has also resulted in a marked reduction in activity, although oral bioavailability may have been slightly enhanced.

d) removal of the benzylic hydroxyl group has resulted in a large reduction in β-agonist activity, as has its conversion to an ether moiety. Chirality at the α-carbon of β-agonists greatly influences activity, R(-)- isomers having higher activities than S(+) - isomers at all ARs. R(-) - isomers of the chiral hydroxyl-bearing carbon of phenoxypropanolamines also have considerably higher affinities for the β-ARs than S(+) - isomers.\textsuperscript{109}

e) alkyl substitution of phenylethanolamines at the β-carbon has resulted in significant reductions in β-agonist activity, although inclusion of this carbon in a cyclohexane ring structure fused to the aromatic ring did not significantly reduce β-agonist activity.

f) a three point contact with the receptor (aliphatic amino nitrogen, benzylic hydroxyl and the aromatic ring) would appear to be very important for receptor binding of both agonists and antagonists, consistent with structural receptor studies discussed previously. This hypothesis has been proposed previously.\textsuperscript{128}

Hormone binding to cellular receptors is a signal to the cell to initiate some particular cellular event. The α- and β-ARs are members of a family of receptor proteins where signal transduction is mediated by heterotrimeric guanine-nucleotide-binding regulatory proteins (G-proteins; for reviews see\textsuperscript{129-137}). These intracellular G-proteins modulate receptor-enzyme interactions rather than receptor-ligand interactions.\textsuperscript{108} G-Proteins are thought to be associated with ≈80% of cellular receptors including those for neurotransmitters (e.g. adrenergic, dopaminergic, cholinergic, muscarinic, purinergic), peptide hormones (e.g. pituitary, hypothalamic), regulatory factors (e.g. bombesin, bradykinin, neuropeptide Y), prostanoids and sensory factors.\textsuperscript{132}

There are several types of G-proteins. Those associated with ARs include:\textsuperscript{138-140}

g) stimulatory G-proteins (G\textsubscript{s}). These activate the enzyme adenylyl cyclase and Ca\textsuperscript{2+} channels and have been associated with all three β-ARs.

h) inhibitory G-proteins (G\textsubscript{i}), of which three are known at present. These inhibit adenylyl cyclase activity and Ca\textsuperscript{2+} channels and stimulate K\textsuperscript{+} channels, and have been associated with α\textsubscript{2} receptors.
i) $G_q$-proteins. These stimulate phospholipases C and $A_2$, and have been associated with $\alpha_1$ receptors.

Other G-proteins subtypes, ($G_o$, $G_p$, and $G_{2/3}$) have also been identified although the function of the latter ($G_{2/3}$) is unknown. The subunits of the G-protein heterotrimer ($\alpha$, $\beta$ and $\gamma$) show considerable diversity within a subunit type, with the suggestion that up to 1 000 different combinations are theoretically possible.\textsuperscript{141}

The $\beta_2$ receptor is often cited as a well-understood example of G-protein-coupled receptors (see G-protein reviews above). Agonist binding has been found to induce a conformational change in the receptor, which then binds to $G_s$ and forms a ternary complex (agonist/receptor/G-protein). This "ternary model" has been used to describe G-protein-mediated signal transduction.\textsuperscript{142,143} In contrast, antagonist binding does not result in G-protein activation. This may have been due to changes in receptor conformation that were unfavourable for ternary complex formation, although it has been suggested that, for G-protein-coupled seven-transmembrane helix receptors, the interface between the receptor and the G-protein may have been blocked by an antagonist.\textsuperscript{144}

Formation of the ternary complex causes receptor-bound $G_s$ to increase its rate of exchange of bound guanosine diphosphate (GDP) for guanosine triphosphate (GTP). The complex of the GTP and the $\alpha$-subunit of the $G_s$-protein (GTP/$G_{s\alpha}$), which hydrolyses slowly back to the original heterotrimer, is then released and diffuses along the intracellular membrane surface to activate adenylyl cyclase. Activated adenylyl cyclase then catalyses the conversion of many ATP molecules to cyclic AMP resulting in increased intracellular cyclic AMP concentration and considerable signal amplification.\textsuperscript{91,145-150}

As a so-called “second messenger”, cyclic AMP mediates post-receptor intracellular events, not only for adrenergic ligands of $\beta$-ARs, but also for other “first messenger” hormones including glucagon, vasopressin, thyrotropin, LH, FSH, and ACTH.\textsuperscript{132} Agonist binding to $\beta$-ARs and the consequent increase in cyclic AMP concentrations causes activation of protein kinase A. This kinase in turn activates hormone-sensitive lipase resulting in lipolysis and mobilization of free fatty acids as well as increasing glycogenolysis, through the activation of glycogen phosphorylase and inhibition of glycogen synthase, in both the liver and skeletal muscle.\textsuperscript{5,37,150-154} In this way, endogenous adrenergic ligands have been understood to exert metabolic effects through $\beta$-ARs. In conjunction with adenylyl cyclase-inhibitory effects mediated by the $\alpha_2$-AR, the ultimate metabolic response to an adrenergic hormone is determined by the concentration and distribution of the two receptor types ($\alpha$ and $\beta$) in the target tissue.
Protein kinase A also recognises particular consensus sequences on the β₂-AR. Upon short-term agonist exposure, the β₂-AR is phosphorylated and hence functionally uncoupled from G₅, a process known as receptor desensitisation (for review see \(^{102,104,105,135,140,155-159}\)). At higher agonist concentrations, agonist-occupied β₂-AR is also phosphorylated (at sites different to those phosphorylated by protein kinase A) by β-adrenergic receptor kinase (BARK), and complete uncoupling appears to require another protein, β-arrestin, which may act by preventing the interaction of the receptor with the G₅ subunit.\(^{104,135,158}\) Phosphorylated receptor is less able to form the ternary complex, resulting in decreased activation of adenylyl cyclase. Chronic agonist exposure leads to a reduction in β₂-AR density at the cell membrane, total receptor number and β₂-AR mRNA, a process termed down-regulation. Desensitisation and down-regulation are therefore regarded as the cellular mechanisms for signal modulation, although these processes are still not entirely understood.

In contrast, the β₃-AR does not contain the same phosphorylation sites as the β₁- or β₂-AR on the third intracellular loop of the receptor protein.\(^{156,160}\) These observations have prompted investigations of desensitisation of the β₃-AR, which found that the β₃-AR was somewhat resistant to agonist-promoted desensitisation or down-regulation.\(^{161-163}\)

### 1.2.3 Mechanism of Energy-Repartitioning

The description of βAAs as “energy-repartitioning” agents has arisen from a repartitioning of nutrients away from fat deposition, toward muscle protein accretion.\(^{8}\) However, this term explains only the overall effect of βAAs and does not explain the mechanism by which this occurs. Despite much investigation, the precise mechanism by which βAAs exert their repartitioning effects remains unclear.

There has been some disagreement as to the precise mechanism by which increased skeletal muscle protein accretion occurs (for review see \(^{37,38}\)). Increased skeletal muscle protein accretion, from in vivo studies, has been attributed to:

a) increased rates of protein synthesis\(^{2,6,11,24,27,29,36,47,65,164-167}\)

b) decreased rates of protein degradation\(^{15,16,19,32,168-171}\)

c) increased protein synthesis and decreased protein degradation.\(^{30,172-174}\)

Comparison between studies has been difficult due to differences in species, dose, length of treatment and method of administration. The measurement of the rates of protein synthesis or degradation have also been difficult (for review see \(^{175-177}\)). Previous studies have suggested that the endogenous adrenergic ligand ADR inhibited muscle protein degradation by inhibiting the release of alanine and glutamine from muscle.\(^{178}\) Others have suggested that the synthetic
catecholamine ISO also inhibited protein degradation. In vitro studies have been equivocal in the clarification of the mechanism, but contractile activity necessary for maintenance of healthy skeletal muscle may have been lacking in such experiments.

Decreased protein degradation following βAA treatment may also be associated with changes in the activities of calcium-dependent proteinases (calpains I and II) and their endogenous inhibitor, calpastatin. However, some studies have reported increased activity of both calpastatin and the calpains, while others have reported decreased activities of the calpains, and it was unclear if the increased protein deposition caused by βAAs was mediated in this way. Other studies have measured the activities of panels of up to 15 proteinases, but found that a number of βAAs had no observable effect on enzyme activity. Overall, the evidence has suggested that the exact mechanism for the increased muscle mass was most likely a combination of both increased rates of protein synthesis and decreased rates of protein degradation.

Likewise, the mechanism involved in decreased fat deposition in response to βAAs has also been unclear (for review see) and could be due to:

d) increased lipolysis

e) decreased lipogenesis

In contrast, other studies have reported that βAA treatment did not result in decreased lipogenesis or increased lipolysis, although an increase in lipogenic enzyme activities has been reported with CB. Both the stimulation of lipolysis and the inhibition of lipogenesis in rat adipocytes, and increased glycerol release and decreased fatty acid synthase activity in the mouse-derived TA1 adipogenic cell line, have been reported for RAC. However, comparisons of in vitro studies of adipocytes with in vivo studies may not be possible, since in vitro culture conditions could possibly influence the results.

Other suggestions to explain decreased fat deposition have included a reduction in fat cell number and modulation of the metabolism of triglyceride-rich lipoprotein and lipoprotein lipase activity. The observed decrease in carcass fat has also been described as a "dilution effect", since the total amount of fat actually remains unchanged and increased protein accretion occurs; hence the amount of fat is effectively "diluted out" resulting in the observed alteration in carcass composition. The reduction in carcass fat has also been thought to be greater in ruminants than in swine. Therefore, the equivocal results of investigations to date have suggested a potentially complex mechanism by which βAAs have altered carcass composition.
1.2.4 Current Uses of β-Adrenergic Agonists

Over-consumption of dietary fat has become a major human health concern in recent years in developed countries\textsuperscript{152,204,205} and the health benefits of reduced dietary fat intake have been emphasised.\textsuperscript{206} This has increased demand for leaner cuts of meat and encouraged producers in some countries to consider alternative production strategies to produce leaner meat, such as the use of growth-promoting substances. This kind of pharmacological manipulation of food production has also offered the producer increased production efficiency through the economic benefits of delivering leaner carcasses and a healthier product for the consumer.

The advantages of using CB and other growth-promoting βAAs are, firstly, a significant reduction in the levels of adipose tissue in the carcasses of treated animals and improved production efficiency and, secondly, growth-promoting effects which are predominantly muscle-specific.\textsuperscript{39,152} By comparison, growth promoting substances such as steroids (now banned for use in livestock in the European Economic Community (EEC)) and growth hormone\textsuperscript{27} are less tissue-specific in their anabolic effects.

However in recent years there has been increased demand on primary producers to deliver food to the market place without the use of so-called “chemicals”,\textsuperscript{207} which has included the recent banning of the use of βAAs by the EEC as growth promotants in livestock because of residual contaminants found in meat.\textsuperscript{208} Indeed, documented cases of human poisoning from consumption of liver which contained CB residues (not removed by cooking) have been reported, causing tremor, tachycardia, dizziness and vomiting,\textsuperscript{209} symptoms associated with the side-effects of CB. CB residues have also been detected above the permitted maximum residue level of 0.5 μg/kg in meat from animals withdrawn from the drug treatment 5 weeks previously.\textsuperscript{208}

Given the legislative constraints and consumer reticence regarding agricultural products grown with the aid of synthetic substances, an attractive alternative would be to mimic the physiological effects of βAAs without using pharmacological agents. The use of veterinary vaccines in animal production has widespread, with the perception they have been perhaps more “consumer friendly” than exogenous “chemicals”. Immunological treatments have been regarded as safe, with positive economic and animal welfare benefits.\textsuperscript{210} For example, a commercially-available vaccine (Vaxstrate\textsuperscript{®}) for the immunological castration of domestic livestock\textsuperscript{211} was developed in the laboratories of the Commonwealth Scientific and Industrial Research Organisation Division of Animal Production (CSIRO DAP).
1.3 Immunological Mimicry

Immunological manipulation of adiposity has been an area of investigation which has received some considerable attention over the last decade or so. The use of anti-hormone antibodies for the immunoneutralization of growth hormone and insulin\cite{210} for the immunoenhancement of growth hormone\cite{212}, antibodies raised against adipocytes to stimulate destruction of adipose tissue\cite{213}, as well as immunomimicry of growth hormone have all been reported (for reviews see\cite{210,214}). The latter approach has involved the \textit{in vivo} generation of anti-idiotypic antibodies which mimicked the original hormone. Mimicry of the effects of growth hormone by anti-idiotypic antibodies has been patented\cite{215}. This methodology of using anti-idiotypic antibodies to mimic the actions of the original ligand formed the basis of research at CSIRO DAP, where the objective was to develop an immunological mimic of CB with energy-repartitioning effects.

1.3.1 Anti-idiotypic Antibodies

Antibodies (Abs), or immunoglobulins (Ig), are large proteins (molecular mass \(\approx 150\) kDa) consisting of two heavy (molecular mass \(\approx 50\) kDa) and two light (molecular mass \(\approx 23\) kDa) chains. Each chain has constant and variable regions and these chains combine to form a molecule with an approximately Y-shaped tertiary structure (Figure 1.5; for review of antibody structure see\cite{216,217}). The variable regions form the antigen binding sites of the Ab. In response to an antigenic challenge, Abs are generated against particular minimum molecular conformations found on the antigen (epitopes or antigenic determinants), for which the antigen binding sites (paratopes) are specific. Abs also contain epitopes, of which three types are broadly defined\cite{218} (illustrated in Figure 1.5):

a) isotopes, which define the major Ig classes and subclasses, and are not immunogenic within a species
b) allotopes, which vary between members of the same species and are immunogenic between these individuals
c) idiotopes, which are located in the variable region of the Ab, and include the antigen binding site, and are immunogenic \textit{to the individual that generated them.}

Idiotopes\cite{219,220} are found in and around the paratope and collectively describe the idiootype or idiotypic Ab (Ab1; for review see\cite{218,221-225}). Later work distinguished between so-called public (shared by several antibodies) and private (specific for a particular antibody) idiotopes.\cite{226,227} The anti-(antibodies) which recognised these particular idiotopes were termed anti-idiotypes or anti-idiotypic Abs (Ab2). This distinction was regarded as purely operational,\cite{228} since the differentiation between that which recognises and that which is being recognised has been largely arbitrary.\cite{229,230}
Figure 1.5 Schematic diagram of an antibody molecule (adapted from\textsuperscript{216})

Some ten years after the discovery of idiotopes, the concept of an "idiotypic network" was proposed. This theory provided an explanation for the regulation of the immune system\textsuperscript{231,232} and has subsequently undergone further refinement\textsuperscript{233,234}. The hypothesis, built on the idioype/anti-idiotype interaction, suggested the existence of an immunological network in equilibrium, regulated by these idiotypic interactions. Antigenic challenge disturbed this equilibrium and resulted in production of Abs with the required specificity, following clonal expansion (for review see\textsuperscript{218,228,235}). The presence of anti-(anti-idiotypes) was seen as a corollary of this hypothesis, although it is unclear how far this immunological network may extend\textsuperscript{218}.

Molecules of small molecular mass (< 1 000 Da, e.g. CB) do not elicit an immune response, although they contain epitopes to which specific Abs may be raised. Covalent attachment (or conjugation) of these small molecular mass molecules (haptenes) to a larger molecule capable of eliciting an immune response (substances known as immunogens, for example, Bovine Serum Albumin, BSA) has produced conjugates which, when formulated into a vaccine and used to immunise animals, have been useful for the production of Abs specific for the original hapten (anti-hapten Abs). A conjugate which can elicit an immune response is then regarded as an antigen (also called an immunogen or immunoconjugate). This approach to the generation of antibodies to small molecular mass compounds not normally recognized by the immune system has been well known for many years (for review see\textsuperscript{236}).

Jerne\textsuperscript{231} also proposed that amongst the heterogeneous population of anti-idiotypic Abs, it was conceivable that some Abs would be specific for the paratope of the idioype, and therefore bear a so-called "internal image" of an external antigen (for review see\textsuperscript{218,228,230,235,237,238}). Indeed, previous reports had shown that the binding of idiotypic Ab to anti-idiotypic Ab was, in some cases, inhibited by hapten.\textsuperscript{239} Confirmation of the existence of internal image Ab2 occurred subsequent to the proposal of the network theory, when Ab2 that were the internal image of retinol-binding protein and insulin were reported.\textsuperscript{240,241} Figure 1.6 below gives a
schematic representation of the generation of internal image Abs.

\[ \text{Carrier Protein} \rightarrow \text{Hapten} \rightarrow \text{Hapten-Protein Conjugate} \rightarrow \text{Idiotype Antibody (Ab1)} \rightarrow \text{Anti-Idiotype Antibody (Ab2)} \]

**Figure 1.6** Schematic diagram depicting the generation of anti-idiotypic antibodies which bear the “internal image” of the original hapten

Immunisation with a particular hapten-protein conjugate generates a heterogeneous population of Ab1, encompassing the many epitopes found on the surface of the conjugate. Some Ab1 will be specific for and complementary to the hapten (anti-hapten Abs), and the hapten binds specifically to the paratope of these Abs. It is possible to isolate particular Ab1 (anti-hapten Ab1) using affinity chromatography techniques.\(^{242,243}\) Immunisation of a naive animal with anti-hapten Ab1 generates a further heterogeneous population of anti-idiotypic Abs, amongst which some will specifically bind the paratope of Ab1 - these are the internal image Ab2. This approach has been known previously\(^{244}\) and was deemed an achievable method for the development of antibodies which could mimic the energy-repartitioning effects of CB.

Ab2 have been classified according to the idiotope recognised in the variable region of Ab1.\(^{228,233,235,245,246}\) Ab2 whose binding to Ab1 cannot be inhibited by hapten are designated Ab2α. Of those Ab2 whose binding to Ab1 is inhibited by hapten, two types are thought to be possible. Ab2 which bind to the paratope of Ab1 are termed Ab2β, and regarded as potential internal image candidates (for review see\(^{229,230,247,248}\)). Those which bind to idiotopes outside the antigen combining site of Ab1, but still inhibit hapten binding (perhaps by steric hindrance) are termed Ab2γ. This delineation highlighted the notion that hapten inhibition of Ab1-Ab2 binding was a necessary but not sufficient condition for internal imagery.\(^{228}\)

### 1.3.2 An Immunological “Internal Image”

Considerable interest in Ab2β (as surrogate antigens for both therapeutic purposes\(^{222,223,230,237,246,249-252}\) and as probes for topological and ligand-binding investigation of cell-surface receptors\(^{225,252-261}\)) has been reported. An anti-idiotypic approach
to cellular receptor characterisation circumvents the problem of obtaining sufficient purified receptor for use as antigen to raise anti-receptor antibodies, when the receptor ligand may be more readily available.\textsuperscript{257} Table 1.2 below lists some examples of Ab2 raised against endogenous hormones and synthetic ligands which bind to cellular receptors.

From these studies, Ab2\textsubscript{B} that were a true “internal image” of the original hapten not only competed with hapten for binding to anti-hapten Ab1 or cellular receptors, but were also shown to mimic particular in \textit{vitro} or in \textit{vivo} effects of the original hapten. The majority of Ab2 in Table 1.2 were tested as ligand mimics \textit{in vitro}, through the use of specific cell lines bearing the receptor in question or receptor preparations in competitive receptor binding assays, as well as measurements of functional cellular responses to Ab2 binding. Relatively few studies have attempted to demonstrate hapten-mimicking properties of Ab2 \textit{in vivo}, other than those cited below:

\begin{itemize}
  \item[a)] Bis Q (a synthetic and potent nicotinic acetylcholinergic agonist) Ab2 induced symptoms (\textit{e.g.} muscle weakness) consistent with the clinical condition myasthenia gravis, an auto-immune disease where antibodies are raised to the patient’s own nicotinic acetylcholinergic receptors.\textsuperscript{265}
  \item[b)] Estradiol stimulates creatine kinase activity when administered to immature female rats. Estradiol Ab2 stimulated a similar increase in creatine kinase activity (BB isozyme) \textit{in vivo}.\textsuperscript{277}
  \item[c)] Progesterone Ab2\textsuperscript{286} was used as surrogate antigen to produce anti-(anti-idiotypic) antibody, or Ab3. The Ab3 were found to behave like anti-progesterone antibodies, and blocked pregnancy \textit{in vivo}.
  \item[d)] GH Ab2 stimulated weight gain in GH-deficient rats.\textsuperscript{283}
\end{itemize}
Table 1.2  A summary of anti-idiotypic antibodies and their characterisation as “internal images” of the original hapten

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Receptor Binding*</th>
<th>Functional Response**</th>
<th>Internal Image</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>✓</td>
<td>↑ α-aminoisobutyric acid uptake</td>
<td>✓</td>
<td>240</td>
</tr>
<tr>
<td>Alpenrolol</td>
<td>✓</td>
<td>↑ adenyl cyclase activity</td>
<td>x</td>
<td>262</td>
</tr>
<tr>
<td>Alpenrolol</td>
<td>✓</td>
<td>inhibited adenyl cyclase activity</td>
<td>✓</td>
<td>264</td>
</tr>
<tr>
<td>Bis Q</td>
<td>✓</td>
<td>muscle weakness in vivo</td>
<td>✓</td>
<td>265</td>
</tr>
<tr>
<td>TSH</td>
<td>✓</td>
<td>↑ adenyl cyclase activity</td>
<td>✓</td>
<td>266</td>
</tr>
<tr>
<td>Morphine</td>
<td>✓</td>
<td>inhibited contraction of vas deferens</td>
<td>✓</td>
<td>268</td>
</tr>
<tr>
<td>Substance P</td>
<td>✓</td>
<td>spasmogenesis inhibition</td>
<td>x</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ phospholipid turnover</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prolactin</td>
<td>✓</td>
<td>NR</td>
<td>-</td>
<td>270</td>
</tr>
<tr>
<td>Angiotensin</td>
<td>✓</td>
<td>NR</td>
<td>-</td>
<td>271</td>
</tr>
<tr>
<td>β-endorphin</td>
<td>✓</td>
<td>↑ cAMP</td>
<td>x</td>
<td>272</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>✓</td>
<td>↑ inositol phosphate and PGE2</td>
<td>✓</td>
<td>273</td>
</tr>
<tr>
<td>Serotonin</td>
<td>✓</td>
<td>no inhibition of 5-HT uptake</td>
<td>x</td>
<td>274</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>✓</td>
<td>plasminogen synthesis activated</td>
<td>✓</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>receptor down regulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>✓</td>
<td>receptor visualisation</td>
<td>✓</td>
<td>276</td>
</tr>
<tr>
<td>Estradiol</td>
<td>✓</td>
<td>↑ creatine kinase in vivo</td>
<td>✓</td>
<td>277</td>
</tr>
<tr>
<td>oLH</td>
<td>✓</td>
<td>↑ progesterone, testosterone</td>
<td>✓</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no effect on testicular size in vivo</td>
<td>x</td>
<td></td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>✓</td>
<td>no immunosuppression</td>
<td>x</td>
<td>279</td>
</tr>
<tr>
<td>Enkephalin</td>
<td>✓</td>
<td>NR</td>
<td>-</td>
<td>280</td>
</tr>
<tr>
<td>Kinin</td>
<td>✓ (Ab1)</td>
<td>NR</td>
<td>-</td>
<td>281</td>
</tr>
<tr>
<td>HS-145</td>
<td>✓</td>
<td>↓ GTP binding, GTPase activity</td>
<td>✓</td>
<td>284</td>
</tr>
<tr>
<td>Okadaic acid</td>
<td>✓ (Ab1)</td>
<td>↓ protein phosphatase activity</td>
<td>✓</td>
<td>285</td>
</tr>
<tr>
<td>Taxol</td>
<td>✓†</td>
<td>tubulin assembly from microtubules</td>
<td>✓</td>
<td>282</td>
</tr>
<tr>
<td>pGH</td>
<td>✓</td>
<td>↑ growth of GH-deficient rats</td>
<td>✓</td>
<td>283</td>
</tr>
</tbody>
</table>

* = Hapten-inhibitable, ** = in vitro unless otherwise stated, † = binds to microtubules, Ab1 = Hapten-inhibited binding to Ab1, NR = not reported, ✓ = Yes, x = No; Alpenrolol: synthetic β-Adrenergic antagonist, Bis Q: synthetic Nicotinic agonist, TSH: Thyrotropin, oLH: ovine Luteinizing Hormone, Cyclosporin A: synthetic immunosuppressive agent, HS-145: synthetic Thromboxane A2 antagonist, Okadaic acid: marine toxin, Taxol: anti-cancer agent, PGE2: prostaglandin E2, 5-HT: 5-hydroxytryptamine, pGH: porcine Growth Hormone
Production of Ab2 that are a true immunological internal image has not been consistently reported. As shown in Table 1.2, Ab2 raised against Alprenolol (a β-adrenoceptor antagonist) have been produced on several occasions, with both agonist and antagonist-like effects observed. The agonist-like action of increased adenyl cyclase activity\(^{262}\) has lead to the suggestion that the ethanolamine side-chain of alprenolol was the key epitope recognised by anti-hapten Ab1, and that an equal probability of either agonist or antagonist activity was possible.\(^{257}\) Lack of a functional internal image has also been observed for other ligands in some reports (refer Table 1.2), while in others it was not consistently observed in all individuals immunised.\(^{262,265,287}\)

These results served to illustrate the heterogeneity of the Ab2 population, and the crucial role of functional assays to identify Ab2 that bear a genuine internal image \textit{i.e.} Ab2β. Immunochemical screening for Ab2β has often been a poor indicator of biological activity,\(^{252}\) and receptor binding did not necessarily result in effector activation. It has also been suggested that Ab2β:

\begin{itemize}
  \item d) are a very small subpopulation of Ab2\(^{223,252,255,288}\)
  \item e) occur only rarely in the immune response\(^{253}\)
  \item f) may exist for some but not all antigens\(^{228}\)
  \item g) are probably of little use in the discovery of previously undescribed receptors.\(^{288}\)
\end{itemize}

The structural nature of the internal image (Ab2β) is still unclear. Ab2β may have resembled hapten more closely in terms of "contact bonds", rather than spatial arrangements of contact residues in the binding site, despite significant topological differences.\(^{237}\) Some degree of paratopic binding flexibility has also been suggested,\(^{223,289,290}\) presumably because the paratope binds hapten and Ab2β equally well. The notion of a paratope (of Ab1) as a cleft or valley for which hapten is complementary has suggested that Ab2β may present a paratope more like a "protuberance" than a cleft,\(^{253}\) while others have suggested that the picture of the paratope as a cleft may be somewhat redundant.\(^{237,248,291}\) Indeed, a flatter paratopic topology has also been suggested.\(^{223}\) Nonetheless, despite the potential structural unrelatedness of hapten and Ab2β, the existence of Ab2β which functionally mimic hapten has been well documented (refer Table 1.2).

Non-protein haptens have provided a structurally unique example of internal imagery, since Ab2β proteins have mimicked the effects of such dissimilar compounds. Ab2 raised against non-protein antigen may be less than optimal structural mimics of the antigen,\(^{222}\) although functional mimicry of synthetic ligands (\textit{e.g.} Bis Q, morphine, Taxol) by Ab2β has been reported (refer Table 1.2). Therefore, Ab2β may be functionally similar to the original hapten,
despite incomplete structural similarity.\textsuperscript{237,248} However, Ab2\B may not demonstrate all functional characteristics of the original hapten because they present an imperfect structural image of this hapten,\textsuperscript{292} lacking important structural characteristics which govern function.\textsuperscript{293} Structural correlates of hapten and Ab2\B have thus been thought to be somewhat difficult to define.\textsuperscript{258} Furthermore, suggestions of paratopic recognition (binding) by Ab2\B of an area on Ab1 larger than that recognised by the actual hapten,\textsuperscript{228} or recognition by Ab1 of patterns or groups of epitopes\textsuperscript{230,237,291} have indicated additional complexities. These suggestions have tended to reinforce the observed heterogeneity of the antibody response, as well as the artificial distinction between paratope and idiotope.\textsuperscript{291}

The ability of Ab2\B to mimic receptor binding of various receptor-specific ligands has suggested that the corresponding Ab1 was somewhat "receptor-like."\textsuperscript{252,256,258} Recognition of Ab2\B as hormone-like by a cellular receptor has been regarded as necessary for the antibody to be an immunological internal image of that hormone.\textsuperscript{257} Therefore, Ab2\B may be either an internal image of the hormone, or be able to recognise structural homologies shared by the receptor and Ab1.\textsuperscript{258} However, Ab2\B may not always be a perfect complement to the ligand binding site of the receptor,\textsuperscript{253} and the nature of the binding of Ab2\B to cellular receptors is still unclear. Indeed, the sheer size of an Ab (molecular mass $\approx 150$ kDa) in comparison to the receptor (e.g. $\beta_2$AR, molecular mass $\approx 64$ kDa) may limit hormone-like receptor binding of Ab2\B, particularly where the ligand binding site is buried within the receptor protein. The generation of Ab2\B that functionally mimicked estradiol\textsuperscript{277} has been particularly notable, since this hormone receptor is \textit{intracellular}, rather than on the cell surface.

The functional significance of idiotypic network regulation of the immune system has continued to be a matter of much discussion, particularly with regards to the regulatory role of idiotopes\textsuperscript{294,295} (for review see\textsuperscript{218,223,291}). However, the transient nature of the Ab2 response has been attributed to the regulatory efforts of the immunological network to re-establish equilibrium after antigenic challenge, probably through generation of anti-(anti-idiotypes), or Ab3, which neutralised Ab2, including Ab2\B.\textsuperscript{255,256,258,260,287} This type of response may also have masked the actual Ab2\B response.\textsuperscript{257} For example, a dramatic reduction in Ab2 titre has been reported after a second boost immunisation with anti-(Bis Q) Ab1, which was not recovered even after several subsequent boosts.\textsuperscript{265} By contrast, antigenic challenge and consequent stimulation of the immunological network at several levels has led to production of Ab2\B through immunisation with a hapten-protein conjugate\textsuperscript{296,297} - a so-called "autoantibody strategy" (for review see\textsuperscript{253}).
1.3.3 Anti-Clenbuterol Antibodies

The initial stage in the generation of Ab2 which could mimic the energy-repartitioning effects of CB would be the production of anti-CB Ab. Recognition by anti-CB Ab of particular structural elements of CB would be important, so that Ab2β elicited by such Abs bear a structural resemblance as complete as possible to CB. CB could be considered to have several epitopes which may be presented to the immune system according to the site of conjugation to the carrier protein. Selection of this site should include consideration of structural features that appear important for ligand binding to the β2-AR (outlined in section 1.2.2). Since hapten conjugation necessarily involves some modification of the hapten, both the site and chemistry of conjugation require consideration.

Maintenance of the structural integrity of the phenylethanolamino moiety would be important; both the benzylic hydroxyl and aliphatic amino groups have been regarded as necessary for β2-AR binding. Ring hydroxyl groups of ISO have also appeared to be structurally important for β2-AR binding, by hydrogen-bonding to receptor protein serine residues, with the implication that ring substituents should apparently have hydrogen-bonding character. However, the structural features important for βAAs to exert energy-repartitioning effects have been unclear. Indeed, the des-amino CB analogue (where hydrogen replaces the 4-amino group) has also been found to have energy-repartitioning effects.298

CB is a relatively small (molecular mass 277 Da) and comparatively rigid molecule, and three broad sites of conjugation could be postulated. These are illustrated in Figure 1.7 with an indication of the epitope of CB presented by each conjugate. Conjugation requires a reactive bridging group through which hapten may be covalently linked to carrier protein (for review see299). This reactive bridging group may already exist in the molecule. Alternatively, a suitable reactive bridging group could be introduced into the hapten, either by further structural modification of the hapten or use of an appropriate analogue. Figure 1.7 illustrates both possibilities.

![Figure 1.7](image-url) Schematic diagram of possible sites of conjugation of CB and the epitopes presented (dotted circled) by these types of conjugates
Conjugate A may be achieved by conversion of the aromatic amino group of CB to the diazonium salt, followed by substitution ortho- to the phenol of the tyrosine residues of the carrier protein.\(^{300}\) This conjugate presents a dichlorophenylethanolamine epitope to the immune system. Conversion of the benzylc hydroxyl to a ketone and then to the oxime derivative is the basis of Conjugate C, and this conjugate would tend to present the dichloroanilino ethylamine epitope. However, this approach effectively removes the structurally necessary benzylc hydroxyl group from the hapten (refer section 1.2.2). Therefore, conjugation through the benzylc hydroxyl group would appear to be undesirable.

Conjugate B represents the use of a functionalised CB analogue to present a third and different epitope. Since the aliphatic amine is also a key structural feature of CB, conjugation through this functional group is again undesirable. Therefore, analogues of CB functionalised on the \textit{N-tert}-butyl group (for example, by conversion of a methyl group to a carboxylic acid group) offer an alternative route to presentation of this epitope. The synthesis of analogues functionalised on the methyl carbons of the \textit{tert}-butylamino group has been investigated by other researchers at CSIRO DAP laboratories.\(^{301}\) Analogues presenting an unaltered benzylc hydroxyl group and a reactive bridging group attached to this hydroxyl-bearing carbon have also been considered; however preliminary investigations have suggested that functionalisation at this carbon may be synthetically difficult, reflected in poor yield of the desired product.\(^{302}\)

Anti-CB antibodies have been previously reported.\(^{300,303-308}\) These antibodies have been generated polyclonally, mainly in rabbits. The immunoconjugate used\(^{300}\) was formed via diazotisation of CB to give a diazo linkage between hapten and carrier protein, as illustrated by Conjugate A in Figure 1.7. However, under these reaction conditions, the aliphatic amino group could also undergo undesirable alterations through the formation of \textit{N}-nitroso compounds.\(^{309,310}\) Consideration of alternative conjugation strategies to avoid possible structural changes to the hapten led to the design of novel analogues of CB, suitably functionalised for conjugation to present an epitope similar to that of Conjugate A.

Conjugation chemistry less likely to alter important structural elements of CB could be the formation of an amide bond, through the \(\varepsilon\)-amino carrier protein lysine residues. Such conjugation chemistry is well known, and requires a hapten containing a reactive carboxylic group, as well as carbodiimide reagents for activation of this moiety prior to conjugation (for review see\(^{299,311,312}\)). Introduction of such a reactive bridging group to the CB molecule could be more readily achieved by substitution of an oxygen for the aromatic nitrogen, and formation of a relatively inert ether moiety containing the requisite functionality. \textit{N}-Alkylation of CB is complicated by the presence of two amino groups in the molecule; one aliphatic, one aromatic.
The simplest example of these haptenic \(O\)-alkyl analogues of CB is the “C2 Hapten” shown in Figure 1.8. Inclusion of a “spacer” to distance the hapten from the carrier protein has been previously reported to improve presentation of the desired epitope to the immune system and, potentially, generate antibodies more specific for the desired hapten (for review see\(^{313}\)). Therefore, a second hapten (C6 Hapten in Figure 1.8) was also targeted as a potentially useful haptenic \(O\)-alkyl analogue of CB, containing a “built in” spacer to further distance the CB epitope from the carrier protein. The simplest phenoxy CB analogue, VUF 8303 (refer section 1.4.1) and the simplest, non-haptenic \(O\)-alkyl CB analogue, \(O\)-Methyl VUF 8303, are model compounds that would also be of interest (Figure 1.8). The \(O\)-Methyl model compound would also be the simplest \(O\)-alkyl analogue which is most “conjugate-like”, in that it has an inert \(O\)-alkyl substitution.

![C2 Hapten and O-Methyl VUF 8303](image)

**Figure 1.8** Novel \(O\)-alkyl CB analogues suitable for protein conjugation (C2 Hapten and C6 Hapten) and model compounds (VUF 8303 and \(O\)-Methyl VUF 8303)

Figure 1.9 below compares the conjugates formed using these \(O\)-alkyl CB analogues (C2 Hapten or C6 Hapten) with conjugation of CB to a carrier protein by diazotisation. Both conjugates would conceivably present a similar CB-like epitope (dotted circle), although the bridge structure between the hapten and the carrier protein would differ.
1.4 O-Alkyl Clenbuterol Analogs

1.4.1 Synthesis

The CB synthesis\(^1\) involved a four-step reaction sequence using 4-aminoacetophenone as starting material. Ring chlorination, formation of the \(\alpha\)-bromoacetophenone (elemental bromine) and reaction with \textit{tert}-butylamine, followed by borohydride reduction of the ketone gave CB, as shown in Scheme 1.1.

A similar strategy for the synthesis of CIM (excluding ring chlorination, and amination with isopropylamine rather than \textit{tert}-butylamine) has also been reported.\(^9\) However, a milder brominating agent was used in that case (cupric bromide), presumably to avoid ring bromination. The O-alkyl haptenic analogues of CB suitable for protein conjugation (C2 Hapten and C6 Hapten) have not been previously reported. Thus, the CB synthetic scheme could provide the basis for development of a synthesis of these new compounds.
Scheme 1.1 Reagents: i, Cl₂, HOAc; ii, Br₂, CHCl₃, reflux; iii, H₂N(CH₃)₃, CHCl₃, reflux; iv, NaBH₄, EtOH

The simplest phenoxy analogue of CB, *N*-tert-butyl-2-(3,5-dichloro-4-hydroxyphenyl)-2-hydroxyethylamine, has been previously reported. This compound, VUF 8303 (Figure 1.10), was prepared by direct elemental chlorination of the non-chlorinated precursor, although yield was not reported. Synthesis of this non-chlorinated precursor, *N*-tert-butynorsynephrine (obtained commercially from Duphar; Figure 1.10) has been known for many years. Here, *N*-tert-butynorsynephrine was prepared by addition of tert-butylamine to the phenacyl bromide intermediate, and protection of the phenol as the benzoyloxy derivative. This reaction sequence was similar to that of CB. Subsequent hydrolysis and catalytic reduction (palladium on charcoal, Pd/C) of the ketone gave *N*-tert-butynorsynephrine.

Figure 1.10 VUF 8303 and *N*-tert-butynorsynephrine

A cognate approach to the formation of a phenylethanolamine structural moiety has been reported for a number of other compounds. One notable difference from the CB synthesis in some cases has been reaction of the requisite phenacyl bromide with secondary *N*-benzyl amines, to improve the yield. Reaction with the required primary amine was found to be unsatisfactory, due to complex reaction product mixtures. In these cases, the aromatic ring contained substituents with oxygen atoms linked directly to the aromatic ring; by comparison CB and CIM have aromatic *amino* substitutions. However, successful addition of primary amines to phenacyl bromides with ring hydroxy substitutions has been reported.
Removal of the N-benzyl group was regularly achieved catalytically using palladium on charcoal (Pd/C), a reagent also used in some cases for ketone reduction.\textsuperscript{317-319,322} The order of reaction of the debenzylolation and ketone reduction has also been reversed, with borohydride ketone reduction prior to catalytic debenzylation.\textsuperscript{318,320,323} Ketone reduction using borohydride has been used in the CB and CIM syntheses\textsuperscript{1,9}, as well as that of other phenylethanolamines.\textsuperscript{324,325} Cyanoborohydride could also be used for the reduction of the ketone.\textsuperscript{326}

Introduction of the reactive bridging group into the target hapten would be a critical reaction step, since this is the structural feature which differs to that of CB. The required functionality could be included through formation of an ether linkage (for review see\textsuperscript{327}). Reaction of a nucleophilic phenoxide ion with an alkyl halide under basic aqueous conditions has been reported.\textsuperscript{328-330} However, the dichloro substitution adjacent to the phenol greatly increased the steric hindrance of the hydroxyl group, with the suggestion that solvation of 2,6-dichlorinated phenoxide ions was sterically inhibited.\textsuperscript{331} Alkylation of 2,4-dichlorophenol with ethyl chloroacetate and sodium metal in butyl alcohol has been reported in 90% yield.\textsuperscript{332} Therefore, O-alkylation of ortho-dichloro-substituted phenol compounds may require more forcing conditions, such as the use of N,N-dimethylformamide (DMF) and K\textsubscript{2}CO\textsubscript{3} in a modified Williamson ether synthesis.\textsuperscript{333} Hence, this reaction may be more conveniently included early in the reaction sequence, since the use of this reaction in the presence of the amino functionality in the molecule would require extra protection/deprotection synthetic steps. Alternatively, the synthetic position of this O-alkylation could precede that of ring chlorination.

Chlorination would appear to be an important synthetic step. Elemental ring chlorination gave VUF 8303,\textsuperscript{314} although the yield was not reported. The corresponding step (reaction i, Scheme 1.1) in the CB synthesis\textsuperscript{1} was low yielding (\approx35%), and the major by-product (2,4,6-trichloroaniline) was the result of displacement of the substituent para- to the amino group by chlorine. Other chlorination reagents have included tert-butylhypochlorite,\textsuperscript{334} trichloroisocyanuric acid\textsuperscript{335} (TICA), and a polymeric reagent which contained a TICA-like moiety.\textsuperscript{336} The C2 Hapten is a phenoxyacetic acid derivative; chlorination of phenoxy acetic acid using elemental chlorine has been reported to give the 2,4-dichloro product initially, and the 2,4,6-trichloro product with an excess of chlorine under both acidic and basic conditions in yields of 50-75%, reactions which are apparently less violent than found for an analogous phenol.\textsuperscript{332} By comparison, inclusion of the ring chlorines in the starting material would be an attractive synthetic alternative, whereby a potentially low yielding chlorination step may be avoided. Indeed, a suitable starting material (3,5-dichloro-4-hydroxyacetophenone) is known,\textsuperscript{337} produced in two steps from 2,6-dichlorophenol, a commercially available starting material.
Bromination of acetophenone intermediates is a well known reaction, proceeding via the enol and autocatalysed by HBr. A variety of brominating reagents have been used, including cupric bromide, N-bromocaprolactam and N-bromosuccinimide. Elemental bromine has been used in the synthesis of molecules containing the phenylethanolamine moiety, and is probably the most straightforward synthetically. The bromination step could also be placed more conveniently after ring chlorination, to avoid bromination of the ring. The phenacyl bromide intermediates formed could then react with the requisite amine to complete the important structural backbone of the target molecules.

The phenylethanolamine structural backbone has also been synthesised via epoxide formation and ring opening with the requisite amine. A similar approach has also been used in the synthesis of β-adrenoceptor antagonists with a phenoxypropanolamine structure. Reductive alkylation of primary amine with the appropriate ketone as well as cyanohydrin intermediates in chiral syntheses have also been utilised for the formation of phenylethanolamine derivatives. However, these syntheses involved more complex chemistries than the CB synthesis; hence the CB synthesis represented a simpler alternative. Any potential commercial use of the haptenic CB analogues in the development of anabolic antibodies would require compounds that were relatively simple to synthesise.

The benzylic carbon of CB is chiral, and the synthesis yielded the racemate (contains both the R(-) and S(+) forms). The R(-) forms of the endogenous phenylethanolamines (ADR, NOR) are more readily recognised by the β2-AR (refer section 1.2.2). In the first instance, however, the O-alkyl analogue target compounds (refer Figure 1.8) would be synthesised as the racemate, similar to CB. A chiral synthesis or separation may not be justified until the utility of these target compounds for the generation of an anabolic anti-idiotypic antibody has been characterised. Indeed, studies of the physiological effects of CB discussed previously have used samples of the drug as the racemate.

1.4.2 Conjugation

Covalent attachment of the haptenic O-alkyl CB analogues could be achieved through reaction of the carboxylic acid group, specifically included in the structure of these molecules, with the ε-amino groups of the lysine residues found in the carrier protein to form an amide linkage. Reaction of carboxyl groups with primary amines is enhanced when the carboxyl group is activated. Activation of the carboxyl group using carbodiimides is a well known reaction (for reviews see), and a number of suitable reagents are available. Two of these reagents are given in Figure 1.11: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) and N,N′-dicyclohexylcarbodiimide (DCC).
Figure 1.11 Structures of the carbodiimide reagents ECDI and DCC

Carbodiimide activation of carboxyl groups has been thought to involve the formation of an O-acylisourea intermediate which then reacted in one of several possible ways (refer Scheme 1.2).\textsuperscript{299} Thus, the original hapten (RCO$_2$\textsuperscript{−}) may be regenerated by hydrolysis, the active intermediate may react with nucleophilic nitrogens such as the ε-amino of lysines or N-terminal amines (which results in hapten-protein conjugation), or the O-acylisourea may rearrange to give the more stable N-acylurea.

Scheme 1.2 Possible Reactions of the O-Acylisourea Intermediate formed by Reaction of Carboxyl groups with Carbodiimides

ECDI has been a useful carbodiimide for protein conjugations owing to its water solubility.\textsuperscript{355} The active intermediate has been generated in situ with carrier protein, at an optimum\textsuperscript{356} pH ≈4-5, and hence directly coupled the hapten to the protein. However, although this intermediate hydrolysed more slowly than it reacted with amine,\textsuperscript{357} it had only a relatively short half-life in aqueous solutions.\textsuperscript{355} The urea by-product formed from ECDI conjugation was also water soluble and hence easily separated from the conjugate by dialysis. When used in large excess, some protein carboxylic acid groups may also be activated, which could lead to some protein cross linking.\textsuperscript{299}

Carbodiimides have also been used indirectly for hapten conjugation. DCC is only soluble in organic solvents, hence use under the predominantly aqueous conditions required by carrier proteins is limited. However, DCC has been used to form an active ester derivative (e.g. N-hydroxysuccinimido; NHS) of the carboxyl-bearing hapten,\textsuperscript{358} which was then reacted with carrier protein.\textsuperscript{299,355} The activated ester derivative was more water soluble than the DCC-
derived O-acylisourea intermediate, and hydrolysed only slowly under aqueous conditions.\textsuperscript{359} This approach has found wide use, since the NHS ester was easily prepared and separated from the highly insoluble dicyclohexyl urea by-product; it was highly activated and reacted with protein amino groups under relatively mild conditions\textsuperscript{299} of pH \(\approx 7-9\). Indeed, the \(\varepsilon\)-amino group of lysine has been regarded as a reasonable nucleophile above pH 8 (\(pK_a = 9.18\));\textsuperscript{311} hence a DCC/NHS ester conjugation may favour a more efficient hapten coupling than other carbodiimides,\textsuperscript{359} since the free base form of the \(\varepsilon\)-amino group necessary for reaction would be more favoured at pH greater than 4-5. Preactivation of hapten may also minimize protein cross-linking which could occur with the ECDI conjugation reagent.

The choice of carrier protein is governed by several factors. Certain local requirements, administered by the Australian Quarantine and Inspection Service (AQIS) regarding imported biologically-derived substances, must be met. BSA (sourced in Australia only) meets these criteria, hence it would be more readily approved for any eventual commercial outcomes of this research. BSA is also more tolerant than other proteins of mixed solvent conditions,\textsuperscript{313} which would be required with the use of DCC-mediated formation of the NHS activated ester. BSA has approximately 59 lysine residues,\textsuperscript{360} a sufficient number to ensure reasonable hapten loading, although not all may be available for conjugation. The number of available amine groups on the carrier protein could be increased by modification of BSA with ethylenediamine.\textsuperscript{361} When carboxylic acid side chains were converted to aminoethyl amides, the modified protein (ED-BSA) contained approximately twice the number of exposed amino groups as BSA, and may have provided more potential sites for conjugation. This could also reduce the potential of the protein to cross link with itself through carbodiimide activation of carboxyl groups. ED-BSA would also be worthy of investigation as a carrier protein.
CHAPTER 2 - SYNTHESIS OF O-ALKYL CLENBUTEROL ANALOGUES

2.1 Introduction

2.1.1 Aim

The aim of this section of the research was to:

a) synthesise four O-alkyl clenbuterol analogues: haptns 5d and 5g, and model compounds VUF 8303 (5e) and O-Methyl VUF 8303 (5c)

b) characterise and confirm the structure of the target compounds and all new compounds synthesised

c) conjugate analogues 5d and 5g to BSA, to provide novel immunoconjugates for the production of anti-clenbuterol antibodies, and to gelatin, to provide microtitre plate coating conjugates for the detection of monoclonal anti-hapten antibodies (raised against the immunoconjugates) in an ELISA, using two different carbodiimide reagents.

2.1.2 Background

O-Alkyl analogues of clenbuterol (CB) suitable for protein conjugation were required for the production of anti-CB antibodies. These antibodies may be candidates for the generation of anti-idiotypic antibodies that mimic the energy-repartitioning effects of CB. The O-alkyl analogues could also be used in the detection and/or isolation of anti-CB antibodies. The structures of the O-alkyl analogue target compounds are given below (Figure 2.1):

![Figure 2.1 O-Alkyl analogue target compounds](image)

The reported synthesis\(^1\) of CB proceeds through four synthetic steps (chlorination, \(\alpha\)-bromination, amination, ketone reduction) from 4-aminoacetophenone (refer Scheme 1.1). A similar approach to the target compounds could be taken, with appropriate modifications to
introduce the required structural differences of the analogues.

Ring chlorination of 4-aminoacetophenone in the CB synthesis has been found to be low yielding (30%), and the inclusion of the ring chlorines in the starting material may be a possible alternative to a chlorination step. Introduction of the alkyl substituent onto the ring oxygen may be possible using a modified Williamson ether synthesis (DMF/K₂CO₃), although the dichloro substitution was an anticipated difficulty. The ether linkage is an important structural element of the target compounds; hence a high yielding and straightforward O-alkylation would be desirable. Addition of the amine moiety could be achieved via nucleophilic substitution of the α-bromo derivative of the requisite ring-oxygen-alkylated acetophenone with either tert-butylamine or N-benzyl-tert-butylamine. The benzyl groups may be removed catalytically (Pd/C) and the carbonyl adjacent to the ring reduced with borohydride (refer section 1.4.1).

Haptenic O-alkyl analogues 5d and 5g may be conjugated to a carrier protein through amide bond formation between lysine residues and the carboxylic acid, using carbodiimide-based reagents (EDCI, or DCC). BSA would be a suitable carrier protein for the preparation of conjugates to be used for immunisation (refer section 1.4.2). Detection of monoclonal anti-hapten antibodies (produced from the analogue-BSA immunoconjugates) by Enzyme-Linked Immunosorbent Assay (ELISA) requires microtitre plate coating conjugates formed using a carrier protein different to that of the immunising conjugate; hence only hapten-specific antibodies would be detected. Therefore, gelatin may be used as the carrier protein for this purpose, and the haptenic analogues conjugated using similar carbodiimide chemistries.
2.2 Discussion

2.2.1 Target Compound Synthetic Scheme

The desired target compounds were synthesised according to the general reaction scheme below (Scheme 2.1):

**Scheme 2.1** Reagents: i, Pyridine, Ac₂O, reflux; ii, AlCl₃, ≈165 °C; iii, DMF, K₂CO₃, NaI (0.1 mol eq.); iv, Br₂, CHCl₃; v, C₆H₅CH₂HNC(CH₃)₂, CHCl₃, reflux; vi, H₂, 5% Pd/C, EtOH-HOAc (9:1), NTP; vii, hydrolysis (if required), 0.1M HCl, H₂O; viii, NaBH₄, EtOH-H₂O; ix, NaBH₃CN, THF-H₂O, pH 5

The starting material (3,5-dichloro-4-hydroxyacetophenone; DCHA) was prepared from 2,6-dichlorophenol (DCP) in two steps, in overall yield of 71%, as previously reported.³³⁷ O-Acylation of DCP in a ten fold excess of acetic anhydride gave 2,6-dichlorophenyl acetate (DCPA) in ≈85% yield, and Fries re-arrangement of this ester using AlCl₃ afforded DCHA in good yield (≈83%). The proton Nuclear Magnetic Resonance (NMR) spectra of these compounds were consistent with their proposed structures, whilst the number of peaks in the carbon-13 NMR spectra was consistent with the number and types of carbon atoms of these compounds. The infra-red (IR) spectra of DCHA showed a broad hump (3300-3000 cm⁻¹) consistent with a phenol, broadened due to hydrogen bonding. The strong absorption at 1656.7 cm⁻¹ confirmed the presence of the carbonyl group, shifted to slightly lower wavenumbers due to conjugation.

The mass spectrum of DCHA showed a prominent molecular ion at m/z 204, consistent with
the theoretical $M^+$. The peak cluster ratio of this molecular ion (100:77:10) was consistent with dichloro substitution, since a dichlorinated molecular or fragment ion should give rise to a cluster of three peaks separated by two mass units ($M+M+2:M+4$) in the approximate ratio$^{362} 100:65.3:10.6$. The base peak at $m/z$ 189 was consistent with the loss of the methyl group $\alpha$-to the carbonyl to give the stable ($ArC=O)\+^+$ ion, characteristic of such aryl ketones. Thus, the mass spectral data was consistent with the structure and other spectral data.

Preparation of a starting material other than DCHA was also considered. Thus the $O$-alkylation of 4-hydroxyacetophenone (4-HA) to give 4-acetylphenoxyacetic acid (4-APA), followed by chlorination, was also investigated according to Scheme 2.2:

\[ \text{HO-CO-C}_6\text{H}_4\text{OH} \xrightarrow{x} \text{HO-CO-C}_6\text{H}_4\text{O-COOH} \xrightarrow{x_{\text{Cl}} \text{H}_2\text{O}} \text{Cl-CO-C}_6\text{H}_4\text{O-COOH} \]

**Scheme 2.2** *Reagents: x, ClCH$_2$CO$_2$H, NaOH, H$_2$O, 80 °C; xi, Cl$_2$, HOAc; xii, C$_3$Cl$_3$N$_3$O$_3$ (TICA), HOAc*

$O$-Alkylation of 4-HA under aqueous alkaline conditions$^{329}$ to 4-APA was slow and did not go to completion, despite a large excess of chloracetic acid and DMF as reaction solvent. Furthermore, chlorination of 4-APA using chlorine$^{1,332}$ gave only a mixture of products by Reverse Phase High Performance Liquid Chromatography (RP HPLC) analysis, and a brown oil which resisted recrystallisation from a variety of solvents. Use of a milder chlorinating agent$^{335,336}$ (trichloroisocyanuric acid, TICA) gave several products by RP HPLC analysis, which could not be separated chromatographically. It was possible that $\alpha$-chlorination of the acetyl group, as well as ring chlorination, may have occurred with both these chlorinating reagents.

### 2.2.2 Etherification

A modified Williamson ether synthesis was used to synthesise the $O$-alkylated intermediates, according to Scheme 2.3 below. The dipolar aprotic solvent DMF was used to enhance phenoxide ion nucleophilicity, as well as a large excess of $K_2CO_3$ to favour phenol deprotonation and neutralisation of the halo acid formed. Preliminary investigations of the reaction of chloracetic acid with DCHA, under basic aqueous conditions, were unsuccessful.
The $O$-alkylated products were formed in yields of 55-95%. Alkyl bromides appeared to react faster than alkyl chlorides, consistent with bromide being a better leaving group than chloride, and esters faster and more smoothly than carboxylic acids. Reaction with tert-butyl 2-chloroacetate gave yields inferior to other halooacetates, despite longer reaction times, and product was isolated as an oil which resisted recrystallisation from a variety of solvents. Increased heating times with benzyl 2-bromoacetate, methyl 2-bromoacetate, benzyl chloride and ethyl 6-bromohexanoate tended to reduce the product yield, and several by-products were observed by RP HPLC analysis. Therefore, the reaction was only gently warmed initially, and thereafter allowed to proceed at room temperature. Furthermore, DCHA was occasionally used in slight excess when removal of the alkyl halide was difficult. Differences in retention time (by RP HPLC analysis) reflected the differences in hydrophobicity of some intermediates: e.g. benzyl ester 1d was retained longer (10.0 min) than methyl ester 1b (7.4 min).

Proton NMR spectra of these $O$-alkylated intermediates showed acetyl and central dichlorinated aromatic ring protons in good agreement between these compounds (ranges of $\delta$ 2.54-2.57 ppm and 7.85-7.90 ppm respectively). Protons $\alpha$- to the ether oxygen, however, showed some differences according to their unique chemical environments, and were observed between $\delta$ 3.94 and 5.12 ppm. Aromatic benzyl protons for benzyl ester 1d occurred as a complex multiplet ($\delta$ 7.32 ppm) which could not be resolved, whereas those of benzyl ether 1e were observed as a doublet and a multiplet ($\delta$ 7.55 and 7.40 ppm). The nature of the doublet at 7.55 was unclear; integration to two protons and a slight downfield shift suggested it corresponded to the ortho- ring protons. However, coupling between these aromatic protons was not resolvable. The ten methylene protons of hexanoate 1f were observed as two triplets and three complex multiplets; the latter could not be fully assigned. The ethyl protons of this ester were observed as the expected triplet/quartet. The spectrum of 1c was in agreement with a previous report.\textsuperscript{363}
In the carbon-13 NMR spectra, acetyl group carbons of these acetophenone intermediates (δ 26.31-26.40 ppm and 194.53-194.74 ppm) agreed well with each other. Definite assignment of the central dichlorinated aromatic ring carbons was assisted by the simplified spectra of compounds 1b and 1c, as well as DEPT (Distortionless Enhancement by Polarisation Transfer) analysis, although a definite assignment of the benzylic aromatic carbons of 1d and 1e was not possible from the present data. However, the number of signals and their spectral positions were consistent with the proposed structures.

The spectral position of alkyl carbons attached to the phenoxy oxygen varied according to their differing chemical environments: for example, the benzylic carbon of 1e (75.24 ppm) was significantly downfield of the analogous methoxy carbon of 1c (60.89). In the spectrum of 1d, the signal at 69.17 was assigned to the methylene carbon α- to the ester carbonyl group, on the basis of similar chemical shift to the analogous carbon in 1b, and the benzylic methylene to the signal at 67.16; hence this was a tentative assignment only. A definite assignment of the central methylenes of hexanoate 1f (δ 24.61, 25.30 and 29.67 ppm) was not possible from the present data, although the number of signals and their spectral positions were consistent with the proposed structure.

Strong absorptions in the IR spectra around 1700 cm⁻¹ confirmed the presence of the carbonyl group. Ethers 1c and 1e showed only one band in this region, whereas esters 1b, 1d and 1f showed two bands, the absorption at longer wave numbers consistent with the unconjugated ester carbonyl. Strong absorptions at approximately 1200 cm⁻¹ and 1000 cm⁻¹ confirmed the presence of the ester group in these compounds, and strong absorptions in the region of 1270 cm⁻¹ to 1280 cm⁻¹ were consistent with the alkyl-aryl ether linkage of all compounds. The broad hump at approximately 3300 cm⁻¹ seen in the spectrum of DCHA was no longer observed, consistent with conversion of the phenol to the ether.

Mass spectra of these compounds showed a prominent molecular ion peak, consistent with the expected mass, except for 1f which showed only a (M + H) peak. The M⁺ peak cluster was consistent with dichloro substitution. Methyl ether 1c showed a base peak at m/z 203 consistent with loss of the O-methyl group; none of the other compounds showed an analogous peak. Benzyl ether 1e also appeared to fragment at the ether linkage, but with charge residing on the benzyl fragment which gave rise to the base peak at m/z 91. A similar observation was made in the spectrum of benzyl ester 1d. Ethyl ester 1f also appeared to fragment at the ester linkage to give the peak at m/z 301 (loss of EtO); the peak cluster ratio confirmed dichloro substitution. A prominent peak in the spectrum of 1f at m/z 143 (no cluster of peaks) was possibly due to fragmentation at the ether linkage and charge remaining on the hexanoate fragment.
Overall, alkylation of DCHA at the phenolic oxygen was conveniently achieved in good yield, and gave the required alkoxy intermediates (1; refer Scheme 2.3), four of which were new compounds (1b, 1d, 1e and 1f). The reaction conditions were relatively mild; by comparison, O-alkylation of the less hindered 2,4-dichlorophenol with ethyl 2-chloroacetate has been previously reported to require the use of sodium metal in butyl alcohol. It was also found to be synthetically advantageous to include ring chlorines in the starting material rather than chlorinate as a synthetic step. NMR (proton and carbon), IR and mass spectral data for the alkylation products were consistent with proposed structures.

2.2.3 α-Bromination

The phenacyl bromide intermediates were prepared using elemental bromine, similar to the bromination of 3,5-dichloro-4-aminoacetophenone in the synthesis of clenbuterol. The reaction is autocatalysed by HBr generated in situ.

\[
\begin{align*}
\text{R} & = \text{MeO}_2\text{CCH}_2 & 2\,\text{b} \\
& = \text{Me} & 2\,\text{c} \\
& = \text{PhCH}_2\text{O}_2\text{CCH}_2 & 2\,\text{d} \\
& = \text{PhCH}_2 & 2\,\text{e} \\
& = \text{EtO}_2\text{C(CH}_2)_5 & 2\,\text{f}
\end{align*}
\]

Scheme 2.4 Reagents: iv, Br$_2$, CHCl$_3$

Bromine was added as a solution in CHCl$_3$ in four equal portions. However several by-products were observed by RP HPLC analysis prior to complete bromine addition. Extra stoichiometric equivalents of bromine tended to produce more by-product, except in the preparation of 2c. These by-products were possibly due to multiple bromination, since α,α-dibromination of p-benzylxoyacetophenone has been previously reported under similar conditions. Alternative bromination conditions using cupric bromide returned only starting material. It was also considered that HBr may be altering other functionalities (such as the ester) or facilitating multiple brominations too rapidly; removal of HBr (brine wash) after the first two bromine additions tended to improve the yield. By contrast, bromination of methyl ether 1c was more tolerant of extrastoichiometric bromine and mid-reaction removal of HBr did not appreciably improve the yield. This suggested that problems with reaction by-product could stem from the ester functionality present in 1b but absent in 1c. However, by-products
with longer retention times than the major products were observed in both reactions which was perhaps consistent with multiple α-bromination.

The phenacyl bromides (2; Scheme 2.4) were prepared in yields of 75-85%, with ≈6-12% of by-products (by RP HPLC analysis). These products were used with little further purification; four recrystallisations from n-hexane removed only about half of the by-product observed in the case of 2b. This was consistent with previous reports\textsuperscript{316,319} where isolation of pure α-bromo compound was afforded with considerable difficulty, often in very poor yield for para-alkoxyacetophenones.\textsuperscript{316} These workers used the compound without rigorous purification, but also preferred the more convenient synthesis of the α,α-dibromo compound instead. Increased retention times (by RP HPLC) of the products were consistent with bromine addition resulting in slightly increased hydrophobicity.

Proton and carbon-13 NMR confirmed α-bromination. In the proton spectrum of 2b, the disappearance of the acetyl methyl group at ≈2.54 ppm (three protons), and a new peak further downfield at ≈4.36 ppm (two protons), was consistent with the two remaining acetyl protons being adjacent to the more electronegative bromine. The number of signals in the carbon-13 spectrum of 2b was consistent with the number and type expected by the proposed structure, as was the additional methylene carbon signal at ≈29.8 ppm and the concomitant absence of a methyl carbon at ≈26.3 ppm. The mass spectrum of 2b showed a molecular ion peak at m/z 354, consistent with the expected mass, while the peak cluster ratio (100:164:75:11) was consistent with a monobromo, dichloro substitution. The base peak at m/z 261 appeared consistent with the loss of CH\textsubscript{2}Br; the peak cluster ratio of this fragment was consistent with the loss of the bromine and retention of the dichloro substitution. The IR spectrum of 2b showed two strong absorptions between ≈1690-1750 cm\textsuperscript{-1}, indicative of the ester and acetophenone carbonyl groups.

2.2.4 Amination

In the synthesis of CB, the tert-butylamino moiety was introduced α- to the carbonyl group by reaction with 3,5-dichloro-4-aminophenacyl bromide (refer Scheme 1.1). HBr liberated from the reaction formed the hydrobromide salt of the excess amine which precipitated from solution. Similar approaches to analogous ketoamines containing oxygenated ring substitutions have been reported.\textsuperscript{315,322,325}

Preliminary investigations of the reaction of methyl ester phenacyl bromide 2b and methyl ether 2c with tert-butylamine gave complex reaction mixtures; numerous products were observed by RP HPLC analysis. Various alterations of reaction conditions (change of solvent, larger and smaller excesses of amine, addition of the phenacyl bromide to the amine) did not improve this
result. No aqueous acid-extractable product was isolated, nor could the brown oil isolated from the organic phase be recrystallised from a variety of solvents. None of the desired product was observed in the white reaction mixture precipitate. These observations were consistent with previous reports on analogous molecules, with the suggestion that the aminoketone product is somewhat unstable, and may undergo self-condensation to give 3-amino pyrroles. Indeed, the unwanted side-reactions may have involved the carbonyl group adjacent to the ring, since both α-bromo methyl ether 2c and α-bromo methyl ester 2b gave similar results. These results tended to suggest that synthetic use of the primary amine for the target compound synthesis was not feasible.

Reaction of para-alkoxy-meta-sulfonamidophenacyl bromides with tert-butylamine has been observed to have similar problems, which were overcome by reaction with N-benzyl-tert-butyl amine to yield one major product. Indeed, for para-alkoxyphenacyl bromides, reaction with N-benzyl amines has been reported as the preferred synthetic route due to poor yield with primary amines, although yields were slightly better than in the case of para-hydroxyphenacyl bromides. Similar uses of the N-benzyl amine in the syntheses of phenylethanolamines have also been previously reported. Therefore, the tert-butylamino moiety was introduced by reaction of the phenacyl bromides with the more sterically hindered secondary amine, N-benzyl-tert-butylamine according to Scheme 2.5 below:

\[ R = \text{MeO}_2\text{CCH}_2 \quad 3\text{b} \]
\[ = \text{Me} \quad 3\text{c} \]
\[ = \text{PhCH}_2\text{O}_2\text{CCH}_2 \quad 3\text{d} \]
\[ = \text{PhCH}_2 \quad 3\text{e} \]
\[ = \text{EtO}_2\text{C(CH}_2\text{)}_5 \quad 3\text{f} \]

**Scheme 2.5 Reagents:** v, C₆H₅CH₂HNC(CH₃)₃, CHCl₃, reflux

The N-benzyl tertiary amino intermediates (3; Scheme 2.5) were prepared using excess amine, in yields between 30-70%. Prolonged heating was found to reduce the product yield. The reaction mixture precipitate collected (90-95% of theoretical amount) was identified as the excess amine by RP HPLC and UV spectroscopy, and contained none of the tertiary aminoketone product. Products were isolated from aqueous acid as their insoluble HCl salts, whereas excess amine remained in solution; thus a convenient separation of product from
excess amine was afforded.

Methyl ester 3b, however, was soluble in aqueous acid. Therefore, a number of separation methods were investigated, including acetylation of excess secondary amine, acid hydrolysis of ester and isolation via an alkaline work up or an ion exchange resin, as well as preparative liquid chromatography and preparative RP HPLC of the ester/amine mixture. None of these approaches were successful, and 3b could not be separated from excess amine. These experiments also tended to suggest some sensitivity of the β-ketoamine product to base, since additional products were observed by RP HPLC analysis after treatment with aqueous base. Conversion of the phenacyl bromide 2b to the ketal with ethylene glycol and subsequent reaction with secondary amine was also unsuccessful, as was conversion of 1b to the ketal, followed by bromination and reaction with amine. Therefore, preparation of target compound 5d was via a benzyl acetate rather than the methyl acetate because the benzyl ester intermediate 3d was more hydrophobic than methyl ester 3b (reflected in a longer retention time by RP HPLC analysis). This facilitated separation of product (insoluble in aqueous acid) from excess N-benzyl-tert-butylamine (soluble in aqueous acid).

Proton NMR spectra of these compounds showed chemical shifts of analogous protons that were in good agreement with each other: tert-butyl protons (δ 1.56-1.57 ppm), dichlorinated aromatic ring protons (δ 7.80-7.82 ppm) and the amino protons (δ 9.13-9.14 ppm). The broad amino proton signals indicated slowly exchanging basic protons, consistent with the compounds as HCl salts, and were exchangeable with D₂O. The N-benzyl aromatic protons were consistently observed as two multiplets in the ratio 3:2 (δ 7.13-7.18 and 7.60-7.62 ppm).

Non-equivalence of the methylene protons either side of the protonated nitrogen was found by the characteristic AB coupling pattern in the proton NMR spectrum of all N-benzyl intermediates, other than 3d, and is illustrated in the Newman projections in Figure 2.2.

![Figure 2.2 Newman projections of the N-benzyl amine intermediates](image)

Each methylene appeared as a doublet of doublets and a broad doublet, however no splitting of the broad amino proton signal was discernible. This pattern collapsed when the amino proton was exchanged with D₂O, consistent with vicinal coupling of the amino proton with the
methylene protons. However, the pattern was not observed when the spectra were run in d$_4$-MeOH. A definite assignment of the observed signals was not possible from the proton and carbon-13 NMR spectra individually. Therefore, two dimensional, C-H correlation (short and long range) NMR experiments were performed, using methyl ether 3c as an exemplar. The assignment of the methylene protons made on the basis of these results is summarised below in Table 2.1.

**Table 2.1** Proton NMR data of methylene protons adjacent to the nitrogen in the N-benzyl amination products (3) (chemical shifts (δ) given in ppm, coupling constants (J) given in Hz, and all samples run in d$_6$-DMSO as their HCl salts)

<table>
<thead>
<tr>
<th>Proton</th>
<th>3c</th>
<th>3d</th>
<th>3e</th>
<th>3f</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCH$_2$Ph</td>
<td>4.58 (1 H, d, J)</td>
<td>4.58 (1 H, br d)</td>
<td>4.58 (1 H, d, J)</td>
<td>4.58 (1 H, d, J)</td>
</tr>
<tr>
<td>12.3 and and</td>
<td>11.5 and</td>
<td>10.8 and</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C=O)CH$_2$N</td>
<td>4.93 (1 H, d, J)</td>
<td>4.94 (1 H, br d)</td>
<td>4.90 (1 H, d, J)</td>
<td>4.19 (1 H, d, J)</td>
</tr>
<tr>
<td>18.4 and and</td>
<td>18.3 and</td>
<td>18.4 and</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.18 (1 H, dd, 5.16 (1 H, br q)</td>
<td>5.15 (1 H, m, 5.17 (1 H, dd,</td>
<td>J 18.5 &amp; 7.3)</td>
<td>J 18.2 &amp; 7.2)</td>
<td>J 18.6 &amp; 7.1)</td>
</tr>
</tbody>
</table>

Interestingly, the methylene proton signal which showed coupling to both the remaining geminal proton and the nitrogen proton (doublet of doublets) appeared further upfield in the case of the N-benzyl methylene protons, but further downfield in the case of the methylene protons adjacent to the ketone. The reason for this was unclear; indeed it was not possible to make a more detailed assignment of the methylene protons from the present data. By contrast, predictions of vicinal coupling constants from the Karplus formulation, Bothner-By and Altona modifications$^{366-368}$ gave J values of between ≈2-12 Hz, compared with the experimentally observed values of ≈7-18 Hz. Further work would be required to investigate these observations and complete a more definite assignment of the spectra; such work was beyond the scope of the present research. Nonetheless, the present data was consistent with the proposed structures.

The two-dimensional NMR correlation experiments also allowed a more definite assignment of the N-benzyl aromatic protons, as well as the peaks in the carbon-13 NMR spectrum of 3c. The assignment of methyl ether 3c, based on these NMR correlation experiments, is illustrated below in Figure 2.3. By analogy, other N-benzyl intermediates were similarly assigned. Analogous carbons of the N-benzyl intermediates, such as methyl carbons of the tert-butyl
group (δ 25.16-25.47 ppm), the central tert-butyl carbon (δ 68.63-68.90 ppm), methylene carbons adjacent to the nitrogen (δ 55.55-55.86 and 56.19-56.45 ppm) and the carbonyl carbons (δ 190.12-190.43 ppm), were in excellent agreement between these intermediates. Two carbons in the spectrum of 3c were coincident in d₄-MeOH, but separable in d₆-DMSO. Overall, the number of different types of carbons was consistent with the number of observed peaks in the spectra.

Figure 2.3 Assignment of 3c (proton NMR chemical shifts (bold) are indicated by arrows, carbon-13 NMR chemical shifts are given adjacent to the corresponding carbon)

IR spectra of these N-benzyl amines showed strong absorptions in the region ≈1680-1695 cm⁻¹ which confirmed the presence of the conjugated ketone, whilst esters 3d and 3f each showed an additional strong band in this region at slightly longer wavenumbers (1735-1760 cm⁻¹), indicative of the ester carbonyl. Strong absorptions at ≈1200 cm⁻¹ and ≈1000 cm⁻¹ in the spectra of 3d and 3f were consistent with the presence of the ester group. Weak absorptions at ≈2600 cm⁻¹ were consistent with these tertiary amines as HCl salts, whilst strong absorptions at ≈1270 cm⁻¹ and ≈1190 cm⁻¹ were again consistent with the alkyl-aryl ether linkage. The confirmatory absorption for ester 3f at ≈1230 cm⁻¹ was not observed, and was possibly not resolvable from neighbouring bands, similar to acetophenone precursor 1f.

Mass spectra of the N-benzyl intermediates consistently showed a base peak at m/z 91, probably due to the loss of the benzyl group as the tropylum ion (C₇H₇)⁺, although there was some evidence that charge was retained on the larger fragment from the observation of an (M - 91) peak in all spectra. With respect to the molecular ion (free base) the prominent peak observed was the (M + H) peak may have corresponded to the protonated amine; the peak cluster was consistent with dichloro substitution. The highly branched tert-butyl group would be expected to fragment by loss of a methyl group, ³⁶² and this was observed in all spectra with peaks at (M - 15), and peak clusters characteristic of dichloro substitution. Ethyl ester 3f
appeared to lose EtO (M - 45, m/z 462), similar to precursor 1f. All spectra showed a large peak at m/z 176, possibly due to cleavage of the C-C bond between the ketone and the adjacent methylene, with charge retained on the nitrogen containing fragment common to all compounds in this group (176 mass units).

All N-benzylamino products were purified by stirring at room temperature in aqueous acid, and washed with organic solvent to give amorphous white solids. The N-benzylamino phenoxyhexanoate 3f was quite waxy and proved difficult to isolate by filtration. Similar observations of analogous keto N-benzyl tertiary amines isolated as amorphous or glassy products with poor recrystallisation properties have been reported. The spectral data was consistent with the proposed structures of these N-benzyl intermediates.

2.2.5 Debenzylation

The use of hydrogenation catalysts (palladium on charcoal; Pd/C) for the removal of benzyl groups has been previously reported in the synthesis of phenylethanolamines, although catalyst can be “poisoned” by basic compounds like amines. Therefore, N-benzylamino intermediates were reacted as HCl salts, in 9:1 EtOH-HOAc as reaction solvent. Furthermore, in the presence of hydrogenation catalysts hydrogenolysis can occur; the benzylic carbonyl group may be reduced completely to the methylene group, via the benzylic alcohol, given sufficiently long reaction time and hydrogen. Indeed, preliminary reactions of p-hydroxyacetophenone with 5% Pd/C showed two products by RP HPLC whilst starting material remained. Given these reactivity considerations, the milder 5% Pd/C catalyst was used rather than the reported 10% palladium on charcoal. Hence, the N-tert-butyl aminoketones were prepared as in Scheme 2.6 below.

\[ \text{Scheme 2.6 Reagents: } \text{vi, H}_2, 5\% \text{ Pd/C, EtOH-HOAc (9:1), Normal Temperature/Pressure (NTP)} \]
Products were formed in quantitative yields in reaction times between 5-55 min. Proton NMR and IR spectral data suggested no catalytic reduction of the ketone; proton NMR data also confirmed no ring dehalogenation, which has been previously reported in the presence of the \( N \)-benzyl linkage and 5% platinum on charcoal.\(^{369}\) The products also showed a similar absorbance to starting material (equimolar amounts) at the detecting wavelength (260 nm). This also suggested that the conjugated ketone was untouched and debenzylolation was more rapid than ketone reduction. Intermediates with both \( O \)-and \( N \)-benzyl groups (3d and 3e) showed two intermediate products (by RP HPLC analysis). Both benzyl groups were smoothly removed, one more easily than the other, although it was not possible from the RP HPLC analysis to determine which this was.

Aqueous base work up was problematic, and isolation of an oil from an alkaline, aqueous phase by solvent extraction showed a complex mixture and very little of originally observed product (by RP HPLC analysis). One solid isolated from this organic extract showed comparable absorbance at the detecting wavelength to both the starting material and the single product, and was evaluated by proton NMR. Only two absorptions were observed: 7.9 ppm (s) and 3.9 ppm (s) in the ratio of 1:3, but the structure of the compound was unclear. The aqueous base sensitivity was observed previously with other \( \beta \)-ketoamines during investigations of phenacyl bromide amination. Isolation of 4d using a cation exchange resin was found to be unacceptable due to poor recoveries. Hence 4d (soluble in aqueous acid) was isolated as the HCl salt by saturation of an aqueous acid solution of 4d with NaCl. Phenol 4e and methyl ether 4c were isolated as their HCl salts by recrystallisation from aqueous acid.

Proton NMR data were in agreement for analogous protons: \textit{tert}-butyl protons (\( \delta \) 1.36-1.37 ppm), aromatic protons (\( \delta \) 8.12-8.23 ppm), methylene group adjacent to the ketone (\( \delta \) 4.63-4.71) and amino protons (\( \delta \) 9.16-9.29 ppm, exchangeable in \( D_2O \)). The \textit{tert}-butyl protons were shifted \( \approx 0.2 \) ppm upfield, consistent with the absence of deshielding effects of the \( N \)-benzyl group. Debenzylolation was confirmed as complete by the absence of any complex multiplets in the aromatic proton region (7-8 ppm). The carboxylic acid protons of 4d and 4g were observed as broad signals, exchangeable in \( D_2O \). By contrast, the phenolic proton of 4e was not readily observed. \( O \)-Debenzylation of 4e was, however, confirmed by the absence of the complex multiplets in the aromatic region of the spectrum. The dichloro substitution of the phenol may have resulted in very slow phenolic hydrogen exchange and therefore a very broad peak, which was not observable.

The \( \alpha \)-keto methylene protons were greatly simplified, in comparison to their tertiary amino precursors. A broad singlet was observed which indicated these protons were equivalent after N-debenzylation. In the case of compounds 4c, 4e and 4f this signal appeared as a broad triplet (\( J \) 5.6-6.3 Hz), and suggested coupling with the adjacent amino protons. Indeed, the
amino proton signal in the spectrum of 4e was resolved as a broad triplet ($J$ 5.5). However, splitting of the amino protons was not observed in the spectra of any other secondary amine ketone intermediates. The previously broad triplet of the $\alpha$-keto methylene protons became a singlet following $D_2O$ exchange, consistent with vicinal coupling between the methylene and amino protons.

Excellent agreement between analogous carbons was observed amongst the debenzylated intermediates in the carbon-13 NMR spectra: tert-buty1 methyl carbons ($\delta$ 25.89-26.20 ppm), the central tert-buty1 carbon ($\delta$ 58.51-58.81 ppm), methylene carbons adjacent to the nitrogen ($\delta$ 48.53-48.94) and the carbonyl carbons ($\delta$ 190.31-190.96 ppm). The $\alpha$-keto methylene carbons were shifted upfield ($\approx$10 ppm) in comparison to the $N$-benzyl precursors, as were the central tert-buty1 carbons, possibly due to removal of the deshielding effects of the $N$-benzyl group. Assignment of the ring carbon attached to the ketone and the chlorine-bearing aromatic carbon were tentative, since the present data did not provide further evidence for a more definite assignment. However, the number of signals observed in each of the spectra was consistent with the number of different types of carbons present.

The aromatic ring carbons of phenol 4e demonstrated some differing effects, in comparison to the O-alkylated compounds 4c, 4d and 4f, due to steric inhibition of resonance.\cite{363,370,371} Thus, in the spectrum of 4e, the carbons ortho- and para- to the phenoxy ring carbon ($\delta$ 124.11 and 127.87 ppm) were observed further upfield than the corresponding carbons in the O-alkylated compounds (e.g. in 4e, $\delta$ 131.58 and 132.49 ppm). Steric interactions between the two ortho- chlorines and the O-alkyl substituent are thought to reduce the extent of electron delocalization due to resonance, in comparison to 4e. Hence the ortho- and para- carbons of the O-alkyl compounds are more deshielded than those of 4e, and occur further downfield of the analogous carbons in 4e. By comparison, the hydrogen-bearing aromatic carbons did not experience this effect, and were in excellent agreement ($\delta$ 130.45-130.76 ppm) across all four compounds.

The IR spectra showed two medium intensity bands in the regions $\approx$2600 cm$^{-1}$ and $\approx$2400 cm$^{-1}$, consistent with the secondary amine products present as their HCl salts. The strong absorption due to the carbonyl group of the $\beta$-ketoamine was also shifted to slightly higher wavenumbers in all compounds. By contrast, the carbonyls of carboxylic acids 4d and 4g, confirmed by the presence of a strong band in the region 1720-1740 cm$^{-1}$, were observed at slightly lower wavenumbers than their ester precursors, 3d and 4f respectively.

The prominent molecular ion peak in the mass spectra of these debenzylated intermediates was the (M + H) peak, although observed only weakly in the spectra of 4c, 4d and 4e. The M$^+$
peak was not observed, but the (M + H) peak cluster was consistent with dichloro substitution. All spectra showed a prominent (M - 15) peak, consistent with loss of a methyl group from the highly branched tert-butyl group. The base peak (m/z 86) probably resulted from cleavage adjacent to the aryl ketone and retention of charge on the common nitrogen-containing fragment (86 mass units). Overall, NMR (proton and carbon), IR and mass spectral data was consistent with the proposed structure.

2.2.6 Hydrolysis/Reduction

The target compounds were prepared from their respective ketone precursors by a borohydride reduction with either NaBH₄ or NaBH₃CN as in Scheme 2.7 below, other than hexanoate ester 4f, which was hydrolysed with aqueous acid prior to ketone reduction.

![Chemical structures](image)

\[ R^1 = \begin{align*}
&\text{Me} \quad 5c \\
&\text{HO}_2\text{CCH}_2 \quad 5d \\
&\text{H} \quad 5e \\
&\text{HO}_2\text{C(\text{CH}_2)_5} \quad 5g
\end{align*} \]

**Scheme 2.7** Reagents: vii, hydrolysis, 0.1M HCl, H₂O, reflux; viii, NaBH₄, EtOH-H₂O; ix, NaBH₃CN, THF-H₂O, pH 5

Hexanoate ester 4f was hydrolysed under both acidic and basic aqueous conditions. Preliminary reactions indicated that final products obtained from either route were identical from proton NMR data. However, previous experiments had found ketoamine intermediates were base sensitive and gave rise to complex mixtures after exposure to aqueous base. Therefore, acid hydrolysis was preferred. Hydrolysis prior to reduction was more convenient; monitoring of the hydrolysis (by RP HPLC) with a conjugated ketone present was more sensitive due to significantly greater absorbance than the corresponding alcohol at the detecting wavelength of 260 nm. Disappearance of the ethyl ester triplet/quartet in the proton NMR spectrum of 4g was consistent with complete hydrolysis.

Reductions of 4c and 4e with borohydride gave target compounds 5c and 5e in good yield (83% and 96% respectively) following recrystallisation from aqueous acid. The amino acids 4d and 4g were reduced with cyanoborohydride to give haptens 5d and 5g in yields of 33% and 48% respectively; yields with cyanoborohydride, despite longer reaction times, were better
than with borohydride. Carboxylic acid 5g was also insoluble in aqueous acid and was thus conveniently isolated as the HCl salt by recrystallisation. However, carboxylic acid 5d was soluble in aqueous acid, and could not be isolated from the crude reaction mixture as the neutral amino acid at pH 7. Therefore, this compound was isolated using a cation exchange resin, although recoveries were not quantitative. Further purification of the product also proved difficult, but recrystallisation from water gave 5d as the neutral amino acid in 33% yield.

Reduction of the ketone to the alcohol yielded the expected ABX pattern (three sets of doublets of doublets) for the three non-equivalent protons ($H_A$, $H_B$ and $H_X$) in the proton NMR spectra of the target compounds. There was excellent agreement in both chemical shift and coupling constants; these results are summarised below in Table 2.2. Predictions of the vicinal coupling constants using the Karplus formulation, and the Bothner-By and Altona modifications\textsuperscript{366-368} gave $J$ values of between $\approx$3-10 Hz, which were in reasonable agreement with the experimental values of between $\approx$3-12 Hz.

### Table 2.2 Summary of proton NMR data for the ABX protons of target compounds (5) (chemical shifts ($\delta$) given in ppm, coupling constants ($J$) given in Hz, all samples run in $d_6$-DMSO as their HCl salts)

<table>
<thead>
<tr>
<th>Proton</th>
<th>5c</th>
<th>5d</th>
<th>5e</th>
<th>5g</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{CH}_3(\text{OH})\text{CH}_A\text{H}_B\text{N}$</td>
<td>3.10 (1 H, dd, $J$ 12.5 &amp; 3.1)</td>
<td>3.12 (1 H, dd, $J$ 12.5 &amp; 3.0)</td>
<td>3.05 (1 H, dd, $J$ 12.5 &amp; 3.2)</td>
<td>3.12 (1 H, dd, $J$ 12.5 &amp; 2.9)</td>
</tr>
<tr>
<td>$\text{CH}_3(\text{OH})\text{CH}_A\text{H}_B\text{N}$</td>
<td>2.95 (1 H, dd, $J$ 12.5 &amp; 9.9)</td>
<td>2.96 (1 H, dd, $J$ 12.5 &amp; 9.9)</td>
<td>2.91 (1 H, dd, $J$ 12.5 &amp; 9.8)</td>
<td>2.96 (1 H, dd, $J$ 12.4 &amp; 10.1)</td>
</tr>
<tr>
<td>$\text{CH}_3(\text{OH})\text{CH}_A\text{H}_B\text{N}$</td>
<td>5.01 (1 H, dd, $J$ 2.9 &amp; 9.8)</td>
<td>4.99 (1 H, dd, $J$ 2.9 &amp; 9.8)</td>
<td>4.93 (1 H, dd, $J$ 3.0 &amp; 9.7)</td>
<td>4.95 (1 H, dd, $J$ 2.7 &amp; 9.8)</td>
</tr>
</tbody>
</table>

The introduction of a hydroxyl-bearing chiral centre also induced non-equivalence of the protons at the protonated nitrogen centre (compounds present as HCl salts), illustrated by the Newman projection in Figure 2.4 below (looking toward the aromatic ring down the N-C bond).

![Figure 2.4 Newman projection of target compounds](image-url)
Therefore, the amino protons were observed as two broad peaks (slow exchange) in the range δ 8.49-9.45 ppm, and were exchangeable with D₂O. Coupling of the amino protons to the adjacent methylene protons was not observed, although the Hₐ and Hₐ signals were considerably sharper in both the D₂O-exchange and d₄-MeOH spectra. Indeed, coupling constants for compounds 5d and 5g were only obtained from their D₂O-exchange spectrum (in d₆-DMSO). These peaks were discernible only as broad doublets or triplets prior to D₂O addition, and amino proton coupling apparently served only to broaden the signals of the adjacent methylene protons. Coupling constants obtained from the d₄-MeOH spectra were in excellent agreement with those from d₆-DMSO/D₂O-exchange spectra.

Carboxylic acid protons of 5d and 5g were observed as very broad signals at δ 13.2 and 12.1 ppm respectively, exchangeable in D₂O. However, the phenolic proton of 5e was not observed, similar to precursor 4e. Signals corresponding to the tert-butyl protons were in excellent agreement across all four target compounds (δ 1.30-1.31 ppm), as were the aromatic protons (δ 7.55-7.56 ppm) and the alcoholic protons (δ 6.38-6.41 ppm), exchangeable in D₂O. However, in the spectrum of 5e, the aromatic and alcoholic protons were slightly upfield of the analogous protons in the other target compounds (δ 7.41 and 6.28 ppm respectively); the reason for this difference was unclear from the present data.

The most significant change in the carbon-13 NMR spectra of these compounds was the disappearance of the carbonyl carbon signal at δ ≈190 ppm, and the appearance of a new hydrogen-bearing carbon (by DEPT) peak in the range δ 69.44-70.46 ppm, consistent with carbonyl reduction. The number of signals in the spectra of all four target compounds was consistent with the number of different types of carbons in the proposed structures, and analogous carbons were in excellent agreement: tert-butyl methyl carbons (δ 26.08-26.12 ppm), tert-butyl central carbons (δ 58.65-58.72 ppm), α-hydroxy methylene carbons (δ 49.44-49.69 ppm), hydroxyl-bearing carbons (δ 69.44-69.58 ppm) and hydrogen-bearing aromatic carbons (δ 127.56-128.28 ppm).

The ortho- and para- carbons of phenol 5e were observed slightly upfield of similar carbons in the O-alkyl target compounds, similar to ketone precursor 4e, due to steric inhibition of resonance of the O-alkylated compounds. The three central methylene carbons of hexanoate 5g gave only two methylene carbon signals, which suggested that the chemical shifts of two of the three central methylene carbons were coincident; proton NMR data from this compound indicated the presence of six central methylene protons.

Reduction of the carbonyl group was also confirmed in the IR spectra by the disappearance of the strong absorption in the region ≈1700 cm⁻¹ due to the α-amino carbonyl. Consistent with
this was the absorption observed in the region 3200-3300 cm\(^{-1}\) due to the newly formed alcohol group and confirmed by the medium absorptions in the region of \(\approx 1200\) cm\(^{-1}\). The two weak to medium absorptions in the region 2400 and 2600 cm\(^{-1}\) were similar to their precursors and consistent with secondary amines present as their HCl salts. A doublet was also observed in the region \(\approx 1600\) cm\(^{-1}\) in the spectrum of 5d, which was consistent with the acid being present as the carboxylate anion. Therefore, these two observations (the amine cation and the carboxylate anion) suggested that 5d was probably isolated as the zwitterion.

Mass spectra of the secondary amine reduction products showed the (M + H) peak rather than the M\(^+\) peak, similar to the ketone precursor, and dichloro substitution was confirmed from the (M + H) peak cluster. The base peak (m/z 86) was identical to the spectra of the ketone precursors, and was probably formed in a similar manner since the aryl ketone and alcohol were expected to fragment similarly. The (M - 33) peak, common to all spectra, was consistent with the simultaneous loss of a methyl group (tert-butyl amines) and water (from the alcohol). Thus, the mass spectral data provided evidence of structure consistent with other spectroscopic data, and with the proposed structure of the target compounds. Despite being a known compound, VUF 8303 (5e) has not been previously characterised spectroscopically, as in the present study.

2.2.7 Hapten-Protein Conjugation

2.2.7.1 Water-Soluble Carbodiimide Conjugation

Conjugates of haptens 5d and 5g to BSA using ECDI were prepared as in Scheme 2.8.

\[
\begin{align*}
\text{BSA} & \quad \text{(CH}_3\text{)}_4\text{NH}_2 + \text{HO}_2\text{C-(CH}_3\text{)}_4\text{O-} & \quad n = 1 \ (5d) \\
\text{Cl'} & \quad \text{OH} & \quad = 5 \ (5g)
\end{align*}
\]

**Scheme 2.8 Reagents:** x, ECDI, pH 6.5, H\(_2\)O-DMF

ECDI conjugations typically use an excess of hapten and a large excess of the ECDI reagent. Reaction of the activated carboxylic acid adduct with the protein amino group is rapid when acid is present as a catalyst, although the protein amino groups are more reactive at higher pH where
a deprotonated lysine is favored. Typically, pH \approx 6 is a compromise between these two factors to give the most favourable reaction conditions. Therefore, to achieve a one-step conjugation reaction, conjugations of haptens 5d and 5g were maintained at \approx pH 6.5 by continual pH adjustment.

The solubility of C2 hapten 5d at the reaction pH (\approx 11 mg/cm^3) was good in mixed solvent (25% DMF in water), although a precipitate of the (presumably) neutral amino acid developed slowly over time. Solutions of hapten 5d were, therefore, prepared immediately prior to reaction by dissolving the hapten (as the HCl salt) in the mixed solvent and adjusting pH to \approx 6.2. Both ED-BSA and BSA tolerated mixed solvent concentrations (final DMF concentration of 20%). The hapten solution, used in excess (1:1 hapten-protein w/w), was added immediately to the aqueous protein solution (40 mg/cm^3, either ED-BSA or BSA) at pH \approx 6.2. A large excess of ECDI was used (10x w/w of the hapten), reaction pH maintained at \approx 6.5, and the conjugate isolated, after dialysis against water, by lyophilisation in yields of greater than 90% based on the weight of the carrier protein.

Gelatin conjugates of hapten 5d were similarly formed using ECDI, since gelatin also tolerated the required mixed solvent conditions. The reaction mixture was kept in a water bath (40°C) to minimise any protein gelling which may occur. Conjugates were isolated after dialysis and lyophilisation in yields similar to those of the BSA conjugates.

Conjugation of the C6 hapten 5g to BSA using ECDI under the above conditions was not possible due to poor solubility of the hapten at the desired reaction pH. This probably resulted from the more hydrophobic nature of this hapten compared with 5d (recall that 5d was soluble in aqueous acid, whereas 5g was recrystallised from aqueous acid). The use of a mixed solvent (1:4:4 DMF-THF-H_2O) gave a solution of 5g (15 mg/cm^3) at pH 5 which precipitated only slowly over time. However, BSA did not tolerate these mixed solvent conditions when dissolved in water, but was more tolerant of the mixed solvent when dissolved in aqueous phosphate buffer.

At higher pH (> 5), hapten 5g was precipitated more swiftly (< 30 s). Therefore, the solution of hapten 5g at pH 5 was preactivated by reaction of the hapten-containing solution (6:3:1 (10 mM phosphate buffer, pH 5)-THF-DMF, 100 mg/cm^3) with ECDI (10:1 ECDI-hapten w/w) prior to addition of the mixture to a solution of BSA (40 mg/cm^3), buffered at pH 8 with 10 mM phosphate buffer. Such an approach has been previously reported.\textsuperscript{372} This method overcame the problem of hapten insolubility at pH > 5, as well as the increased tolerance of the mixed solvent by BSA, when dissolved in the phosphate buffered solution. Again, hapten was used in a large excess (1:1 hapten-BSA w/w).
The preactivation was done for only two minutes and the solution showed no signs of any precipitated solid; the hapten-ECDI activated adduct may be more water soluble than the hapten itself because of the cationic amino moiety of ECDI (refer Figure 1.11). Preactivated hapten was added dropwise to the BSA solution (pH 8), since rapid addition resulted in precipitation of considerable amounts of an unidentified white solid. The turbid reaction mixture was cleared by adjustment to pH 6.5 after 30 minutes; a further portion of ECDI was added prior to overnight stirring. Dialysis and lyophilisation afforded the conjugates as white solids in yields of ≈90% of the initial weight of carrier protein. The corresponding gelatin conjugate was not synthesised, since the analogous conjugate formed using the DCC reagent was used instead for the required ELISAs.

2.2.7.2 Activated Ester Conjugation

Conjugation of haptens 5d and 5g to carrier protein using DCC essentially comprises a hapten preactivation step: DCC-mediated formation of the NHS ester of the carboxylic acid, followed by reaction of this activated ester with ε-amino moiety of the lysine residues of the carrier protein results in hapten conjugation by amide bond formation. This type of conjugation is well-known,\textsuperscript{311,312} and reaction of the active NHS ester occurs preferentially at aliphatic amines (e.g. ε-amino of lysine) at a pH of at least 8. This alternative conjugation approach may couple hapten more efficiently to the carrier protein than the ECDI-mediated conjugations, and therefore couple more hapten per mole of carrier. Cross-linking of carrier protein, which could occur with ECDI conjugations, may be also be minimised through this hapten preactivation using DCC and NHS.

Initial investigations into the required reaction conditions for the DCC-mediated conjugations utilised 3,4-dimethoxytyramine (DMT) as a model amine. The aliphatic amine present in the hapten was protected by protonation (HCl salt) during active ester formation, an approach that has not been previously reported for conjugations of haptens proceeding via active ester formation. The scheme for this model reaction is shown in Scheme 2.9 below. DMT was selected as a model amine since it has a strong absorption in the UV spectrum, which allowed easy detection of the expected adduct by RP HPLC analysis using a variable wavelength detector.
Scheme 2.9 *Reagents:* xi, DCC, 4:1 THF-DMF; xii, DMT, 4:1 THF-DMF

Initial investigations of the DCC-mediated reaction of 5d with DMT demonstrated that:

a) a single major product (retention time 7.7 min) with a large absorbance was observed by RP HPLC when the solution of the activated ester was added to a two times excess of DMT. By contrast, addition of the DCC solution to a mixture of NHS, 5d and DMT resulted in a more complex reaction mixture and showed a number of product peaks;

b) addition of an equimolar aliquot of the DCC solution to a mixture of NHS and 5d (in 4:1 THF-DMF) resulted in a precipitate of dicyclohexylurea after 3-5 min (indicative of active ester formation or DCC hydrolysis) and was virtually complete after 20 min. This solid was conveniently removed by centrifugation;

c) after 2 h the reaction of the active ester with DMT was virtually complete, and little of hapten 5d was observed by RP HPLC;

d) reaction of a “blank” preactivation solution (i.e. NHS and DCC but no hapten 5d) did not show the major product when hapten 5d was included;

e) when the reaction mixture of the activated ester of 5d and DMT was re-analysed at DMT $\lambda_{\text{max}}$ (280 nm) only the same single major product seen previously was again observed by RP HPLC.

These observations suggested that preactivation of the hapten was superior to generation of the activated ester in the presence of the amine. The reaction of the active ester intermediate with DMT was very selective since only a single major product was observed. Preactivation of hapten was virtually complete within 20 min of addition of DCC.
This preactivation was adopted for the DCC-mediated reaction of hapten 5d with carrier protein. The carrier protein was buffered at pH 8 (with 10 mM phosphate buffer) to favour deprotonation of the lysine residues; the phosphate-buffered protein solution also tolerated increased levels of mixed solvent in comparison to unbuffered protein. ED-BSA did not tolerate the mixed solvent conditions, and was therefore unsuitable. However, BSA tolerated the addition of activated hapten ester in mixed solvent, provided the protein solution already contained small amounts of the mixed solvent.

DCC-mediated conjugation was likely to be somewhat more efficient than the ECDI-mediated conjugation. Hence the ratio of protein to hapten 5d that was initially used was 4:1 w/w (compared to 1:1 w/w for ECDI conjugation). However, only half of the activated ester solution could be added before large amounts of a white solid precipitated and a ratio of protein to hapten 5d of 10:1 w/w was found to be more satisfactory. The reaction mixture was adjusted to pH 7.5 after 30 min prior to overnight stirring. However, after dialysis against water for approximately 30 h, a small amount of fine white precipitate had formed in the dialysis bag. Dialysis against 10 mM phosphate buffer (pH 7.2) caused this precipitate to dissolve and led to recovery of a weight of conjugate (as a white solid) greater than the initial amount of protein, presumably due to phosphate salt inclusion.

The need for a reduced hapten:protein ratio, and dialysis against dilute (~10 mM) phosphate buffer to maintain conjugate solubility suggested that the DCC-mediated conjugation of 5d may have been more efficient than the corresponding ECDI conjugations. The DCC-produced conjugates were less soluble during dialysis than those produced from ECDI-mediated coupling, and suggested perhaps an increased hapten loading, a result consistent with a more efficient coupling reaction. However, ECDI could also have reacted with tyrosine residues and led to formation of ionic O-alkyl isoureas; hence ECDI conjugation may have been as efficient as DCC conjugation, but produced a somewhat more water-soluble conjugate. Conditions similar to the DCC-mediated conjugation of 5d to BSA were used for the preparation of the hapten 5d-gelatin conjugate.

Conjugates of C6 hapten 5g to both BSA and gelatin were similarly formed using a protein:hapten ratio of 10:1 (w/w). Conjugates were isolated as white solids, after dialysis against dilute (~10 mM) phosphate buffer to maintain conjugate solubility, in weights greater than the initial weights of the carrier proteins. However, conversion of hapten 5g to the NHS ester, evidenced by precipitation of dicyclohexylurea after 10-12 min, was significantly longer than that of 5d (3-5 min). Hence a total activation time of 20 min was allowed to ensure complete activation of hapten.

Several methods for the characterisation of conjugates have been reported including use of
radiolabelled hapten,\textsuperscript{373,374} UV spectrophotometry,\textsuperscript{375,376} gel electrophoresis\textsuperscript{377} and, recently, mass spectrometry using electrospray ionisation techniques.\textsuperscript{378} These methods aim to characterise the average number of hapten molecules conjugated per carrier protein molecule. By comparison the electrospray ionisation/mass spectrometry technique can identify a range of conjugates with differing hapten densities.

Use of radiolabelled analogues of the two novel haptens was not possible since the synthesis of these radiolabels was beyond the scope of the present research. Gel electrophoresis was also considered unlikely to yield much useful information regarding the hapten density in the case of the ECDI-derived conjugates. The ECDI reagent would be likely to promote some activation of carboxyl residues of the carrier protein, leading to cross-linking of the protein. High molecular mass cross-linked proteins formed in this way would make identification of the number of hapten molecules more difficult since the increase in molecular mass would be relatively smaller. A hapten density of approximately 10 would lead to a change in molecular mass of $\approx 3\,000$ Da of BSA (molecular mass $\approx 60\,000$ Da). Densities lower than this would be increasingly difficult to quantify, and distinguishing between a hapten density of $n$ and $n + 1$ with a hapten of molecular mass $\approx 300$ Da would be problematic. Thus the use of gel electrophoresis would be likely to yield, at best, only a very approximate estimate of the hapten density.

The use of UV spectrophotometry for a determination of hapten density relies on haptens with UV chromophores which will significantly change the absorbance coefficient of the carrier protein, which may not always be applicable to the conjugate under evaluation.\textsuperscript{360} The haptens \textbf{5d} and \textbf{5g} do not have suitable UV chromophores, and no difference in UV absorbance between BSA and the conjugates formed was observed. This indicated that hapten density of the novel conjugates could not be readily determined using UV spectrophotometry.

Electrospray ionisation is regarded as a gentle ionisation technique in mass spectrometry, able to produce multicharged ions of proteins of considerable molecular mass.\textsuperscript{379,380} In conjunction with a high resolution mass spectrometer, this method has been reported\textsuperscript{378} as able to distinguish between hapten densities of $n$ and $n + 1$. However, this technique is still developing and proteins like BSA, and conjugates thereof, are at the upper limit of what may be detected. Furthermore, access to the specialised equipment required was not available.

With respect to the conjugates formed, hapten density may be of interest, but may not completely informative about the nature of the conjugates formed. Indeed, a hapten density of between 8 and 25 per carrier protein molecule is considered to be in the range from which good antibody titres can be produced,\textsuperscript{312} but provides no information about the nature of the haptenic epitope. However, the model reaction of the activated NHS ester of \textbf{5d} with excess DMT produced only one major product, which tended to suggest that conjugation of this hapten with
BSA using this method was reasonably specific.

This model reaction showed only a single major product by RP HPLC (UV detection, \( \lambda = 240 \) and 280 nm), with strong absorbance and a longer retention time than either 5d or DMT; the reaction was almost complete after 2 h. The active NHS ester of 5d would react with an available nucleophile, and two major nucleophiles exist in this reaction mixture - the primary amine of DMT (present as the free base and in excess) and the secondary \( N\text{-}tert \)-butyl amino group of the hapten itself (protected as the HCl salt). If the activated hapten reacted with DMT, the product would appear to be more hydrophobic than either 5d or DMT (and hence have a longer retention time) and a strong UV absorbance at the single wavelength UV detector (due to the strong absorbance of DMT in the UV). Both these observations were made. Conversely, if the activated ester of 5d reacted with itself, then the resulting product would not have the strong UV absorbance of the DMT adduct.

Therefore, it appeared that the activated NHS ester of 5d reacted selectively with the primary aliphatic amine of DMT, and the clenbuterol-like portion of the hapten remained unaltered; hence the \( N\text{-}tert \)-butylamino group was apparently protected as the HCl salt. Therefore, conjugation of 5d to BSA via an active ester intermediate could be expected to be similarly selective and provide immunoconjugates which contained intact clenbuterol-like epitopes. Furthermore, the immunogenicity and ability of these conjugates to elicit clenbuterol-specific antibodies would only be demonstrated following immunisation with these novel conjugates and characterisation of the antibody response and specificity.
2.3 Conclusion

The desired O-alkyl analogues of CB, haptens 5d and 5g, and model compounds 5c and 5e, were synthesised in a five-step reaction sequence, using 3,5-dichloro-4-hydroxyacetophenone (DCHA) as starting material, and represents the first synthesis of O-alkyl CB analogues. DCHA was synthesised in two steps from 2,6-dichlorophenol in a yield of 71%, and the requisite target compounds were formed in reasonable overall yields of between 5.5-27.8%. The synthetic pathway was based on that of the parent compound CB, with the following modifications:

a) removal of a chlorination step by inclusion of the required chlorines in the starting material. This was found to be more synthetically convenient than direct chlorination of the phenoxy ring system.

b) introduction of the required alkyl substituent onto the phenoxy oxygen through the use of a modified Williamson ether synthesis. O-Alkylation was readily achieved in very good yields with a variety of alkyl halides, and represented successful adaption of this reaction as a convenient route to O-alkylated derivatives of DCHA.

c) use of the secondary amine, N-benzyl-tert-butylamine, rather than tert-butylamine, to introduce the tert-butylamino substituent of the clenbuterol analogues. Consistent with previous reports, amination of para-alkoxyphenacyl bromides with tert-butylamine resulted in a mixture of products, whereas the use of N-benzyl secondary amines gave the required intermediates in reasonable yield.

Three of the target compounds were not previously reported (5c, 5d and 5g) and the fourth, 5e (VUF 8303), has been previously synthesised by the chlorination of commercially-acquired 1-(4-hydroxyphenyl)-2-(tert-butylamino)ethanol. The synthesis of 5e, therefore, represents a previously unreported complete synthesis and characterisation of this compound, as well as demonstrating the utility of the synthetic route for the formation of these O-alkyl analogues of CB. All new compounds (12 intermediates, other than the phenacyl bromides, and 3 target compounds), as well as those previously reported, were fully characterised by proton and carbon-13 NMR, infra-red and mass spectroscopic data, these providing strong evidence consistent with the proposed structures. Two dimensional NMR experiments on the N-benzyl intermediate 3c provided data for a more definite assignment of the proton and carbon-13 NMR spectra of this compound and, by analogy, the NMR spectra of the other tertiary amine intermediates.
Conjugates of the analogues 5d and 5g to BSA and gelatin were formed using carbodiimide-based chemistry by linkage of an activated carboxyl group to the \( \varepsilon \)-amino group of the lysine residues of BSA. These conjugates have not been previously reported. The model reaction of the NHS ester of C2 Hapten (5d) with dimethoxytyramine (DMT) tended to suggest a reasonably specific reaction of the activated ester with primary amine, since only a single major product (by RP HPLC analysis) was formed in this reaction. Therefore, this conjugation reaction would appear able to conjugate a structurally intact clenbuterol-like epitope to a carrier protein. In contrast, the only reported clenbuterol conjugate uses diazotisation chemistry, which could alter important structural features of CB.
2.4 Experimental

The infra-red spectra were recorded on a Bio Rad FTS-7 spectrophotometer, and absorptions are reported in cm$^{-1}$.

$^1$H and $^{13}$C NMR data are reported as chemical shifts (δ ppm) relative to tetramethylsilane reference. The $^1$H NMR were recorded on a Brucker AC300F (300 MHz) or a Brucker AM500 (500 MHz) at the University of NSW (School of Chemistry) unless otherwise stated. The $^{13}$C NMR were recorded on the same instrument at 125 MHz and the substitution of the carbon atoms determined by Distortionless Enhancement Polarisation Transfer (DEPT) techniques. NMR peaks are designated as follows: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m) and broad (br). Exchangeable protons were determined by D$_2$O exchange. The central dichlorinated ring is abbreviated as Ar. ArC indicates carbons which belong to this central dichlorinated aromatic ring. ArC indicates a carbon attached to an aromatic ring carbon.

Low Resolution mass spectra were recorded on an AEI MS-12 mass spectrometer at 70 eV and an ion source temperature of 200 °C at the University of NSW (School of Chemistry) Observed peaks are reported with the mass:charge ratio (m/z), and the % relative abundance given in brackets (base peak 100%). High Resolution mass spectra were recorded on a VG AutoSpec Q mass spectrometer at 70 eV and an ion source temperature of 220 °C at the University of NSW (Biomedical Mass Spectrometry Unit).

Melting points in glass capillaries were recorded on a Gallenkamp melting point apparatus and are uncorrected.

Suppliers of chemicals used in this section are given in Appendix 1, as is the detailed description of the Reversed Phase HPLC (RP HPLC) equipment. A saturated aqueous solution of NaCl is referred to as brine. Organic solvents were redistilled and dried over molecular sieves (type 4Å, minimum 48h) prior to use.
2,6-dichlorophenyl acetate.\textsuperscript{337} To a 3-necked, 250 cm\(^3\) round-bottomed flask was added 2,6-dichlorophenol (20.10 g, 123.3 mmol), acetic anhydride (116 cm\(^3\), 1.23 mol), pyridine (12 cm\(^3\), 151.3 mmol) and a few anti-bump granules. A water-cooled condenser and drying tube (CaCl\(_2\)) were fitted and the reaction heated at reflux for 4 h. The cooled reaction mixture was distilled under vacuum to remove excess low boiling liquids (b.p. 42-46 °C/20 mmHg). The remaining brown reaction mixture was transferred to a 50 cm\(^3\) distillation flask filled with glass wool and fitted with an air cooled condenser. Distillation under vacuum yielded the \textit{title compound} as a pale green liquid (21.46 g, 85%) b.p. 128-129 °C/20 mmHg (lit.\textsuperscript{337} 125-126 °C/17 mmHg); \(\nu_\text{max( neat)}/\text{cm}^{-1}\) 1774.0 (C=O); \(\delta_H\) (500 MHz, CDCl\(_3\)) 2.40 (3 H, s, CH\(_3\)), 7.13 (1 H, t, J 8.14, p-ArH), 7.35 (2 H, d, J 8.14, m-ArH); \(\delta_C\) (125 MHz, CDCl\(_3\)) 20.17 (1 C, C=OCH\(_3\)), 127.14 (1 C, p-ArC), 128.60 (2 C, m-ArC), 128.91 (2 C, Cl), 144.06 (1 C, ArCO(C=O)), 167.25 (1 C, C=O); \(m/z\) 204 (14%, M\(^+\)), 162 (100, M - C=OCH\(_2\)), 43 (94, C=OCH\(_3\)^+).

3,5-dichloro-4-hydroxyacetophenone.\textsuperscript{337} To a 250 cm\(^3\) round-bottomed flask was added 2,6-dichlorophenyl acetate (25.00 g, 122.0 mmol), fresh AlCl\(_3\) (20.00 g, 150.0 mmol), the flask fitted with a drying tube (CaCl\(_2\)) and the mixture heated in an oil bath (165 °C) for 45 min. The initially light tan reaction mixture turned a deep burgundy/red colour and became progressively more viscous. White vapour was also evolved. A teflon-coated magnetic stirring bar was added to the cooled reaction mixture along with a mixture of HCl (12 M, 25 cm\(^3\)) and crushed ice (100 g). The flask was stoppered and the mixture stirred for several hours till the solidified reaction mixture was dispersed as a yellow precipitate. This mixture was extracted with EtOAc (1 x 200 cm\(^3\), 1 x 100 cm\(^3\), 1 x 50 cm\(^3\)). The golden yellow extracts were washed with brine (2 x 50 cm\(^3\)) and extracted with 5% Na\(_2\)CO\(_3\) (7 x 50 cm\(^3\)). The combined pink aqueous extracts were acidified (pH 2.5, 10% HCl) and a pink precipitate collected (30.4 g). Recrystallisation from benzene and drying of the light pink needle-like crystals over silica yielded the \textit{title compound} (20.806 g, 83%), m.p. 163-165 °C, (lit.\textsuperscript{337} 164.5-165.5 °C); \(\nu_\text{max(Nujol)}/\text{cm}^{-1}\) 3100 (ArOH), 1656.7 (C=O); \(\delta_H\) (500 MHz, d\(_6\)-DMSO) 2.52 (3 H, s, CH\(_3\)), 7.88 (2 H, s, ArH), 11.13 (1 H, br, OH); \(\delta_C\) (125 MHz, d\(_4\)-MeOH) 26.54 (1 C, C=OCH\(_3\)), 123.60 (2 C, ArCl), 130.33 (2 C, ArCH), 131.19 (1 C, ArC(C=O), 155.29 (1 C, ArC-OH), 197.19 (1 C, C=OCH\(_3\)); \(m/z\) 204 (33%, M\(^+\)), 189 (100, M - Me), 161 (18, M - C=OCH\(_3\)), 43 (62, C=OCH\(_3\)^+).

\textit{Methyl 2-(2,6-dichloro-4-acetylphenoxy)acetate 1b}. To a conical flask containing a stirred solution of 3,5-dichloro-4-hydroxyacetophenone (3.000 g, 14.64 mmol) in dry DMF (3.4 cm\(^3\)), was added K\(_2\)CO\(_3\) (4.05 g, 29.28 mmol) and methyl 2-bromoacetate (1.628 cm\(^3\), 17.57 mmol). To the flask was fitted a small reflux condenser, and then set in a shallow oil bath (≈60-80 °C) with stirring. Reaction was monitored by RP HPLC (gradient program: 55%
A 45% B to 100% B: 5 min. Hold 100% B: 10 min. $\lambda = 260$ nm). After 30 min no starting material ($t_R$ 4.4 min) and only one product peak ($t_R$ 7.4 min) were observed. The reaction mixture was diluted with H$_2$O (50 cm$^3$), extracted with EtOAc (3 x 50 cm$^3$, 1 x 20 cm$^3$), the organic phase washed with brine (4 x 20 cm$^3$), dried (Na$_2$SO$_4$) and concentrated in vacuo to give a dark brown oil. Recrystallisation from n-hexane gave shiny, off-white, plate-like crystals of 1b (2.448 g, 60%), m.p. 86-87 °C; $\nu_{\text{max}}$(Nujol)/cm$^{-1}$ 1759.2 (O-(C=O)), 1693.5 (ArC=O); $\delta_H$(500 MHz, CDCl$_3$) 2.54 (3 H, s, CH$_3$), 7.85 (2 H, s, Ar CH), 4.68 (2 H, s, ArOCH$_2$), 3.81 (3 H, s, CH$_3$OC=O); $\delta_C$(125 MHz, CDCl$_3$) 26.35 (1 C, C=OCH$_3$), 129.50 (2 C, ArCCl), 129.10 (2 C, ArCH), 134.37 (1 C, ArC(C=O)), 153.87 (1 C, ArCOCH$_2$), 194.53 (1 C, C=OCH$_3$), 68.98 (1 C, ArOCH$_2$), 52.32 (1 C, CH$_3$OC=O), 167.88 (1 C, CH$_3$OC=OCH$_2$).

Methyl 2-(4-bromoacetyl-2,6-dichlorophenoxy)acetate 2b. A solution of Br$_2$ in dry CHCl$_3$ was freshly prepared at a concentration of 1.69 mmol/cm$^3$. To an aluminium foil-covered 5 cm$^3$ round bottom flask, magnetically stirred, was added a solution of 1b (1.000 g, 3.61 mmol) in dry CHCl$_3$ (2 cm$^3$). One quarter (0.534 cm$^3$) of the theoretical amount of bromine solution was added; the tan colour of the reaction mixture discharged immediately. The second, third and fourth quarters of bromine solution were added rapidly with immediate colour discharge. The reaction mixture was promptly concentrated in vacuo to give an oil which was taken up in EtOAc (30 cm$^3$) and washed with brine (2 x 15 cm$^3$) until the washings were neutral to pH paper. The organic phase was dried (Na$_2$SO$_4$) and concentrated in vacuo to give a crude light tan oil (1.378 g). By RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. $\lambda = 260$ nm) this product contained $\approx$75% 2b ($t_R$ 8.5 min), with $\approx$13% 1b ($t_R$ 7.4 min), and $\approx$12% of an unidentified by-product ($t_R$ 9.3 min). This product was recrystallised four times from n-hexane, to give pale-tan, flakes of 2b (401 mg, 31%). By RP HPLC analysis (conditions as previously), this product contained $\approx$94.7% 2b, 1.5% 1b and 3.8% of an unidentified by-product. m.p. 83-85 °C; $\nu_{\text{max}}$(Nujol)/cm$^{-1}$ 1752.2 (O-(C=O)), 1698.9 (ArC=O); $\delta_H$(500 MHz, CDCl$_3$) 4.36 (2 H, s, CH$_2$Br) 7.91 (2 H, s, Ar CH), 4.73 (2 H, s, ArOCH$_2$), 3.83 (3 H, s, CH$_3$OC=O); $\delta_C$(125 MHz, CDCl$_3$) 29.84 (1 C, C=OCH$_2$Br), 129.84 (2 C, ArCCl), 129.78 (2 C, ArCH), 131.21 (1 C, ArC(C=O)), 154.60 (1 C, ArCOCH$_2$), 188.28 (1 C, C=OCH$_2$Br), 69.06 (1 C, ArOCH$_2$), 52.42 (1 C, CH$_3$OC=O), 167.84 (1 C, CH$_3$OC=OCH$_2$); $m/z$ 354 (6%, M$^+$), 261 (7, M - CH$_2$Br).

3,5-dichloro-4-methoxycacetophenone 1c. To a conical flask heated in a shallow oil bath at 40 °C containing a stirred solution of 3,5-dichloro-4-hydroxyacetophenone (5.031 g, 24.40 mmol) in dry DMF (10.5 cm$^3$) was added K$_2$CO$_3$ (15.1 g, 97.60 mmol), and methyl iodide (3 cm$^3$, 48.48 mmol). The solution was left stirring for 30 min and a further portion of K$_2$CO$_3$ (5.0 g) and MeI (3 cm$^3$) added in that order and stirring/heating continued for a further 45 min. The reaction mix showed only one spot by TLC (silica, 100% chloroform: R$_f$ 0.53)
and no starting DCHA. The mixture was diluted with CHCl₃ (80 cm³), filtered, concentrated in vacuo and the light brown oil taken up in EtOAc (100 cm³). The organic phase was washed with brine (2 x 40 cm³), 5% Na₂CO₃ (1 x 50 cm³, 1 x 25 cm³), brine again (2 x 25 cm³), dried (Na₂SO₄), concentrated in vacuo and dried over silica gel to a golden/yellow solid (5.25 g). The solid was chromatographed (silica, n-hexane-chloroform 1:1) to give a pale white solid 1c (5.216 g, 97%) m.p. 66-67 °C; νmax(Nujol)/cm⁻¹ 1681.1 (C=O); δH(500 MHz, CDCl₃) 2.55 (3 H, s, CH₃), 7.86 (2 H, s, ArH), 3.94 (3 H, s, CH₃OAr; δC(125 MHz, CDCl₃) 26.40 (1 C, C=OCH₃), 129.85 (2 C, ArCl), 129.07 (2 C, ArCH), 133.92 (1 C, ArC(C=O), 156.17 (1 C, ArCOCH₂), 194.74 (1 C, C=OCH₃), 60.89 (1 C, ArOCH₃); m/z 218 (33%, M⁺), 203 (100, M - Me), 43 (65, C=OCH₃⁺) (Found M⁺, 217.9901. C₉H₈Cl₂O₂ requires M, 217.9901).

3,5-dichloro-4-methoxyphenacyl bromide 2c. A solution of Br₂ in dry CHCl₃ was freshly prepared at a concentration of 1.99 mmol/cm³. To an aluminium foil-covered 50 cm³ round bottom flask containing a magnetic stirring bar was added a solution of 1c (2.500 g, 11.4 mmol) in dry CHCl₃ (4 cm³). One quarter of the theoretical amount of bromine solution (1.430 cm³) was added to the solution and the stoppered flask stirred at room temperature (1.25 h), at which time the tan colour of the reaction mix discharged. The second, third and fourth quarters of the bromine solution were then added rapidly with immediate colour discharge after each addition. By RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) ≈12% of 1c remained and a further portion of the bromine solution was added (0.69 cm³), the colour discharging in 20-30 s. The reaction mix was then promptly concentrated in vacuo, the resulting oil taken up in EtOAc (50 cm³), washed with brine (4 x 15 cm³) until the washings were neutral to pH paper, dried (Na₂SO₄) and concentrated in vacuo to give a white/pale brown solid of crude 2c (3.271 g). By RP HPLC (conditions as previously) this product contained ≈89% 2c (tR 10.0 min), with approximately equal proportions of 1c (tR 9.1 min) and an unidentified by-product present (tR 10.9 min). The material was used directly without further purification.

1-(3,5-dichloro-4-methoxyphenyl)-2-(N-benzyl-N-tert-butylamino)ethanone hydrochloride 3c. A magnetically stirred 150 cm³ round bottom flask fitted with a water-cooled condenser and a drying tube (CaCl₂), containing N-benzyl-tert-butylamine (11.66 g, 71.46 mmol) in dry CHCl₃ (5 cm³), was heated in an oil bath at reflux (2 min). To this hot solution was added a solution of crude 2c (7.189 g, ≈83% as phenacyl bromide 2c, ≈19.7 mmol) in dry CHCl₃ (10 cm³) and set at reflux for 60 min. A white precipitate formed after 10 min and the solution had turned yellow, and at 60 min the solution was an orange colour. Analysis of the mixture by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) showed ≈15% of 2c (tR 10.0 min) remaining and only one product (tR 7.6 min), so a further portion of amine (1.152 g, 7.06 mmol) was added and
heating continued for 60 min. The cooled reaction mixture was diluted with EtOAc (50 cm³) and filtered to remove N-benzyl tert-butylamine hydrobromide as a white solid (5.513 g). The filtrate was extracted with 1 M HCl (2 x 40 cm³). No product was detected in the first extract by RP HPLC analysis. However, a white precipitate from the second extract was collected at the pump (5.935 g), stirred (60 min) with 1 M HCl (20 cm³) then stirred (1.5 h) with hot EtOAc (30 cm³) to yield 3c as a clean white solid (5.093 g, 62%). m.p. 165-167 °C (decomp.); RP HPLC (conditions as previously, t_R = 7.6 min); ν_max(Nujol)/cm⁻¹ 2635.4 (HN⁺), 1681.6 (C=O); δ_H(500 MHz, d_6-DMSO) 1.57 (9 H, s, Bu³); 4.14 and 4.58 (1 + 1 H, dd, J 9.1 and 12.4, and d, J 12.3, NCH₂C₆H₅≥), 7.14-7.16 (3 H, m, m- & p-C₆H₅CH₂N), 7.62 (2 H, d, J 7.0, o-C₆H₅CH₂N), 4.93 and 5.18 (1 + 1 H, d, J 18.4, and dd, J 7.3 and 18.5, C=OCH₂N), 7.82 (2 H, s, ArH), 3.98 (3 H, s, CH₃OAr), 9.14 (1 H, br s, NH); δ_C(125 MHz, d₄-MeOH) 25.42 (3 C, NCMMe₃), 68.90 (1C, NCMMe₃), 55.85 (1C, NCH₂C₆H₅≥), 133.76, 131.30, 130.57 and 131.38 (6 C, NCH₂C₆H₅≥), 56.45 (1C, C=OCH₂N), 190.43 (1C, C=OCH₂N), 139.38 (1 C, ArC(C=O), 130.70 (2 C, ArCCl), 130.26 (2 C, ArCH), 158.89 (1 C, ArCOCH₂), 61.91 (1 C, ArOCH₃); m/z 380 (9%, M + H), 364 (7, M - Me), 288 (6, M - C₇H₇≥), 203 (50, M - CH₂N(Bu¹)CH₂C₆H₅≥), 176 (37, CH₂N(Bu¹)CH₂C₆H₅≥), 120 (72, CH₂NCH₂C₆H₅≥), 91 (100, C₇H₇≥) (Found M⁺, 379.1082. C₂₀H₂₃Cl₂NO₂ requires M, 379.1106).

1-(3,5-dichloro-4-methoxyphenyl)-2-(tert-butylamino)ethanone hydrochloride 4c. Into a 250 cm³ magnetically stirred 3-necked round bottom flask was placed 3c (2.500 g, 6.00 mmol) and EtOH-HOAc (9:1, 250 cm³). The solution was bubbled with nitrogen for 5 min, 5% Pd/C (250 mg) added, and the flask connected to a low pressure hydrogenation apparatus. The system and flask were evacuated and hydrogen gas admitted to the system. The mixture was stirred vigorously and monitored periodically by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), aliquots being centrifuged prior to analysis. After a period of 15 min (H₂ absorption = 145 cm³, theoretical = 147 cm³) only the expected products (4c, t_R 2.4 min; toluene, t_R 8.75 min) were present. The reaction mix was then filtered and the filtrate concentrated in vacuo to give a pale yellow solid. Recrystallisation from 0.5 M HCl and drying over silica gel gave 4c as a white solid (1.811 g, 93%), m.p. 180-190 °C (decomp.); ν_max(Nujol)/cm⁻¹ 2621.3 and 2410.7 (H₂N⁺), 1704.4 (C=O); δ_H(500 MHz, d_6-DMSO) 1.37 (9 H, s, Bu³), 4.69 (2 H, br t, J 5.9 and 6.3, C=OCH₂N), 8.23 (2 H, s, ArH), 3.93 (3 H, s, ArOCH₃), 9.29 (2 H, br s, NH₂); δ_C(125 MHz, d₄-MeOH) 26.22 (3 C, NCMMe₃), 58.81 (1C, NCMMe₃), 48.94 (1C, C=OCH₂N), 190.96 (1C, C=OCH₂N), 132.49 (1 C, ArC(C=O), 131.58 (2 C, ArCCl), 130.76 (2 C, ArCH), 158.82 (1 C, ArCOCH₂), 61.91 (1 C, ArOCH₃); m/z 290 (6%, M + H), 274 (35, M - Me), 203 (7, M - CH₂NHBu¹), 86 (100, [CH₂NHBu¹]+) (Found M⁺, 289.0634. C₁₃H₁₇Cl₂NO₂ requires M, 289.0636).
1-(3,5-dichloro-4-methoxyphenyl)-2-(tert-butylamino)ethanol 5c. To a 150 cm³ round bottom flask was added a solution of 4c (550 mg, 1.53 mmol) in EtOH-H₂O (2:1, 7.5 cm³). To this magnetically stirred solution was added NaBH₄ (20% w/w aqueous solution, 5 x 58 mm³ aliquots) over 1.5 h, at which time no 4c (tₚ 8.5 min) and only one product (5c, tₚ 10.8 min) were observed by RP HPLC (isocratic: 75% A 25% B, λ = 240 nm). The solution was diluted with H₂O (35 cm³), the milky solution extracted with EtOAc (2 x 30 cm³), and re-extracted (30 cm³) after adjustment to pH ≈9 and saturation of solution with NaCl. The organic extracts were dried (Na₂SO₄) and concentrated in vacuo to give an oil (485 mg). The reduction product 5c was isolated as the HCl salt from aqueous acid as a white solid and dried over silica gel (417 mg, 83%), m.p. 196-198 °C; νₓₓₓₓ(Nujol)/cm⁻¹ 3274.9 (COH), 2642.4 and 2411.9 (HN⁺); δₓₓₓₓ(500 MHz, d₆-DMSO) 1.31 (9 H, s, Bu₁), 3.10 (1 H, dd, J 12.5 and 3.1, CHₓₓₓₓ(OH)CHₓₓₓₓHₓₓₓₓBₓₓₓₓN), 2.95 (1 H, dd, J 12.5 and 9.9, CHₓₓₓₓ(OH)CHₓₓₓₓHₓₓₓₓBₓₓₓₓN), 5.01 (1 H, dd, J 2.9 and 9.8, CHₓₓₓₓ(OH)CHₓₓₓₓHₓₓₓₓBₓₓₓₓN), 6.41 (1 H, br, CHOₓₓₓₓH), 7.55 (2 H, s, ArHₓₓₓₓ), 3.82 (3 H, s, ArOCHₓₓₓₓ₃), 8.63 and 9.35 (2 H, br, NH₂ₓₓₓₓ); δₓₓₓₓ(125 MHz, CDClₓₓₓₓ₃) 28.92 (3 C, NCMeₓₓₓₓ₃), 50.85 (1C, NCMeₓₓₓₓ₃), 49.90 (1C, CH(OH)CHₓₓₓₓHₓₓₓₓN), 70.46 (1C, ArCH(OH)CHₓₓₓₓHₓₓₓₓN), 140.59 (1 C, ArCCH(OH)), 129.22 (2 C, ArCCI), 126.18 (2 C, ArCH), 151.28 (1 C, ArCOCHₓₓₓₓ₃), 60.65 (1 C, ArOCHₓₓₓₓ₃); m/z 292 (7%, M + H), 258 (10, M - [Me + H₂O]), 86 (100, [CH₂NHBBıt⁺]) (Found M⁺ + H, 292.0842. C₁₃H₂₀Cl₂NO₂ requires M + H, 292.0871; found M⁺ - [Me + H₂O], 258.0452. C₁₃H₂₀Cl₂NO₂ requires M - [Me + H₂O], 258.0452).

Benzyl 2-(2,6-dichloro-4-acetylphenoxy)acetate 1d. To a conical flask containing a stirred solution of 3,5-dichloro-4-hydroxyacetophenone (6.000 g, 29.26 mmol) in dry DMF (12 cm³), was added K₂CO₃ (8.04 g, 58.14 mmol) followed by NaI (440 mg) and benzyl 2-bromoacetate (4.632 cm³, 29.26 mmol). The flask was stoppered, covered in foil and stirred at room temperature. Reaction was monitored by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), and at 46.5 h no starting material (tₚ 4.4 min) and only one product peak (tₚ 10.0 min) were observed. The reaction mixture was diluted with H₂O (150 cm³), extracted with EtOAc (3 x 100 cm³, 1 x 50 cm³), the organic phase washed with brine (4 x 50 cm³), dried (Na₂SO₄) and concentrated in vacuo to give a dark brown oil (10 g). Recrystallisation from n-hexane gave pale tan needle-like crystals of 1d (5.972 g, 56%), m.p. 43-44 °C; νₓₓₓₓ(Nujol)/cm⁻¹ 1754.8 (O-C=O), 1693.5 (ArC=O); δₓₓₓₓ(500 MHz, CDClₓₓₓₓ₃) 2.55 (3 H, s, CH₃), 7.87 (2 H, s, ArH), 4.76 (2 H, s, ArOCH₂), 5.26 (2 H, s, C₆H₅CH₂OC=O), 7.32 (5 H, m, C₆H₅₃); δₓₓₓₓ(125 MHz, CDClₓₓₓₓ₃) 26.34 (1 C, C=OCH₃), 129.47 (2 C, ArCCI), 129.10 (2 C, ArCH), 134.32 (1 C, ArC(C=O), 154.01 (1 C, ArCOCCH₂), 194.54 (1 C, C=OCH₃), 67.16 (1 C, ArOCH₂), 69.17 (1 C, C₆H₅CH₂OC=O), 167.33 (1 C, C₆H₅CH₂OC=OCH₂), 128.59, 128.55, 128.45 and 134.97 (6 C, C₆H₅CH₂OC=O); m/z 353 (7%, M + H), 352 (39, M⁺), 91 (100, C₇H₇⁺), 43 (22, C=OCH₃); (Found M⁺, 352.0277. C₁₇H₁₄Cl₂O₄ requires M, 352.0269).
Benzyl 2-(4-bromoacetyl-2,6-dichlorophenoxy)acetate 2d. A solution of Br₂ in dry CHCl₃ was freshly prepared at a concentration of 2.44 mmol/cm³. To an aluminium foil-covered 100 cm³ round bottom flask, magnetically stirred, was added a solution of 1d (8.323 g, 23.57 mmol) in dry CHCl₃ (30 cm³). One quarter (2.414 cm³) of the theoretical amount of bromine solution was added, the flask stoppered, and the solution stirred at room temperature until the tan colour of the reaction mixture discharged (30 min). The second quarter of bromine solution was added rapidly with immediate colour discharge. The reaction mixture was promptly concentrated in vacuo to give an oil which was taken up in EtOAc (150 cm³) and washed with brine (3 x 50 cm³) until the washings were neutral to pH paper. The organic phase was dried (Na₂SO₄) and concentrated in vacuo to give a crude light tan oil. The oil was redissolved in dry CHCl₃ (30 cm³) and the third and fourth quarters of the bromine solution added rapidly with a 15 min interval. The reaction mixture was then immediately concentrated in vacuo, the oil taken up in EtOAc (150 cm³), washed with brine (3 x 50 cm³) until the washings were neutral, dried (Na₂SO₄) and concentrated in vacuo to give a tan-coloured oil (10.304 g). By RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) this product contained ≈76% 2d (tₚ 10.5 min), with approximately equal proportions of 1d (tₚ 10.0 min), and an unidentified by-product (tₚ 11.1 min). The material was used directly without further purification. In a separate experiment the by-product increased with further additions of Br₂ at the expense of product 2d.

Benzyl 2-((N-benzyl-N-tert-butylaminoacetyl)-2,6-dichlorophenoxy)acetate hydrochloride 3d. A magnetically stirred 150 cm³ round bottom flask fitted with a water-cooled condenser and a drying tube (CaCl₂), containing N-benzyl-tert-butylamine (15 cm³, 13.215 g, 80.94 mmol) and dry CHCl₃ (25 cm³), was heated in an oil bath at reflux (2 min). To this hot solution was added a solution of crude 2d (10.304 g, 76% as phenacyl bromide 2d, 17.9 mmol) in dry CHCl₃ (15 cm³) and set at reflux for 3 h. A white precipitate formed after 30 min and the solution turned yellow, and at 3 h the solution was an orange colour. Analysis of the mixture by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) showed approximately 10-15% of 2d (tₚ 10.5 min) remaining and one major product (tₚ 9.5 min). Further heating had been shown to decrease the yield, so this burnt orange reaction mixture was cooled, diluted with EtOAc (125 cm³), and filtered to remove N-benzyl-tert-butylamine hydrobromide (5.19 g) as a white solid. The filtrate was extracted with 1 M HCl (3 x 80 cm³) and the white precipitate which formed in the third extraction was collected by filtration. No product was detected in the first two extracts by RP HPLC analysis. A further 1 M HCl extraction yielded more white precipitate (total 10.653 g). The combined solids were stirred vigorously in 1 M HCl (100 cm³) for 60 min, filtered, the precipitate collected at the pump, and washed by stirring successively in EtOAc (75 cm³) and Et₂O (75 cm³) to yield a clean white solid HCl salt of the addition product 3d (7.566 g, 76%). m.p. 135-137 °C (decomp.); RP HPLC (conditions as previously, tₚ = 9.5 min);
4-(tert-butylaminoacetyl)-2,6-dichlorophenoxyacetic acid hydrochloride 4d. Into a 1 dm$^3$ magnetically-stirred 3-necked round bottom flask was placed 3d (6.500 g, 11.8 mmol) and EtOH-HOAc (9:1, 650 cm$^3$). The solution was bubbled with nitrogen for 5 min, 5% Pd/C (650 mg) added, and the flask connected to a low pressure hydrogenation apparatus. The system and flask were evacuated and hydrogen gas admitted. The mixture was stirred vigorously and monitored periodically by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), aliquots being centrifuged prior to analysis. After a period of 1.1 h (H$_2$ absorption = 615 cm$^3$, theoretical = 578 cm$^3$) only the expected products (4d, $t_R$ 1.3 min; toluene, $t_R$ 8.75 min) were present. The reaction mixture was filtered, the filtrate concentrated in vacuo to give an oil, which was taken up in EtOAc (100 cm$^3$) and extracted with 1 M HCl (2 x 100 cm$^3$). The aqueous phase was again filtered to remove small amounts of Pd/C and concentrated in vacuo to give a white solid. This solid 4d was washed by stirring in EtOAc (150 cm$^3$) for 30 min, then recovered by filtration (3.767 g; 86%); m.p. 178-185 °C (decomp.); $v_{\text{max}}$(Nujol)/cm$^{-1}$ 2638.9 and 2437.7 (H$_2$N$^+$), 1741.0 (COOH), 1705.2 (ArC=O); $\delta_H$(500 MHz, d$_6$-DMSO) 1.36 (9 H, s, Bu$^t$), 4.71 (2 H, br s, ArC=OCH$_2$N), 8.22 (2 H, s, ArH), 4.77 (2 H, s, ArOCH$_2$H), 9.18 (2 H, br s, NH$_2$), 13.22 (1 H, br, COOH); $\delta_C$(125 MHz, d$_4$-MeOH) 25.90 (3 C, NCM$e_3$), 58.55 (1C,NCMe$_3$), 48.65 (1C, C=OCH$_2$N), 190.58 (1C, C=OCH$_2$N), 132.47 (1 C, ArC(C=O), 130.97 (2 C, ArCCI), 130.48 (2 C, ArCH), 156.36 (1 C, ArCOCH$_2$), 70.00 (1 C, ArOCH$_2$), 170.90 (1 C, HOC=OCH$_2$); m/z 334 (9%, M + H), 318 (68, M - Me), 259 (29, M - H$_3$NBu$_t$), 247 (30, M - CH$_2$NHCH$_2$C$_6$H$_5$), 86 (100, [CH$_2$NHBu$^t$]$^+$) (Found M$^+$, 333.0539. C$_{14}$H$_{17}$Cl$_2$NO$_4$ requires M, 333.0535).

4-(2-tert-butylamino-1-hydroxyethyl)-2,6-dichlorophenoxyacetic acid 5d. To a magnetically stirred 100 cm$^3$ round bottom flask was added a solution of 4d (3.907 g, 10.7
mmol) in aqueous HCl (pH 5, 65 cm³) and THF (65 cm³), then NaBH₃CN (1.6 g). Additional quantities of hydride were added at 16 h (0.6 g), 24 h (0.6 g), and 48 h (0.6 g) with periodic adjustment of pH to 4-5. After 64 h the reaction mixture was heated at reflux for an additional 2.75 h. No starting material 4d (tᵣ 3.1 min) and a single product (5d, tᵣ 4.1 min) were observed by RP HPLC (isocratic: 85%A 15%B. λ = 240 nm). This mixture was adjusted to pH 1 and applied to a cation exchange resin. The resin was washed to neutral with water and eluted with 0.1 M NH₃. The combined elutions were concentrated in vacuo several times with fresh portions of water to give a yellow/white solid (1.690 g), which was recrystallised from water to yield the neutral amino acid 5d as fine microneedle-like crystals (1.170 g, 33%). m.p. >230 °C (decomp.); νₘₐₓ(Nujol, neutral 5d)/cm⁻¹ 3319.9 (COH), 2683.5 and 2436.9 (H₂N⁺), 1619.5 and 1596.4 (COO⁻); δ_H(500 MHz, d₆-DMSO) 1.31 (9 H, s, Bu₃), 3.12 (1 H, dd, J 12.5 and 3.0, CHₓ(OH)CHₓAHB₃N), 2.96 (1 H, dd, J 12.5 and 9.9, CHₓ(OH)CHₓAHB₃N), 4.99 (1 H, dd, J 2.9 and 9.8, CHₓ(OH)CHₓAHB₃N), 6.44 (1 H, br s, CHOH), 7.56 (2 H, s, ArH), 4.56 (2 H, s, ArOCH₂), 8.55 and 9.27 (2 H, br s, NH₂), 13.2 (1 H, br, COOH); δ_C(125 MHz, d₄-MeOH) 26.08 (3 C, NCMe₃), 58.72 (1C,NCMe₃), 49.44 (1C, CH(OH)CH₂N), 69.44 (1C, ArCH(OH)CH₂N), 141.63 (1 C, ArCCl(OH)), 130.65 (2 C, ArCCl), 128.28 (2 C, ArCH), 151.42 (1 C, ArCOCH₂), 70.12 (1 C, ArOCH₂), 171.60 (1 C, HOC=OCH₂); m/z 356 (14%, M + H), 302 (39, M - [Me + H₂O]), 262 (36, M - H₂NBu⁵), 86 (100, [CH₂NHBu⁺]) (Found M⁺ - [Me + H₂O], 302.0376. C₁₄H₁₉Cl₂NO₄ requires M - [Me + H₂O], 302.0351).

3,5-dichloro-4-benzyloxyacetophenone 1e. To a conical flask containing a stirred solution of 3,5-dichloro-4-hydroxyacetophenone (6.000 g, 29.26 mmol) in dry DMP (12 cm³) was added K₂CO₃ (8.04 g, 58.14 mmol) followed by NaI (440 mg) and benzyl chloride (3.372 cm³, 29.26 mmol). The flask was stoppered, covered in foil and stirred at room temperature. The reaction was monitored by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), and after 46.5 h no starting material (tᵣ 4.3 min) and only one product peak (tᵣ 11.0 min) were observed. The reaction mix was diluted with H₂O (100 cm³), the aqueous phase extracted with EtOAc (3 × 100 cm³), the organic extracts washed with brine (3 × 50 cm³), dried (Na₂SO₄) and then concentrated in vacuo to give a light brown oil (9.2 g). Recrystallisation from n-hexane, gave creamy white needle-like crystals of 1e (6.222 g, 72%), m.p. 73-74 °C; νₘₐₓ(Nujol)/cm⁻¹ 1685.5 (ArC=O); δ_H(500 MHz, CDCl₃) 2.54 (3 H, s, CH₃), 7.85 (2 H, s, ArH), 5.12 (2 H, s, C₆H₅CH₂OAr), 7.40 (3 H, m, m- & p-C₆H₅), 7.55 (2 H, d, J 8.1, o-C₆H₅); δ_C(125 MHz, CDCl₃) 26.38 (1 C, C=OCH₃), 130.24 (2 C, ArCCl), 129.11 (2 C, ArCH), 134.08 (1 C, ArC(C=O), 154.91 (1 C, ArCOCH₂), 194.72 (1 C, C=OCH₃), 75.24 (1 C, ArOCH₂), 128.65, 128.56, 128.54 and 135.74 (6 C, C₆H₅CH₂OC=O); m/z 295 (10%, M + H⁺), 294 (29, M), 91 (100, C₇H₇⁺), 43 (10, C=OCH₃⁺) (Found M⁺, 294.0212. C₁₅H₁₂Cl₂O₂ requires M, 294.0214).
3,5-dichloro-4-benzzyloxyphenacyl bromide 2e. A solution of Br₂ in dry CHCl₃ was freshly prepared at a concentration of 1.58 mmol/cm³. To an aluminium foil-covered 50 cm³ round bottom flask containing a magnetic stirring bar was added a solution of 1e (6.851 g, 23.21 mmol) in dry CHCl₃ (15 cm³). One quarter of the theoretical amount of bromine solution (3.653 cm³) was added to the solution and the stoppered flask stirred at room temperature (10-15 min), at which time the tan colour of the reaction mix discharged. The second, third and fourth quarters of the bromine solution were then added rapidly with immediate colour discharge after each addition. The reaction mix was then promptly concentrated in vacuo to give an oil which was taken up in EtOAc (200 cm³) and washed with brine (3 x 75 cm³) until the washings were neutral to pH paper. The organic phase was dried (Na₂SO₄) and concentrated in vacuo to give crude 2e as a light-tan oil (8.325 g, theoretical yield 8.696 g for mono bromination). By RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) this product contained ≈75% 2e (tᵣ 11.7 min), and approximately equal proportions of starting material 1e (tᵣ 10.9 min) and an unidentified by-product (tᵣ 12.6 min). The material was used directly without further purification. In a separate experiment the by-product was shown to increase with further additions of Br₂ at the expense of product 2e.

1-(3,5-dichloro-4-benzzyloxyphenyl)-2-(N-benzyl-N-tert-butylamino)ethanone hydrochloride 3e. A magnetically stirred, 100 cm³ round bottom flask, fitted with a water-cooled condenser and a drying tube (CaCl₂), containing N-benzyl-tert-butylamine (12.90 cm³, 11.368 g, 69.6 mmol) in dry CHCl₃ (25 cm³), was heated in an oil bath at reflux (2 min). To this hot solution was added a solution of crude 2e (8.325 g, ≈75% as phenacyl bromide 2e, 23.2 mmol) in dry CHCl₃ (30 cm³), and set at reflux for 3.75 h. A white precipitate formed after 30 min and the solution turned yellow, and after 3.75 h the solution was an orange colour. Analysis of the mixture by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) showed ≈10-15 % of 2e remained and one major product (tᵣ 10.3 min). In a separate experiment it was found that further heating decreased the yield, so the reaction mixture was cooled, diluted with EtOAc (200 cm³) and filtered to remove N-benzyl-tert-butylamine hydrobromide (5.525 g) as a white solid. The filtrate was then washed with 1 M HCl (2 x 100 cm³), and no product was detected in these aqueous acid washings by RP HPLC analysis. The organic filtrate was further washed with brine (2 x 50 cm³), dried (Na₂SO₄) and concentrated in vacuo to give a brown oil that contained a white precipitate. To this oil was added Et₂O (50 cm³), a yellow/white solid collected by filtration and washed with EtOAc and Et₂O (3.408 g). This solid was stirred in 0.1 M HCl (30 cm³) for 60 min, then in hot EtOAc (50 cm³) for 30 min to yield a clean white solid 3e (3.327 g, 29%). m.p. 145-148 °C (decomp.); RP HPLC (conditions as previously, tᵣ 10.3 min); νₚₐₖₜ(Nujol)/cm⁻¹ 2633.7 (HN⁺), 1694.4 (ArC=O); δH(500 MHz, d₆-DMSO) 1.57 (9 H, s, Bu¹); 4.12 and 4.58 (1 + 1 H, m, J 9.3 and 11.9, and d, J 11.5, NCH₂C₆H₅), 7.13-7.18 (3 H, m, m- & p-C₆H₅CH₂N),
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7.60 (2 H, d, J 6.8, o-C₆H₅CH₂N), 4.90 and 5.15 (1 + 1 H, d, J 18.3, and m, J 7.2 and 18.2, C=OCH₂N), 7.82 (2 H, s, ArH), 5.15 (2 H, s, C₆H₅CH₂OAr), 7.39-7.45 (3 H, m, m-
& p-C₆H₅CH₂O), 7.51 (2 H, d, J 7.6, o-C₆H₅CH₂O), 9.13 (1 H, br s, NH); δC (125 MHz, d₄-MeOH) 25.47 (3 C, NCMe₃), 68.88 (1C, NCMe₃), 55.85 (1C, NCH₂CH₂H₃), 133.76, 131.32, 130.57 and 131.66 (6 C, NCH₂C₆H₅), 56.43 (1C, C=OCH₂N), 131.42 (1 C, ArC(C=O), 130.68 (2 C, ArCCl), 130.27 (2 C, ArCH), 157.43 (1 C, ArCOCH₂), 76.79 (1 C, ArOCH₂), 130.15, 130.06, 129.78 and 137.28 (6 C, C₆H₅CH₂OC=O); m/z 456 (52%, M + H), 440 (9, M - Me), 364 (16, M - C₇H₇), 176 (39, C₆H₅N(Buᵗ)CH₂C₆H₅⁺), 120 (51, CH₂NHCH₂C₆H₅⁺), 91 (100, C₇H₇⁺) (Found M⁺, 455.1367. C₂₆H₂₇Cl₂NO₂ requires M, 455.1419).

1-[(3,5-dichloro-4-hydroxyphenyl)-2-(tert-butylamino)ethaneone hydrochloride 4e. Into a 1 dm³, magnetically stirred 3-necked round bottom flask was placed 3e (3.327 g, 6.6 mmol) and dissolved in EtOH-HOAc (9:1, 300 cm³). The solution was bubbled with nitrogen for 5 min, 5% Pd/C (250 mg) added, and the flask connected to a low pressure hydrogenation apparatus. The system and flask were evacuated and hydrogen gas admitted. The mixture was stirred vigorously and monitored periodically by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), aliquots being centrifuged prior to analysis. After a period of 40 min (H₂ absorption = 325 cm³, theoretical = 323 cm³) only the expected products (4e, tᵣ 1.4 min; toluene, tᵣ 8.75 min) were present. No starting material 3e (tᵣ 10.3 min) was observed. The reaction mixture was then filtered and the filtrate concentrated in vacuo to give a yellow/white solid. This solid was added to EtOAc (100 cm³), heated for 10 min, then cooled and collected by filtration. The solid was recrystallised from 1 M HCl and dried over silica gel to give 4e (2.033 g, 98%) as a clean, white powder. m.p. > 200 °C (decomp.); νmax(Nujol)/cm⁻¹ 2639.5 and 2429.8 (H₂N⁺), 1693.7 (ArC=O); δH (500 MHz, d₆-
DMSO) 1.36 (9 H, s, Buᵗ), 4.63 (2 H, br t, J 5.6 and 6.0, C=OCH₂N), 8.12 (2 H, s, ArH), 9.17 (2 H, br t, J 5.5 NH₂); δC (125 MHz, d₄-MeOH) 26.20 (3 C, NCMe₃), 58.73 (1C, NCMe₃), 48.53 (1C, C=OCH₂N), 190.31 (1C, C=OCH₂N), 127.87 (1 C, ArC(C=O), 124.11 (2 C, ArCCl), 130.46 (2 C, ArCH), 156.62 (1 C, ArCOH); m/z 276 (7%, M + H), 260 (90, M - Me), 242 (17, M - [Me + H₂O]), 236 (33), 189 (4, M - CH₂NHBuᵗ), 86 (100, [CH₂NHBuᵗ]⁺) (Found M⁺, 275.0478. C₁₅H₁₅Cl₂NO₂ requires M, 275.0480).

1-[(3,5-dichloro-4-hydroxyphenyl)-2-(tert-butylamino)ethanol hydrochloride 5e. To a 150 cm³ round bottom flask was added a solution of 4e (1.000 g, 3.20 mmol) in aqueous NaOH (0.001 M, 100 cm³). To this magnetically stirred solution was added NaBH₄ (200 mg), a further portion (250 mg) added at 1.25 h, and the solution stirred overnight. Further portions of NaBH₄ (total 800 mg) were added between 17 and 23 h. After 48 h no starting material 4e (tᵣ 3.3 min) and only one product 5e (tᵣ 2.8 min) were observed by RP HPLC (isocratic: 80% A 20%B. λ = 240 nm). The solution was diluted with H₂O (50 cm³), adjusted to pH = 9,
and a white precipitate collected by filtration. Recrystallisation from 1 M HCl yielded 5e as fine microneedle-like crystals (0.969 g, 96%). m.p. 219-221 °C (lit.314 229-230 °C); \( \nu_{\text{max}}(\text{Nujol})/\text{cm}^{-1} \) 3453, 3359.4 and 3210.9 (OH), 2665.7 and 2435.0 (H\(_2\)N\(^+\)); \( \delta_{\text{H}} \) (500 MHz, d\(_2\)-DMSO) 1.30 (9 H, s, Bu\(^t\)), 3.05 (1 H, dd, J 12.5 and 3.2, CH\(_X\)(OH)CH\(_A\)H\(_B\)N), 2.91 (1 H, dd, J 12.5 and 9.8, CH\(_X\)(O H)CH\(_A\)H\(_B\)N), 4.93 (1 H, dd, J 3.0 and 9.7, CH\(_X\)(OH)CH\(_A\)H\(_B\)N), 6.28 (1 H, br, CHO\(\text{OH}\)), 7.41 (2 H, s, Ar\(\text{H}\)), 9.26 and 9.45 (2 H, br, NH\(_2\)); \( \delta_{\text{C}} \) (125 MHz, d\(_4\)-MeOH) 26.12 (3 C, NCMe\(_3\)), 58.65 (1C,NCMe\(_3\)), 49.69 (1C, CH(OH)CH\(_2\)N), 69.58 (1C, ArCH(OH)CH\(_2\)N), 135.54 (1 C, ArCCH(OH)), 123.81 (2 C, Ar\(\text{C}l\)), 127.56 (2 C, Ar\(\text{CH}\)), 150.73 (1 C, Ar\(\text{COH}\); m/z 278 (20%, M + H), 244 (20, M - [Me + H\(_2\)O]), 86 (100, [CH\(_2\)NHBut\(^t\)+]) (Found M\(^+\) - [Me + H\(_2\)O], 244.0297. C\(_{12}\)H\(_{17}\)Cl\(_2\)NO\(_2\) requires M - [Me + H\(_2\)O], 244.0296).

**Ethyl 6-(4-acetyl-2,6-dichlorophenoxy)hexanoate 1f.** To a conical flask containing a stirred solution of 3,5-dichloro-4-hydroxyacetophenone (4.425 g, 21.58 mmol) in dry DMF (9 cm\(^3\)) was added K\(_2\)CO\(_3\) (6.00 g, 43.39 mmol) followed by NaI (354 mg) and ethyl 6-bromohexanoate (3.199 cm\(^3\), 17.97 mmol). The flask was stoppered, covered in foil and stirred at room temperature. The reaction was monitored by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. \( \lambda = 260 \) nm), and at 168 h the single product (\( t_R \ 11.5 \) min) to starting material (\( t_R \ 4.4 \) min) ratio had maximised. The reaction mixture was diluted with H\(_2\)O (50 cm\(^3\)), extracted with EtOAc (1 x 200 cm\(^3\), 1 x 100 cm\(^3\), 1 x 50 cm\(^3\)), the extracts washed with brine (3 x 50 cm\(^3\)), dried (Na\(_2\)SO\(_4\)) and concentrated in vacuo to give crude 1f as a light brown oil (6.289 g, theoretical yield 6.239 g) which resisted crystallisation from a variety of organic solvents. RP HPLC (conditions as previously, \( t_R = 11.5 \) min); \( \nu_{\text{max}}(\text{Nujol})/\text{cm}^{-1} \) 1733.9 (O-(C=O)), 1692.4 (ArC=O); \( \delta_{\text{H}} \) (500 MHz, CDCl\(_3\)) 2.54 (3 H, s, CH\(_3\)), 7.85 (2 H, s, Ar\(\text{H}\)), 4.06 (2 H, t, J 6.4, (CH\(_2\))\(_2\)CH\(_2\)OAr), 2.33 (2 H, t, J 7.4, EtO\(_2\)CC\(_2\)H\(_2\)), 1.56, 1.71 and 1.86 (2 + 2 + 2 H, m, CH\(_2\)(CH\(_2\))\(_3\)CH\(_2\)), 4.11 (2 H, q, J 7.1, CH\(_3\)CH\(_2\)OC=O), 1.24 (3 H, t, J 7.1, CH\(_3\)CH\(_2\)OC=O); \( \delta_{\text{C}} \) (125 MHz, CDCl\(_3\)) 26.31 (1 C, C=OCH\(_3\)), 129.86 (2 C, ArC\(_{\text{Cl}}\)), 129.00 (2 C, Ar\(\text{CH}\)), 133.68 (1 C, ArC(C=O)), 155.46 (1 C, Ar\(\text{COCH}\)), 194.67 (1 C, C=OCH\(_3\)), 73.71 (1 C, ArOCH\(_2\)), 24.61, 25.30, 29.67 (3 C, CH\(_2\)(CH\(_2\))\(_3\)CH\(_2\)), 34.15 (1 C, EtOC=OCH\(_2\)), 173.50 (1C, EtOOC=O), 60.17 (1C, CH\(_3\)CH\(_2\)OC=O), 14.18 (1C, CH\(_3\)CH\(_2\)OC=O); m/z 347 (18%, M + H), 301 (82, M - EtO), 143 (84, [EtO\(_2\)C(CH\(_2\))\(_3\)+], 69 (100), 43 (45, C=OCH\(_3\)+) (Found M\(^+\), 346.0738. C\(_{16}\)H\(_{20}\)Cl\(_2\)O\(_4\) requires M, 346.0739).

**Ethyl 6-(4-bromoacetyl-2,6-dichlorophenoxy)hexanoate 2f.** A solution of Br\(_2\) in dry CHCl\(_3\) was freshly prepared at a concentration of 2.97 mmol/cm\(^3\). To an aluminium foil-covered 100 cm\(^3\) round bottom flask containing a magnetic stirring bar was added a solution of crude 1f (4.741 g, 13.47 mmol) in dry CHCl\(_3\) (50 cm\(^3\)). One quarter (1.150 cm\(^3\)) of the theoretical amount of bromine solution was then added to the light tan-coloured solution, and
the stoppered flask stirred at room temperature (≈7 h) at which time the tan colour of the reaction mixture discharged. The second quarter of bromine solution was then added with immediate colour discharge. The reaction mixture was promptly concentrated in vacuo to give an oil that was taken up in EtOAc (150 cm³) and washed with brine (3 x 50 cm³) until the washings were neutral to pH paper. The organic phase was dried (Na₂SO₄) and concentrated in vacuo to give a crude light tan oil. The oil was redissolved in dry CHCl₃ (30 cm³), the third quarter of the bromine solution added rapidly and after colour discharge (≈6 h), the fourth quarter added. The reaction mixture was then promptly concentrated in vacuo to give an oil which was taken up in EtOAc (150 cm³) and washed with brine (3 x 50 cm³) until the washings were neutral to pH paper. The organic phase was dried (Na₂SO₄) and concentrated in vacuo to give crude 2f as a light-tan oil (5.914 g, theoretical yield 5.740 g for mono bromination). This oil resisted crystallisation from a variety of solvents. Analysis by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) showed this product contained ≈77% 2f (tᵣ 10.5 min), with < 5% of starting material 1f (tᵣ 11.3 min) and ≈18% of an unidentified by-product (tᵣ 11.8 min). The material was used directly without further purification.

**Ethyl 6-(4-(N-benzyl-N-tert-butylaminoacetyl)-2,6-dichlorophenoxy)hexanoate hydrochloride 3f.** A magnetically stirred 150 cm³ round bottom flask fitted with a water-cooled condenser and a drying tube (CaCl₂), containing N-benzyl-tert-butylamine (7.489 cm³, 6.598 g, 40.4 mmol) in dry CHCl₃ (5 cm³), was heated in an oil bath at reflux (2 min). To this hot solution was added a solution of crude 2f (5.914 g, 77% as phenacyl bromide 2f, 10.4 mmol) in dry CHCl₃ (10 cm³), and set at reflux for 1.5 h. A white precipitate formed after 15 min and the solution had turned yellow. After 1.5 h a further portion of amine (1.00 cm³) was added and heating continued for a further 60 min. Analysis of the mixture by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm) showed approximately 5 % of 2f remaining and one major product (tᵣ 10.7 min). The resulting burnt orange reaction mixture was cooled, diluted with EtOAc (150 cm³) and filtered to remove N-benzyl tert-butylamine hydrobromide (3.425 g) as a white solid. The filtrate was washed with 1 M HCl (3 x 75 cm³) and brine (3 x 50 cm³), dried (Na₂SO₄) and concentrated in vacuo to give a brown oil containing a white precipitate (8.651 g). No product was detected in the aqueous acid washings by RP HPLC. The brown oil was heated in Et₂O (100 cm³), cooled, and the orange supernatant decanted. The remaining white solid was heated in a fresh portion of Et₂O (50 cm³), recovered by filtration, dissolved in CHCl₃, concentrated in vacuo, and the product recovered as an amorphous pale yellow solid 3f (3.250 g, 44%). m.p. 134-139 °C; RP HPLC (conditions as previously, tᵣ = 10.7 min); ν max (Nujol)/cm⁻¹ 2610.3 (HN⁺), 1735.4 (O-(C=O)), 1682.9 (ArC=O); δ H (500 MHz, d₆-DMSO) 1.56 (9 H, s, Bu⁻), 4.13 and 4.58 (1 + 1 H, dd, J 9.1 and 12.2, and d, J 10.8, NCH₂C₆H₅), 7.13-7.17 (3 H, m, m- & p-C₆H₅CH₂N), 7.60 (2 H, br t, J 4.1 and 3.4, o-C₆H₅CH₂N), 4.19 and 5.17 (1 + 1 H,
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d, J 18.4, and dd, J 7.1 and 18.6, (C=OCH₂N), 7.81 (2 H, s, ArH), 4.05 (2 H, t, J 6.3, (CH₂)₂CH₂OAr), 2.32 (2 H, t, J 7.2, EtO₂CCH₂), 1.55 and 1.77 (2 + 4 H, m, CH₂(CH₂)₃CH₂), 4.05 (2 H, q, J 7.1, CH₃CH₂OC=O), 1.17 (3 H, t, J 7.1, CH₃CH₂OC=O), 9.13 (1 H, br s, NH); δ_C(125 MHz, d₄-MeOH) 25.16 (3 C, NCMe₃), 68.63 (1C,NCMe₃), 55.55 (1C, NCH₂C₆H₅), 133.48, 131.02, 130.29 and 130.37 (6 C, NCH₂C₆H₅), 56.19 (1C, C=OCH₂N), 190.12 (1C, C=OCH₂N), 130.86 (1 C, ArC(C=O), 130.37 (2 C, ArCCl), 129.97 (2 C, ArCH), 157.90 (1 C, ArCOCH₂), 75.15 (1 C, ArOCH₂), 25.73, 26.41, 30.77 (3 C, CH₂(CH₂)₃CH₂), 34.98 (1 C, EtOC=OCH₂), 175.32 (1C, EtOC=O), 61.38 (1C, CH₃CH₂OC=O), 14.54 (1C, CH₃CH₂OC=O); m/z 508 (84%, M + H), 492 (28, M - Me), 462 (60, M - EtO), 416 (50, M - C₇H₇), 176 (95, CH₂N(Bu)+CH₂C₆H₅+), 120 (95, CH₂NCH₂C₆H₅+), 91 (100, C₇H₇+) (Found M⁺, 507.1923. C₂₇H₃₅Cl₂NO₄ requires M, 507.1943).

Ethyl 6-(4-(tert-butylaminoacetyl)-2,6-dichlorophenoxy)hexanoate hydrochloride 4f. Into a 1 dm³ magnetically stirred 3-necked round bottom flask was placed 3f (2.611 g, 4.79 mmol) and EtOH-HOAc (9:1, 250 cm³). The solution was bubbled with nitrogen for 5 min, 5% Pd/C (250 mg) added, and the flask connected to a low pressure hydrogenation apparatus. The system and flask were evacuated and hydrogen gas admitted. The mixture was stirred vigorously and monitored periodically by RP HPLC (gradient program: 55% A 45% B to 100% B: 5 min. Hold 100% B: 10 min. λ = 260 nm), aliquots being centrifuged prior to analysis. After a period of 55 min (H₂ absorption = 95 cm³, theoretical = 118 cm³) only the expected products (4f, t_R 2.6 min; toluene, t_R 8.75 min) were present. The reaction mixture was then filtered, the filtrate concentrated in vacuo to give a yellow oil, which was stirred in Et₂O (250 cm³) overnight. This mixture was then filtered to give a waxy, clean white solid 4f (1.634 g, 75%). m.p. 142-150 °C; ν_max(Nujol)/cm⁻¹ 2623.5 and 2416.9 (H₂N⁺), 1735.3 (O=C=O), 1703.3 (ArC=O); δ_H(500 MHz, δ₄-DMSO) 1.36 (9 H, s, Bu), 4.70 (2 H, br t, J 5.6, C=OCH₂N), 8.22 (2 H, s, ArH), 4.09 (2 H, t, J 6.3, (CH₂)₂CH₂OAr), 2.32 (2 H, t, J 7.2, EtO₂CCH₂), 1.56 and 1.79 (4 + 2 H, m, CH₂(CH₂)₃CH₂), 4.05 (2 H, q, J 7.2, CH₃CH₂OC=O), 1.18 (3 H, t, J 7.2, CH₃CH₂OC=O), 9.16 (2 H, br s, NH₂); δ_C(125 MHz, d₄-MeOH) 25.89 (3 C, NCMe₃), 58.52 (1C,NCMe₃), 48.60 (1C, C=OCH₂N), 190.63 (1C, C=OCH₂N), 132.00 (1 C, ArC(C=O), 131.35 (2 C, ArCCl), 130.45 (2 C, ArCH), 157.67 (1 C, ArCOCH₂), 75.13 (1 C, ArOCH₂), 25.74, 26.44, 30.80 (3 C, CH₂(CH₂)₃CH₂), 35.00 (1 C, EtOC=OCH₂), 175.32 (1C, EtOC=O), 61.38 (1C, CH₃CH₂OC=O), 14.55 (1C, CH₃CH₂OC=O); m/z 418 (55%, M + H), 402 (53, M - Me), 316 (59), 86 (100, [CH₂NHBu]+) (Found M⁺, 417.1453. C₂₀H₂₉Cl₂NO₄ requires M, 417.1474).

6-(4-(tert-butylaminoacetyl)-2,6-dichlorophenoxy)hexanoic acid hydrochloride 4g. To a 100 cm³ round bottom flask was added 4f (1.921 g, 4.2 mmol) and aqueous HCl (1 M, 30 cm³), a reflux condenser fitted and the mixture set at reflux for 2 h, then left overnight at room
temperature. A yellow/white solid was collected by filtration, stirred in Et2O (35 cm3), and refiltered to yield a white solid. This solid was recrystallised from aqueous acid and dried over KOH to give 4g as a white solid (1.284 g, 71%), m.p. =160-168 °C; RP HPLC (isocratic 50%A 50%B λ=260 nm, tR 2.0 min); \( v_{\text{max}}(\text{Nujol})/\text{cm}^{-1} \) 2627.2 and 2422.3 (H2N\(^+\)), 1720.7 (COOH), 1703.0 (Ar=C=O); \( \delta_H(\text{MHz}, \text{d}_6\text{-DMSO}) \) 1.36 (9 H, s, Bu\(^t\)), 4.70 (2 H, br s, C=OCH2N), 8.22 (2 H, s, ArH), 4.09 (2 H, t, J 6.3, (CH2)3CH2OAr), 2.25 (2 H, t, J 7.0, HO2CC CH2), 1.55 and 1.80 (4 + 2 H, m, CH2(CH2)3CH2), 9.19 (2 H, br s, NH2), 12.03 (1 H, br s, COOH); \( \delta_C(\text{MHz}, \text{d}_4\text{-MeOH}) \) 25.90 (3 C, NCMe3), 58.51 (1C,NCMe3), 48.60 (1C, C=OCH2N), 190.63 (1C, C=OCH2N), 131.96 (1 C, ArC(O)=O), 131.37 (2 C, ArCCl), 130.43 (2 C, ArCH), 157.88 (1 C, ArCOCH2), 75.15 (1 C, ArOCH2), 25.76, 26.46, 30.83 (3 C, CH2(CH2)3CH2), 34.84 (1 C, HOC=OCH2), 177.34 (1C, HOC=O); m/z 390 (43%, M + H), 374 (74, M - Me), 86 (100, [CH2NHBu\(^t\)]\(^+\)). (Found M\(^+\), 389.1152. C18H25Cl2NO4 requires M, 389.1161).

6-(4-(tert-butylamino-1-hydroxyethyl)-2,6-dichlorophenoxy)hexanoic acid hydrochloride 5g. To a magnetically stirred solution of 4g (1.08 g, 2.53 mmol) in aqueous HCl (10-5 M, 5 cm3) and THF (8 cm3) was added NaBH3CN (200 mg). Further portions were added at 2 h (200 mg) and at 27 h (200 mg). No starting material (tR 2.0 min) and only one product (tR 1.3 min) was observed by RP HPLC (isocratic: 50%A 50%B. \( \lambda = 240 \text{ nm} \)) after 69 h. THF was removed in vacuo, and the solution diluted with water (35 cm3). This mixture was then stirred with CH2Cl2 (30 cm3) for 30 min, yielding a white solid insoluble in either phase. The solid was collected by filtration (794 mg wet) and recrystallised from 1 M HCl to yield 5g as a white solid (410 mg). A second crop from the mother liquors was also collected (115 mg) and combined with the first crop (total 48%). m.p. 67-71 °C; \( v_{\text{max}}(\text{KBr})/\text{cm}^{-1} \) 3431.0, 2672.4 and 2525.9 (H2N\(^+\)), 1732.8 (COOH); \( \delta_H(\text{MHz}, \text{d}_6\text{-DMSO}) \) 1.30 (9 H, s, Bu\(^t\)), 3.12 (1 H, dd, J 12.5 and 2.9, CHX(OH)CH\(_2\)HBN), 2.96 (1 H, dd, J 12.4 and 10.1, CH\(_X\)O(CH\(_2\))CH\(_2\)NB), 4.95 (1 H, dd, J 2.7 and 9.8, CH\(_X\)C(OH)CH\(_2\)BN), 6.38 (1 H, br s, CHOH), 7.55 (2 H, s, ArH), 3.96 (2 H, t, J 6.3, (CH2)3CH2OAr), 2.24 (2 H, t, J 7.0, HO2CCCH2), 1.53 and 1.63 (4 + 2 H, m, CH2(CH2)3CH2), 8.49 and 9.14 (2 H, br s, NH2), 12.1 (1 H, br, COOH); \( \delta_C(\text{MHz}, \text{d}_4\text{-MeOH}) \) 26.09 (3 C, NCMe3), 58.69 (1C,NCMe3), 49.51 (1C,Ch(OH)CH2N), 69.48 (1C, CH(OH)CH2N), 140.71 (1 C, ArCC(OH)), 130.95 (2 C, ArCCl), 128.13 (2 C, ArCH), 152.77 (1 C, ArCOCH2), 74.76 (1 C, ArOCH2), 25.85 and 31.09 (3 C, CH2(CH2)3CH2), 35.16 (1 C, HOC=OCH2), 177.73 (1 C, HOC=OCH2); m/z 392 (11%, M + H), 358 (57, M - [Me + H2O]), 318 (9, M - H2NBU\(^t\)), 279 (54), 244 (93), 86 (100, [CH2NHBU\(^t\)]\(^+\)). (Found M\(^+\) + H, 392.1370. C18H27Cl2NO4 requires M + H, 392.1395; found M\(^+\) - [Me + H2O], 358.0975. C18H27Cl2NO4 requires M - [Me + H2O], 358.0977).
Conjugation of 5d to bovine serum albumin (BSA) by intermediate active ester formation. BSA (100 mg) was dissolved in a sodium phosphate buffer (0.1 M, pH 8, 2.0 cm³) and 4:1 THF-DMF (1.68 cm³). Compound 5d (HCl salt, 20 mg, 59 μmol) was dissolved in DMF (40 mm³) and THF (160 mm³) in a centrifuge tube (Eppendorf, 1.5 cm³) and a magnetic stirring bar added. To this solution of hapten 5d was added NHS (7.5 mg, 65 μmol) and a solution of DCC in THF (1 M, 59 mm³, 59 μmol) and the solution stirred vigorously for 20 min. The reaction mixture was centrifuged (2 min) to remove dicyclohexylurea, and an aliquot of the supernatant (128 mm³; 38 μmol of 5d) added dropwise to the stirred BSA solution. The solution was stirred at room temperature for a further 2 h, the pH adjusted to 7.2, and allowed to react overnight. This solution was diluted with H₂O (50 cm³) and the THF removed in vacuo prior to dialysis, initially against H₂O (8 dm³). After 30 h a white precipitate had formed, which was resolubilized by dialysis against a sodium phosphate buffer (10 mM, pH 7.2, 8 dm³), with regular exchanges for 3 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (145 mg).

Conjugation of compound 5d to gelatin by intermediate active ester formation. Gelatin (Bloom 300, 100 mg) was dissolved in a sodium phosphate buffer (0.1 M, pH 8, 2.0 cm³) and 4:1 THF-DMF (1.68 cm³). Compound 5d (HCl salt, 20 mg, 59 μmol) was dissolved in DMF (40 mm³) and THF (160 mm³) in a centrifuge tube (Eppendorf, 1.5 cm³) and a magnetic stirring bar added. To this solution of hapten was added NHS (7.5 mg, 65 μmol) and a solution of DCC in THF (1 M, 56 mm³, 59 μmol), and the solution stirred vigorously for 20 min. The reaction mixture was centrifuged (2 min) to remove dicyclohexylurea, and an aliquot of the supernatant (128 mm³; 38 μmol of 5d) added dropwise to the warm (40 °C), stirred gelatin solution. This solution was slowly cooled to room temperature, and stirred for 24 h. This solution was diluted with H₂O (50 cm³) and dialysed against H₂O (8 dm³) with regular exchanges for 4 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (96 mg).

Conjugation of compound 5d to bovine serum albumin (BSA) using a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). BSA (100 mg) was dissolved in H₂O (2.4 cm³) adjusted to pH 6.5 with saturated aqueous NaHCO₃. Compound 5d (HCl salt, 100 mg, 297 μmol) was dissolved in H₂O (4.0 cm³) and DMF (1.88 cm³), adjusted to pH 6.5 with saturated aqueous NaHCO₃, and added to the protein solution. To this mixture was added ECDI (1 g) and the mixture stirred whilst pH was maintained at ≈6 with addition of 1 M HCl. After 3 h a further portion of ECDI (1 g) was added and the solution stirred overnight. This solution was diluted with H₂O (75 cm³) and dialysed against H₂O (8 dm³) with regular exchanges for 4 d. Filtration of the slightly cloudy mixture, followed by lyophilisation of the dialysate yielded the conjugate as a white solid (100 mg).
Conjugation of compound 5d to ethylenediamine-modified bovine serum albumin (ED-BSA) using a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). The carboxylic groups of bovine serum albumin (BSA, Fraction V) were modified to an N-aminoethylcarboxamide moiety by reaction with ECDI (0.1 M) in the presence of a large excess of ethylenediamine (1.0 M) at pH 4.7 essentially according to a general procedure for the diimide modification of protein carboxyl groups. The modified protein (ED-BSA) contained twice the number of exposed amino groups as BSA, and was kindly donated by Dr. Robin Rigby (CSIRO DAP).

ED-BSA (50 mg) was dissolved in H_2O (1.2 cm^3) and adjusted to pH 7 with saturated aqueous NaHCO_3. Compound 5d (HCl salt, 50 mg, 149 µmol) was dissolved in H_2O (3.0 cm^3) and DMF (1.3 cm^3), adjusted to pH 6.5 with saturated aqueous NaHCO_3, and added to the protein solution. To this mixture was added ECDI (500 mg) and the mixture stirred whilst pH was maintained at 6.25 with addition of 1 M HCl. After 1.5 h a further portion of ECDI (500 mg) was added, the pH adjusted to 6.5 and the solution stirred overnight. This solution was diluted with H_2O (50 cm^3) and dialysed against H_2O (8 dm^3) with regular exchanges for 4 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (47 mg).

Conjugation of compound 5d to gelatin using a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride). Gelatin (Bloom 300, 50 mg) was dissolved in H_2O (1.025 cm^3), then set in a water bath (≈40 °C). DMF (0.475 cm^3) was added with stirring, and the solution adjusted to pH 6.5 with saturated aqueous NaHCO_3. Compound 5d (HCl salt, 50 mg, 149 µmol) was dissolved in H_2O (2.0 cm^3) and DMF (2.0 cm^3), adjusted to pH 6.5 with saturated aqueous NaHCO_3, and added to the protein solution. To this mixture was added ECDI (500 mg) and the mixture stirred whilst pH was maintained at ≈6 with addition of 1 M HCl. After 8 h a further portion of ECDI (500 mg) was added and the solution stirred overnight. This solution was diluted with H_2O (50 cm^3) and dialysed against H_2O (8 dm^3) with regular exchanges for 4 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (42 mg).

Conjugation of compound 5g to bovine serum albumin (BSA) using intermediate active ester formation. BSA (100 mg) was dissolved in a sodium phosphate buffer (0.1 M, pH 8, 2.0 cm^3) and 4:1 THF-DMF (1.68 cm^3). Compound 5g (HCl salt, 34.5 mg, 88 µmol) was dissolved in DMF (60 mm^3) and THF (240 mm^3) in a centrifuge tube (Eppendorf, 1.5 cm^3) and a magnetic stirring bar added. To this solution of hapten 5g was added NHS (12.8 mg, 111 µmol) and a solution of DCC in THF (1 M, 88 mm^3, 88 µmol) and the solution stirred vigorously for 20 min. The reaction mixture was centrifuged (2 min) to remove dicyclohexylurea, and an aliquot of the supernatant (128 mm^3; 38 µmol of 5g) added dropwise to the stirred BSA solution and allowed to react overnight. This solution was diluted with H_2O
(40 cm³) and dialysed, initially against H₂O (8 dm³). After 48 h a white precipitate had formed, which was resolubilized by dialysis against a sodium phosphate buffer (10 mM, pH 7.2, 8 dm³), with regular exchanges for 3 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (142 mg).

**Conjugation of compound 5g to gelatin using intermediate active ester formation.** Gelatin (Bloom 300, 100 mg) was dissolved in a sodium phosphate buffer (0.1 M, pH 8, 2.0 cm³) and 4:1 THF-DMF (1.68 cm³). Compound 5g (HCl salt, 34.5 mg, 88 μmol) was dissolved in DMF (60 mm³) and THF (240 mm³) in a centrifuge tube (Eppendorf, 1.5 cm³) and a magnetic stirring bar added. To this solution of hapten 5g was added NHS (12.8 mg, 111 μmol) and a solution of DCC in THF (1 M, 88 mm³, 88 μmol) and the solution stirred vigorously for 20 min. The reaction mixture was then centrifuged (2 min) to remove dicyclohexylurea, and an aliquot of the supernatant (128 mm³; 38 μmol of 5g) added dropwise to the warm (40 °C), stirred gelatin solution. This solution was slowly cooled to room temperature and stirred for 24 h. This solution was diluted with H₂O (40 cm³) and dialysed, initially against H₂O (8 dm³). After 48 h a white precipitate had formed, which was resolubilized by dialysis against a sodium phosphate buffer (10 mM, pH 7.2, 8 dm³), with regular exchanges for 3 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (131 mg).

**Conjugation of compound 5g to bovine serum albumin (BSA) using a water-soluble carbodiimide.** BSA (100 mg) was dissolved in a sodium phosphate buffer (0.2 M, pH 8, 2.0 cm³). Compound 5g (HCl salt, 100 mg, 250 μmol) was dissolved in DMF (80 mm³), THF (320 mm³) and a sodium phosphate buffer (10 mM, pH 5, 600 mm³), and the solution adjusted to pH ≈5 with saturated aqueous NaHCO₃. To this solution of hapten 5g was added ECDI (1000 mg), the solution allowed to stand at room temperature for 2 min, then added dropwise over a period of 5 min to the protein solution (final pH 8.4). This turbid mixture was stirred for 30 min, then adjusted to pH 6.5 with 1 M HCl, giving a clearer solution. A further portion of ECDI (1000 mg) was added and the solution stirred overnight. This solution was diluted with H₂O (50 cm³) and dialysed against H₂O (8 dm³) with regular exchanges for 4 d. Lyophilisation of the dialysate yielded the conjugate as a white solid (96 mg).
CHAPTER 3 - PHYSIOLOGICAL STUDY OF O-ALKYL CLENBUTEROL ANALOGUES IN RODENTS

3.1 Introduction

3.1.1 Aim

The aim of this study of the *in vivo* physiological effects of CB and the O-alkyl analogues VUF 8303 (5e), O-Methyl VUF 8303 (5c), C2 Hapten (5d) and C6 Hapten (5g) (synthesised in Chapter 2) was:

a) to characterise the effects of these analogues on whole body growth (weight gain and carcass composition), skeletal and non-skeletal tissue, the skeletal muscle $\beta_2$-ARs (receptor density and affinity) and a metabolic hormone (insulin) and major metabolites (glucose and fatty acids).

b) to evaluate the effects on physiological function of changes in chemical structure of CB, and the implications for the use of these CB analogues in the generation of anti-idiotypes that bear the physiological “internal image” of CB.

3.1.2 Background

Clenbuterol (CB), a $\beta$-adrenergic agonist ($\beta$AA), has growth-promoting effects in a variety of species (refer Chapter 1). Specifically, a reduction in adipose tissue deposition and a concomitant increase in protein accretion has produced leaner carcasses, and led to the description of clenbuterol as a sympathomimetic energy-repartitioning agent. Characterisation of other whole body effects of $\beta$AAs like skeletal muscle hypertrophy and increased weight gain have also been reported (refer Chapter 1). Other $\beta$AAs, such as cimaterol (CIM) and ractopamine (RAC), have also shown similar repartitioning effects.

It has been found that physiological effects of hormones can be mimicked immunologically by anti-idiotypic antibodies (for review see). These antibodies have been said to bear the internal image of the original hormone. Research in this laboratory has sought to generate anti-idiotypic antibodies that bear the “internal image” of CB, with respect to energy-repartitioning effects. This research initially required production of specific anti-CB antibodies, by immunisation with a CB-protein conjugates (*i.e.* the CB-protein conjugate is used as the antigen). Consideration of alternative conjugation strategies led to the design and synthesis of O-alkyl analogues of CB, for the purpose of forming protein conjugates (refer Chapter 2).
These O-alkyl analogues, C2 hapten 5d and C6 hapten 5g, and the two model compounds, VUF 8303 5e and O-methyl VUF 8303 5c, and CB are shown in Figure 3.1.

\[ \text{Z} = \text{OH (VUF 8303, 5e)} \\
= \text{OCH}_3 \text{ (O-Methyl VUF 8303, 5e)} \\
= \text{OCH}_2\text{CO}_2\text{H (C2 Hapten, 5d)} \\
= \text{O(CH}_3\text{CO}_2\text{H (C6 Hapten, 5g)} \\
= \text{NH}_2 \text{ (Clenbuterol)} \]

**Figure 3.1** Clenbuterol and O-alkyl analogues

The use, therefore, of the haptenic CB analogues for the generation of anti-idiotypes that may mimic the physiological effects of CB necessitates the in vivo characterisation of these analogues in comparison to CB, the parent drug. Furthermore, three of the four CB analogues were new (i.e. hapten 5d, 5g and model compound 5c), and the physiological actions of these compounds have not been previously characterised. Therefore, this study also provided the opportunity to investigate the effect on physiological function of chemical modifications to the structure of CB, for a simple series of CB analogues in which the aromatic amine was replaced with a hydroxyl or O-alkyl substituent. Rats provided a convenient animal model to evaluate the physiological effects of the CB analogues since they are inexpensive, easily handled, and experiments using them require only small amounts of the compounds.

The physiological effects of CB in rats have been previously reported in several studies.\(^{11,15,17,18,25,28,29,32,381}\) The present protocol was based on these studies with respect to the dose, duration of treatment, and range of tissues taken at the conclusion of the treatment. The hindlimb skeletal muscles taken were representative of the different muscle types, whilst non-skeletal tissues contained different populations of β-adrenoceptor (β-AR) subtypes. Investigations of the effects of βAA growth promotants on blood levels of energy substrates and metabolic hormones have been inconclusive\(^{23,25,53,59,60,382}\) so only a single blood sample was taken for this purpose (post mortem).

Skeletal muscle ARs are thought to be predominantly β\(_2\),\(^{383,384}\) and the muscle hypertrophic effects of CB are thought to be β\(_2\)-mediated.\(^{26,381}\) Therefore, the effects of CB and the four analogues on the characteristics of the β\(_2\)-AR in hindlimb muscles, namely receptor density (B\(_{\text{max}}\)) and affinity (K\(_D\)), were also investigated in the present study. Similar characterisations of the β\(_2\)-ARs following chronic treatment with CB, isoprenaline and terbutaline have been previously reported.\(^{26,381,385}\)
3.2 Materials and Methods

Suppliers of materials, equipment and animals are given in Appendix 1.

3.2.1 Experimental Animals

Female Wistar rats weighing 175.7 ± 1.3 g were used for this study. The animals had free access to food and water, *ad libitum*, and were housed three per box with a 12:12 hr light:dark cycle. Lights were switched on at 0600 hr. The study was replicated with n=3 animals per compound per replicate (total n=6 per compound). Prior to commencement of the injections, the rats were individually adapted to both human contact and the experimental environment by removing and weighing the animals, then readmitting them to their boxes, every other day for 12 days. At the end of this 12 day settling period the bodyweight (BW) of the animals was 203.6 ± 1.5 g.

3.2.2 Study Protocol

This experiment was approved by the CSIRO DAP Animal Care and Experimental Ethics Committee (Protocol No. 93049).

The animals were given daily subcutaneous injections (0.2 cm³, in saline) of either CB (1 mg/kg BW CB free base), one of the four structural analogues (molar equivalent of 1 mg/kg BW CB free base) or saline (n=6 per group) for 22 days. Animals were weighed daily and the weight recorded.

On Day 23, the animals were killed by stunning and cervical dislocation. The following tissues were then removed, washed in saline, blotted and weighed, then frozen immediately in liquid nitrogen before storage at -70 °C:

**Hindlimb Skeletal Muscles** (representative of the different muscle types):

a) Soleus (Sol): mainly slow-twitch, oxidative muscle fibres
b) Plantaris (Plant) and Extensor Digitorum Longus (EDL): mixture of fast-twitch, oxidative-glycolytic and glycolytic fibres
c) Gastrocnemius (Gastroc): mixture of fast-twitch, oxidative-glycolytic and slow-twitch oxidative fibres
d) Quadriceps (Quads): mainly fast-twitch oxidative-glycolytic fibres
Non-Skeletal Tissues (which are known to contain β-ARs):

   e) Heart
   f) Lungs
   g) Kidneys
   h) Interscapular Brown Adipose Tissue
   i) Liver

A sample of trunk blood (1-2 cm³) was also taken, stored at 4 °C prior to centrifugation and collection of the plasma on the same day, and finally stored at -20 °C. The remainder of the gastrointestinal tract was removed along with the head, paws, tail and skin, and the carcass weighed before storage at -70 °C.

3.2.3 Analytical Methods

3.2.3.1 Total Carcass Fat

The frozen carcasses were dried in vacuo by lyophilisation, weighed, homogenized in a commercial blender and stored in sealed glass jars. Total Fat determinations were carried out using a chloroform/methanol extraction procedure on subsamples oven-dried overnight (100 °C) to constant weight. Briefly, samples (singlets) were extracted at reflux with 2:1 chloroform-methanol (v/v) for four hours in a Soxhlet apparatus. Total Fat was calculated as below (on a dry matter basis):

\[
\text{Total Fat (\%) = \{[Weight \ Fat] ÷ [Sample Weight (post oven dry)]\} \times 100}
\]

3.2.3.2 Total Carcass Protein

Whole body protein content was estimated on samples of the dried carcass from the nitrogen (N) content (protein being N X 6.25) on a dry matter basis. Briefly, the frozen carcasses were dried in vacuo, weighed, homogenized in a commercial blender and stored in sealed glass jars. Total Protein determinations were measured by the Kjeldahl method on a Kjitec Auto 1030 Analyzer interfaced with a personal computer. The samples (in triplicate) were digested in sulfuric acid containing a selenium catalyst, and after treatment with alkali, the liberated ammonia steam distilled and titrated against standard acid.

3.2.3.3 Plasma Non-Esterified Fatty Acids (NEFA)

Plasma samples were assayed for NEFA (duplicates) using a commercially available
colorimetric assay kit, and performed according to the manufacturers instructions. The assay is based on acylation of Coenzyme A by the fatty acids in the presence of acyl-CoA synthetase, ATP and Mg$^{2+}$ cations. The acyl-CoA produced is oxidised by acyl-CoA oxidase with the generation of hydrogen peroxide. Hydrogen peroxide in the presence of added peroxidase allows the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)aniline with 4- aminoantipyrine to form a purple colored adduct with an absorption maxima at 550 nm, thus allowing colorimetric determination of the amount of NEFA. Ascorbic acid can interfere with the hydrogen peroxide formation, so ascorbate oxidase is also included in the assay buffers.

3.2.3.4 Plasma Glucose

Plasma samples were assayed (triplicates) for glucose using the the ABBOTT Quickstart™ Glucose assay and ABBOTT Quickstart™ VP System. The assay is based on the phosphorylation of glucose by hexokinase to give glucose-6-phosphate, which in the presence of glucose-6-phosphate dehydrogenase reduces NAD$^+$ to NADH. The change in absorbance measured at 340/380 nm is proportional to the amount of glucose in the sample.

3.2.3.5 Plasma Insulin

Plasma insulin was measured in triplicates with a double antibody radioimmunoassay using guinea pig anti-insulin antiserum. The first antibody preparation was raised in guinea pigs against porcine insulin. This antiserum does not distinguish between human and porcine insulin and has a high affinity for rat insulin. Rat insulin does not have a dilution curve parallel to human or porcine insulin. The final dilution of the antiserum used was 1:200 000-300 000 in a final assay volume of 400 mm$^3$. Rat insulin was assayed against standards using the A$^{14}$-mono$^{[125]}$iodotyrosyl human insulin as radioactive tracer. The standard buffer consisted of 5% (w/v) bovine serum albumin (BSA insulin-free) in 40 mM sodium phosphate buffer, pH 7.4 with 0.02% (w/v) thimerosal as preservative. 25-100 mm$^3$ of the plasma samples was used and serially diluted standards were prepared. To the samples and standards, 100 mm$^3$ of the antibody was added and after an incubation period of 24 hr at 4 °C, 100 mm$^3$ aliquot of $^{125}$I-insulin (6 000-7 000 cpm) was added to the assay mixture and further incubated for 24 hr at 4 °C.

Separation of the bound antibody from the free tracer was achieved by adding 100 mm$^3$ of sheep anti-guinea pig immunoglobulin G and normal guinea pig serum and further incubated at 20 °C for 6 hr. Following this, 2 cm$^3$ of 6% (w/v) polyethylene glycol (PEG) was added to precipitate the hormone-antibody complex. The tubes were centrifuged for 30 min at 3500 rpm, decanted and gamma counted (NE 1600 gamma Counter). A radioimmunoassay processing program called ASSPRO (Garvan Medical Research Institute, Sydney, Australia)
based on a cubic spline function fit was used to calculate the standard dose response curve. The assay had an intra-assay coefficient of variation (CV) of 5% and inter-assay CV of 15% at 12 mU/dm³.

3.2.3.6  β-Adrenergic Receptor Assay

3.2.3.6.1  Receptor Isolation

β₂-ARs from either the quadriceps (quads) or the soleus/plantaris/gastrocnemius (SPG) bundle were isolated by a 7-step ultracentrifugation procedure,³⁸⁸,³⁸⁹ modified for use at CSIRO DAP Laboratories. Muscle samples from each of the six rats per group were combined for receptor isolation, and samples were maintained at 4 °C throughout the isolation. Frozen muscle samples were quickly cut into thin strips and homogenized once for 3 seconds using an Ultra-Turrax probe set at half-maximum speed in 4 volumes of 0.75 M KCl plus 5 mM histidine, pH 7.4 (Buffer 1). This brief homogenization was to disrupt the tissue and allow extraction of contractile proteins. The homogenate was then sedimented (14 000 g/20 min/4 °C), the pellet resuspended in Buffer 1 (4 volumes) and resedimented at the same speed. This pellet was gently resuspended and washed in 4 volumes of 10 mM NaHCO₃ plus 5 mM histidine, pH 7.4 (Buffer 2) and then sedimented (14 000 g/20 min/4 °C). The pellet was again gently resuspended in the minimum volume of Buffer 2 (∼20% v/v suspension) and then homogenized three times for 30 s with the Ultra-Turrax set at half-maximum speed, with cooling of the homogenate in an ice bath between each homogenization. This suspension was centrifuged to remove nuclei, cell debris and mitochondria (14 000 g/20 min/4 °C), and then the supernatant was centrifuged (45 000 g/35 min/4 °C). The resulting pellet was completely resuspended in freshly prepared 0.25 M sucrose plus 5 mM histidine, pH ∼7.4 (Buffer 3) and centrifuged (17 000 g/20 min/4 °C) to remove any mitochondria which might still be present. The supernatant was then centrifuged (100 500 g/30 min/4 °C). This resulted in a firm, brown pellet and a white rim of a fluffy, flocculent material, the latter containing the isolated receptors present in the sarcolemmal membrane. This fluffy, flocculent material was removed by gentle aspiration, and gentle washing with a known minimum volume (3 cm³ maximum) of 50 mM Trizma, plus 10 mM MgCl₂ and 0.9% NaCl, pH 7.7 (Buffer 4). Both the receptor preparation and the final supernatants were retained for protein determination and stored at -70 °C. Based on protein determinations, the receptor preparations were diluted to 0.1 mg/cm³ protein concentration with Buffer 4 in readiness for the receptor binding assay.

3.2.3.6.2  Protein Determination

Receptor preparations and final supernatants were assayed (duplicates) for total protein using a
commercially available colorimetric assay kit, based on the method of Bradford.\footnote{400} The assay is a dye-binding assay, where the dye changes colour in response to various concentrations of protein. The absorbance maximum for an acidic solution of the dye (Coomassie Brilliant Blue G-250) changes from 465 nm to 595 nm when binding to protein occurs. This dye binds primarily to aromatic and basic amino acid residues, especially arginine. Standards are prepared using BSA.

3.2.3.6.3 Saturation Studies

The receptor density and affinity of the \( \beta_2 \)-AR preparations were determined by equilibrium binding studies\footnote{26} with \(^{125}\text{I}-(\pm)\text{iiodcyanopindolol} ([^{125}\text{I}] \text{CYP}).\footnote{391} Validation of this assay at CSIRO DAP Laboratories has characterised the receptors isolated from rodent hindlimb skeletal muscle (as described above) as primarily \( \beta_2 \)-ARs from the order of affinity of standard adrenergic ligands (ISO>ADR>>NOR). Establishment of this assay at this laboratory also optimised the range of label concentrations, concentration of receptor protein needed and incubation times.

The assay buffer used was Buffer 4 containing 5 \( \mu \text{M} \) iodoacetamide as preservative. The samples were assayed in triplicates of triplicates. Reaction was commenced by addition of receptor preparations (diluted to 0.1 mg protein/cm\(^3\) in Buffer 4; 100 mm\(^3\)/tube) with varying concentrations of \(^{125}\text{I}] \text{CYP} (200000-3000 \text{ dpm or 165-2 pM; 50 mm}^3\text{/tube}) in a final volume of 250 mm\(^3\). Non-Specific Binding was determined in separate tubes by the inclusion of (-)-propanolol (5 \( \mu \text{M; 50 mm}^3\text{/tube})). After incubation for 1.5 h at 37 °C on a shaking water bath (120 cycles/min) the reaction was stopped by the addition of ice-cold Buffer 4 (5 cm\(^3\)). The mixtures were immediately filtered through pre-soaked (Buffer 4) Whatman GF/B Glass Microfilters (25 mm diameter) under vacuum, and the filters washed with further portions of ice-cold Buffer 4 (3 x 5 cm\(^3\)). Reaction quenching and filtration took no longer than 30 s for any one tube. The radioactivity remaining on the filters was quantitated in an LKB 1277 gamma counter (counting efficiency 68.0\%). Results were analysed by the LIGAND computer program\footnote{392} (non-linear least squares curve fitting). Correction for the decay of \(^{125}\text{I}] \text{CYP} was based on the assumption that radioactive decay led to a product that was inactive at \( \beta \)-ARs.\footnote{393}

3.2.3.7 Statistical Analysis of Results

All results are expressed as mean ± SEM. Statistical comparisons between groups were made using one-way analysis of variance (ANOVA) with posthoc determinations. Statistical analyses were performed using a commercial general purpose statistics package (Statview SE ± graphics) and by the CSIRO Institute of Animal Production and Processing Biometrics Unit (Prospect, Sydney, Australia) with a commercial general purpose statistics package (Genstat 5).
3.3 Results

3.3.1 Whole Body Effects

The initial and final bodyweights and the changes in bodyweight over the 22-day treatment period for control and treated rats are presented in Table 3.1. On Day 1 there were no significant differences in body weight across all six treatment groups. After 22 days treatment, significant differences in final bodyweight were observed with the CB and VUF 8303 groups, in comparison to saline. CB and VUF 8303 also showed significantly greater changes in the bodyweight ($\Delta$ BW): 36% and 18% respectively in comparison to saline. None of the other treatment groups showed any significant bodyweight changes. Figure 3.2 shows bodyweights for the control and treatment animals during the 22-day treatment period.

Table 3.1 Effect of clenbuterol and analogues on whole body characteristics (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial BW (g)</th>
<th>Final BW (g)</th>
<th>$\Delta$ BW (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>198.2 ± 1.7</td>
<td>242.8 ± 3.7</td>
<td>44.6 ± 2.4</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>213.5 ± 3.5</td>
<td>274.3 ± 5.3*</td>
<td>60.8 ± 2.1*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>214.5 ± 1.8</td>
<td>267.0 ± 3.7*</td>
<td>52.5 ± 2.7*</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>207.2 ± 3.6</td>
<td>253.2 ± 3.3</td>
<td>46.0 ± 3.6</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>211.3 ± 2.5</td>
<td>254.8 ± 2.1</td>
<td>43.5 ± 3.7</td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>206.7 ± 5.2</td>
<td>249.7 ± 5.5</td>
<td>43.0 ± 1.4</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (F-test)  BW = Bodyweight

Figure 3.2 Effect of clenbuterol analogues on cumulative bodyweight during the treatment period (each data point represents the mean of six animals)
3.3.2 Skeletal Tissue Effects

The effects of the six treatments on various hindlimb skeletal muscle weights are given in Table 3.2. CB significantly increased the weight of the slow-twitch soleus by 35%. Increases were also observed for VUF 8303 (11%), O-Methyl VUF 8303 (14%) and C2 Hapten (8%) although these increases were not significantly different from saline. Large significant increases in the weight of the plantaris were also observed with CB (73%), VUF 8303 (77%), O-Methyl VUF 8303 (74%) and C2 Hapten (46%).

None of the four analogues or CB were found to have an effect on the weights of the gastrocnemius muscle. In contrast, in the O-Methyl VUF 8303 and C2 Hapten groups there were insignificant decreases of muscle weights of 18% and 13% respectively. Significant weight increases of the EDL muscle were observed with CB (29%), VUF 8303 (12%) and O-Methyl VUF 8303 (13%). None of the drug treatments influenced the wet weights of the quadriceps muscles.

Table 3.2 Effect of clenbuterol and analogues on hindlimb skeletal muscle (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Sol  (g)</th>
<th>Plant (g)</th>
<th>Gastroc (g)</th>
<th>Quads (g)</th>
<th>EDL (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.132 ± 0.012</td>
<td>0.149 ± 0.010</td>
<td>2.239 ± 0.109</td>
<td>1.822 ± 0.049</td>
<td>0.125 ± 0.011</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>0.178 ± 0.009*</td>
<td>0.258 ± 0.031*</td>
<td>2.233 ± 0.093</td>
<td>2.204 ± 0.102</td>
<td>0.161 ± 0.006*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>0.147 ± 0.003</td>
<td>0.264 ± 0.015*</td>
<td>2.315 ± 0.074</td>
<td>2.131 ± 0.074</td>
<td>0.140 ± 0.008*</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>0.150 ± 0.003</td>
<td>0.259 ± 0.016*</td>
<td>1.845 ± 0.099</td>
<td>1.891 ± 0.073</td>
<td>0.141 ± 0.002*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>0.143 ± 0.006</td>
<td>0.218 ± 0.009*</td>
<td>1.958 ± 0.099</td>
<td>1.946 ± 0.088</td>
<td>0.127 ± 0.007</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>0.136 ± 0.008</td>
<td>0.177 ± 0.016</td>
<td>2.177 ± 0.135</td>
<td>1.905 ± 0.051</td>
<td>0.135 ± 0.004</td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (F-test)

3.3.3 Non-Skeletal Tissue Effects

Table 3.3 shows the effects of CB and analogues on various non-skeletal tissue. VUF 8303 was the only treatment to have any effect on the heart weight in comparison to saline and a significant 21% increase was observed. There was a slight but insignificant increase of 14% in the CB treatment group. A significant increase in the liver weight was observed for CB (10%), VUF 8303 (18%) and O-Methyl VUF 8303 (12%). Only the CB treatment group showed any significant increase in kidney weight (10%). No significant differences were observed in any treatment groups in comparison to saline for the weight of the lungs or BAT.
Table 3.3  Effect of clenbuterol and analogues on various non-skeletal tissue (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Heart (g)</th>
<th>Liver (g)</th>
<th>Lungs (g)</th>
<th>Kidneys (g)</th>
<th>BAT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>0.847 ± 0.027</td>
<td>9.232 ± 0.416</td>
<td>1.512 ± 0.125</td>
<td>1.985 ± 0.076</td>
<td>0.465 ± 0.056</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>0.969 ± 0.043</td>
<td>10.175 ± 0.279*</td>
<td>1.765 ± 0.141</td>
<td>2.179 ± 0.051*</td>
<td>0.510 ± 0.057</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>1.024 ± 0.074*</td>
<td>10.862 ± 0.337*</td>
<td>1.694 ± 0.175</td>
<td>2.039 ± 0.063</td>
<td>0.488 ± 0.041</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>0.912 ± 0.027</td>
<td>10.335 ± 0.286*</td>
<td>1.715 ± 0.200</td>
<td>2.046 ± 0.078</td>
<td>0.512 ± 0.026</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>0.858 ± 0.027</td>
<td>9.955 ± 0.234</td>
<td>1.556 ± 0.089</td>
<td>1.902 ± 0.025</td>
<td>0.474 ± 0.023</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>0.869 ± 0.028</td>
<td>9.066 ± 0.329</td>
<td>1.357 ± 0.058</td>
<td>1.858 ± 0.041</td>
<td>0.449 ± 0.034</td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (F-test)

3.3.4 Carcass Effects

Table 3.4 shows the effects of saline, CB and the four analogues on the carcass composition. The wet weight of the carcass was significantly different in the CB and VUF 8303 treatment groups, and 19% and 13% increases, respectively, were observed. None of the other treatment groups gave significant results. No significant differences were found in the dried carcass weights among the six groups.

The water content of the wet carcasses (% Water) was significantly different from saline across all treatment groups, although the increases were very small (3-7%). The CB treatment group was the only one to show any significant decrease in % Fat (30%; on a Dry Matter Basis). VUF 8303 and C6 Hapten treatment groups showed very modest decreases (6% and 8% respectively) that were not significant. The % Protein (Dry Matter Basis) was observed to increase by 20% in the CB treatment group, and this increase was significant. The other treatment groups did not show any significant differences from the control group.
Table 3.4  Effect of clenbuterol and analogues on carcass composition (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>Wet Weight (g)</th>
<th>Dry Weight (g)</th>
<th>% Water</th>
<th>% Fat#</th>
<th>% Protein#</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>106.8 ± 1.4</td>
<td>37.8 ± 1.1</td>
<td>64.7 ± 0.6</td>
<td>33.6 ± 1.7</td>
<td>52.8 ± 1.5</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>126.8 ± 2.6*</td>
<td>39.0 ± 0.9</td>
<td>69.3 ± 0.2*</td>
<td>23.5 ± 1.4*</td>
<td>63.5 ± 1.1*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>121.2 ± 1.1*</td>
<td>39.3 ± 1.4</td>
<td>67.6 ± 0.9*</td>
<td>31.5 ± 1.2</td>
<td>56.3 ± 1.4</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>112.3 ± 1.0</td>
<td>37.5 ± 0.6</td>
<td>66.6 ± 0.5*</td>
<td>32.1 ± 1.4</td>
<td>56.9 ± 0.6</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>116.3 ± 0.5</td>
<td>38.8 ± 0.4</td>
<td>66.6 ± 0.4*</td>
<td>34.2 ± 1.1</td>
<td>53.5 ± 1.1</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>109.6 ± 2.3</td>
<td>35.9 ± 0.7</td>
<td>67.2 ± 0.5*</td>
<td>31.0 ± 1.6</td>
<td>54.9 ± 1.2</td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (F-test)

# on Dry Matter Basis

3.3.5 Plasma NEFA, Glucose and Insulin

Table 3.5 shows the effects of the six treatments on the levels in plasma of NEFA, glucose and insulin at the end of the treatment period. No significant differences were observed in the levels of these substrates in plasma at the end of the treatment period, in comparison to saline. However, considerable between-animal differences were observed within all groups, reflected in high averages of intra-group coefficients of variation (CV) of ≈34% for glucose, and ≈30% for NEFA. Insulin levels were below the minimum level of detection.

Table 3.5  Effect of clenbuterol and analogues on plasma NEFA, glucose and insulin at the end of the 22 day treatment period (n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>NEFA (mM)</th>
<th>Glucose (mM)</th>
<th>Insulin (mU/dm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.11 ± 0.13</td>
<td>9.30 ± 1.68</td>
<td>&lt; 3.3</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>0.93 ± 0.11</td>
<td>9.93 ± 1.86</td>
<td>&lt; 3.3</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>1.78 ± 0.13</td>
<td>11.22 ± 1.13</td>
<td>&lt; 3.3</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>1.97 ± 0.31</td>
<td>9.12 ± 1.03</td>
<td>&lt; 3.3</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>1.87 ± 0.22</td>
<td>8.45 ± 0.54</td>
<td>&lt; 3.3</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>1.53 ± 0.24</td>
<td>13.58 ± 2.70</td>
<td>&lt; 3.3</td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (F-test)
3.3.6 \( \beta \)-Adrenergic Receptor Effects

3.3.6.1 Receptor Isolation Recoveries

Receptors were isolated from the soleus/plantaris/gastrocnemius bundle (SPG) or the quadriceps (QUADS), and the total protein concentration of both the isolated receptor preparation and the final supernatant was determined. These results are shown in Table 3.6.

**Table 3.6** Protein concentrations of isolated receptors (Preparation) and final isolation supernatants (Supernat.)

<table>
<thead>
<tr>
<th>Group</th>
<th>SPG (Preparation) ((\mu g/cm^3))</th>
<th>SPG (Supernat.)  ((\mu g/cm^3))</th>
<th>QUADS (Preparation) ((\mu g/cm^3))</th>
<th>QUADS (Supernat.) ((\mu g/cm^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>310</td>
<td>260</td>
<td>1240</td>
<td>270</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>360</td>
<td>550</td>
<td>1360</td>
<td>400</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>860</td>
<td>450</td>
<td>1400</td>
<td>340</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>760</td>
<td>500</td>
<td>1120</td>
<td>510</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>880</td>
<td>280</td>
<td>1400</td>
<td>280</td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>840</td>
<td>260</td>
<td>1560</td>
<td>370</td>
</tr>
</tbody>
</table>

Saturation binding studies were carried out on both the receptor preparation and the final supernatant at an equal concentration of protein (0.1 mg protein/cm\(^3\)). The saturation curves obtained from similar muscles and treatment group were compared to assess what proportion of protein in the waste volumes was actually isolated receptors. This procedure provided a further evaluation of the quality of the isolation protocol, since the majority of receptors should have been sedimented following the final centrifugation step, leaving very little receptor protein in the final supernatant volume. Representative saturation curves for both the soleus/plantaris/gastrocnemius bundle and quadriceps muscle from saline treatment group are given in Figures 3.3 and 3.4 as examples.
Figure 3.3  Representative saturation curve from assay of β₂-AR isolated from SPG muscle (saline treatment group)

Figure 3.4  Representative saturation curve from assay of β₂-AR isolated from Quads muscle (saline treatment group)

At equivalent protein concentrations, the final supernatant contained ≈10% of the [¹²⁵I]CYP-saturable binding sites found in the actual receptor preparation. Similar results were observed for all treatment groups and both muscle types. The results indicated that although the final supernatant had protein concentrations comparable to the receptor preparations, most of this protein was not receptor protein. Therefore, the majority of the receptors were able to be isolated from the final centrifugation step, and losses in the final supernatant were acceptably small. Furthermore, although the preparation of receptors from the quadriceps had a protein concentration greater than those from the SPG muscle bundle, the receptor preparation from the
latter appeared to have a larger number of \([^{125}I]\)CYP-saturable binding sites (higher specific binding) and hence a greater proportion of receptor protein present. This conclusion was confirmed by the higher receptor density \((B_{\text{max}})\) for the receptor preparation of the SPG bundle in comparison with the quadriceps receptor preparation (refer section 3.3.6.2).

### 3.3.6.2 Receptor Density and Affinity

Table 3.7 shows the effects of the six treatments on the \(\beta_2\)-AR characteristics \(B_{\text{max}}\) (Receptor Density) and \(K_D\) (Dissociation Constant) of receptors isolated from the soleus/plantaris/gastrocnemius (SPG) muscle bundle and the quadriceps (Quads) muscle.

**Table 3.7** Effect of clenbuterol and analogues on receptor density \((B_{\text{max}})\) and receptor dissociation constant \((K_D)\) of \(\beta_2\)-ARs isolated from soleus/plantaris/gastrocnemius (SPG) and quadriceps (QUADS) muscle bundles

<table>
<thead>
<tr>
<th>Group</th>
<th>SPG (B_{\text{max}}) (fmol/mg protein)</th>
<th>SPG (K_D) (pmol)</th>
<th>QUADS (B_{\text{max}}) (fmol/mg protein)</th>
<th>QUADS (K_D) (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>268.5 ± 10.1</td>
<td>21.3 ± 4.5</td>
<td>147.3 ± 3.0</td>
<td>15.1 ± 1.5</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>193.7 ± 3.2*</td>
<td>17.0 ± 0.2</td>
<td>41.4 ± 23.3*</td>
<td>131.9 ± 33.0*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td>183.6 ± 9.4*</td>
<td>17.0 ± 1.9</td>
<td>148.5 ± 4.2</td>
<td>17.5 ± 2.0</td>
</tr>
<tr>
<td>O-Methyl</td>
<td>195.6 ± 0.5*</td>
<td>17.0 ± 0.5</td>
<td>102.3 ± 3.9</td>
<td>23.7 ± 2.2</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>243.0 ± 2.3</td>
<td>20.2 ± 0.9</td>
<td>132.8 ± 4.9</td>
<td>14.2 ± 1.4</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>305.6 ± 5.5</td>
<td>20.0 ± 1.2</td>
<td>134.2 ± 4.0</td>
<td>15.1 ± 1.0</td>
</tr>
</tbody>
</table>

* P < 0.05 versus saline (Dunnet’s T-test)

With respect to the SPG \(\beta_2\)-ARs, the CB, VUF 8303 and O-Methyl VUF 8303 treatment groups showed significant decreases in \(B_{\text{max}}\) of 28%, 32% and 27% respectively in comparison to saline. No other treatment groups were significantly different from saline, although the C6 Hapten group showed a modest increase of 14%. No significant differences in \(K_D\) were observed in any treatment groups in comparison to saline, although the same treatment groups that showed a decrease in \(B_{\text{max}}\) also showed a decrease in \(K_D\) of 20%.

With respect to the quads, the triplicate values of \(B_{\text{max}}\) and \(K_D\) obtained from the CB treatment group varied considerably, reflected in the SEM. Scatchard plots of the data prior to analysis (using LIGAND) also showed a much wider scatter of experimental points. By comparison, Scatchard plots of data from other treatment groups were close to linearity. Examples of these
Scatchard plots are given below in Figure 3.5. Therefore some doubt was cast on the accuracy of the mean of these results. However, despite the errors, the CB treatment group showed a statistically significant decrease, in comparison to saline, in $B_{\text{max}}$ (72%) and increase in $K_D$ (770%).

![Scatchard plots from receptor saturation studies](image)

**Figure 3.5** Scatchard plots from receptor saturation studies

The analysis of variance of the $B_{\text{max}}$ and $K_D$ values for $\beta_2$-ARs (Quads) was, therefore, repeated for all treatment groups except CB, since the large errors observed in the CB treatment group may have masked any other significant differences present. These results are presented in Table 3.8. It was observed from these results that only the $O$-Methyl VUF 8303 treatment group showed significant changes in $B_{\text{max}}$ (31% decrease) and $K_D$ (80% increase), and these were smaller than those observed in the CB group (Table 3.7). No other treatment groups were significantly different to saline.

**Table 3.8** Effect of clenbuterol analogues on receptor density ($B_{\text{max}}$) and receptor dissociation constant ($K_D$) of $\beta_2$-ARs isolated from soleus/plantarisis/gastrocnemius (SPG) and quadriceps (QUADS) muscle bundles (CB group excluded from ANOVA)

<table>
<thead>
<tr>
<th>Group</th>
<th>QUADS</th>
<th>$B_{\text{max}}$ (fmol/mg protein)</th>
<th>$K_D$ (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td></td>
<td>147.3 ± 3.0</td>
<td>15.5 ± 0.9</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td>148.5 ± 4.2</td>
<td>19.1 ± 2.0</td>
</tr>
<tr>
<td>$O$-Methyl</td>
<td></td>
<td>102.3 ± 3.9*</td>
<td>27.8 ± 4.3*</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td></td>
<td>132.8 ± 4.9</td>
<td>15.8 ± 1.8</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td></td>
<td>134.2 ± 4.0</td>
<td>16.0 ± 1.1</td>
</tr>
</tbody>
</table>

* $P < 0.05$ versus saline (Dunnet's T-test)
3.4 Discussion

The purpose of this study was to characterise the physiological effects of the four CB analogues as in Figure 3.1. CB was used as a positive control and comparisons of these five treatment groups were made against saline (n=6 per group), after the 22-day treatment period. A summary of these effects is given in Table 3.9.

Table 3.9 Summary of physiological effects of clenbuterol analogues

<table>
<thead>
<tr>
<th>Group</th>
<th>BW / ΔBW</th>
<th>Muscle</th>
<th>Organs</th>
<th>Carcass</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clenbuterol</td>
<td>↑ Body Wgt↑</td>
<td>Soleus</td>
<td>Liver</td>
<td>↑ Protein</td>
<td>↑ KD (Quads)</td>
</tr>
<tr>
<td></td>
<td>↑ Wgt Gain</td>
<td></td>
<td></td>
<td>↑ Protein</td>
<td>↓ B(_{\text{max}}) (SPG)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ EDL</td>
<td>Kidney</td>
<td>↑ Fat</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VUF 8303</td>
<td>↑ Body Wgt↑</td>
<td>EDL</td>
<td>Heart</td>
<td>NS</td>
<td>↓ B(_{\text{max}}) (SPG)</td>
</tr>
<tr>
<td></td>
<td>↑ Wgt Gain</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>↑ Plantaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-Methyl</td>
<td>NS</td>
<td>↑ EDL</td>
<td>Liver</td>
<td>NS</td>
<td>↓ B(_{\text{max}}) (SPG)</td>
</tr>
<tr>
<td>VUF 8303</td>
<td></td>
<td>↑ Plantaris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>NS</td>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>NS</td>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

The results from the CB treatment group were in good agreement with similar previously reported studies of this compound in rats. For example, increased weights of the soleus (35%), plantaris (73%) and EDL (29%) muscles, increased bodyweight gain (36%) and carcass protein concentration (20%), and decreased carcass fat concentration (30%), observed in the present study were comparable with published results as shown below:

a) 11-20% increases in the weight of the soleus\(^{15,25,28,32}\)
b) 11-25% increases in the weight of the plantaris\(^{15,28,32}\)
c) 9-20% increases in the weight of the gastrocnemius\(^{11,15,18,25,28,29,32}\)
d) 15-39% increases in the weight of the EDL\(^{15,25,28,32}\)
e) 5-30% increases in bodyweight gain\(^{11,18,25,28}\)
f) 7-20% increases in carcass protein concentration\(^{11,17,18,22,25,28,29,32}\)
g) decreases in carcass fat of 15-36%,\(^{17,18,28,29,32}\)

Several factors could explain these variations in muscle weights, bodyweight gains and carcass composition: differences in species used,\(^{3,394}\) dose level (0.2-2 mg/kg BW/day), treatment period (14-21 days), and route of drug administration (subcutaneous injection, diet, continuous infusion). The effect of CB on muscle mass has been demonstrated over a wide range of
dietary concentrations (0.1 to 10 mg/kg diet),\textsuperscript{15} which suggested that the effects are not dose-dependent within this range. Nevertheless, despite the noted variability of the physiological effects of βAAs,\textsuperscript{395} the effects on weight gain, muscle hypertrophy and carcass composition were clearly characteristic of CB.

Of the CB analogues tested, only the phenolic compound VUF 8303 showed similar effects to CB with respect to weight gain and muscle hypertrophy for EDL and plantaris muscles, although the soleus muscle was observed to increase non-significantly by 11%. O-Methyl VUF 8303 and C2 Hapten treatment groups showed increases in the weight of the plantaris muscle, but were not significantly different to saline in all other respects. However, the very dramatic effects of CB on carcass composition (decreased adipose and increased protein accretion), which has given rise to the description of CB as an energy-repartitioning agent, were not observed in any of the other treatment groups. This result suggested that the aromatic amino substituent of CB was a structural feature important for energy-repartitioning properties, since replacement of this amino group with either a hydroxyl, methoxy, oxyacetic acid or 6-oxyhexanoic acid moiety resulted in almost complete loss of the characteristic physiological effects of CB.

Cardiac hypertrophy in rats in response to chronic CB treatment has been reported in some studies,\textsuperscript{15,24,29} but not in others,\textsuperscript{11,28} including the present study. It has been suggested that this was consistent with the increase in tachycardia (β\textsubscript{1} effect) observed with growth-promoting doses of CB in sheep.\textsuperscript{41} However, despite a significant increase in heart weight observed for the VUF 8303 treatment group, it was difficult to conclude that the loss of CB-like effects for this compound could have been due to a reduced β\textsubscript{2}/β\textsubscript{1} selectivity, since the effect of CB on heart weight in previous studies was inconsistent.

Weight gain over a βAA treatment period has not been accounted for entirely by changes in carcass composition,\textsuperscript{26} and this was reflected in the significant weight gain but the lack of change in carcass composition observed for the VUF 8303 treatment group. Therefore, although weight gain has been used widely in studies of physiological effects of βAAs it would appear that it does not necessarily reflect important changes in carcass composition.

Chronic treatment with CB and the four analogues did not give rise to significant effects on the two energy substrates, NEFA and glucose, and the important metabolic hormone, insulin, although any small effects present may have been masked by the high intra-group CV (refer section 3.3.5). Several investigations on the effects of βAAs on metabolic substrates and hormones have been reported\textsuperscript{23,25,53,59,60,382} but results from these studies have been inconclusive. A 7-day dietary treatment (25 ppm CB) of female Wistar rats\textsuperscript{23} found no change in NEFA levels, a modest negative change in glucose (5.6%) and a more substantial negative
change in insulin (27.9%). Similar changes in insulin were observed in some of the above studies, although in one study no effect on insulin was observed\textsuperscript{396} which led to the conclusion that the physiological effects of CB were not mediated by insulin.

In the present study, blood samples were taken from trunk blood, and plasma was not collected and frozen until all animals had been sacrificed and tissues removed. This blood may have been diluted by other body fluids which, coupled with any delay in sample freezing resulting in hormone degradation, may have led to only low measurable levels of the hormone. The trunk blood samples collected in the present study were likely to have been lysed and hence were perhaps more heterogeneous in nature. This could have contributed to the higher intra-group CV.

With regard to the effects of CB on the \( \beta_2 \)-AR, the observed decrease in receptor density in the SPG muscle bundle of 28% was in good agreement with previously reported work, where decreases of between 40-50% were observed for the same muscle bundle\textsuperscript{26,29,381} following CB treatment. In the latter study,\textsuperscript{26} the non-selective \( \beta \)-antagonist, sotalol, attenuated both the muscle growth and reduction in receptor density observed with CB treatment. In the same study, it was also concluded that since the \( \beta_2 \)-selective antagonist, ICI 118 551, attenuated muscle growth, then the anabolic effects of CB were mediated by \( \beta_2 \)-ARs. However, effects of this antagonist treatment on receptor density were not reported.

In the present study, VUF 8303 and \( O \)-Methyl VUF 8303 treatment groups both showed a similar decrease in \( \beta_2 \)-AR density to that observed for CB, but a reduced hypertrophic effect on the SPG muscle bundle. Increases in the soleus muscles of these two treatment groups were less than those observed with CB and were not significantly different from saline, whilst similar significant increases were observed in the plantaris across all treatment groups. These results showed that the reduction in \( \beta_2 \)-AR density caused by the VUF 8303 and \( O \)-Methyl VUF 8303 treatment in the SPG bundle was not entirely reflected in the observed increase in muscle mass. This suggested that such reductions in receptor density may not always be related to increases in muscle mass.

These reductions in receptor density in the SPG bundle were similar to the 72% decrease in \( \beta_2 \)-AR density observed in the quadriceps of CB-treated rats. However, no significant change in muscle size, compared with saline, was observed. An increase of \( \approx \)500% in the receptor dissociation constant (\( K_D \)), which represents an increased tendency for the ligand/receptor complex to dissociate, was also observed. This kind of characterisation of the \( \beta_2 \)-ARs found in the quadriceps following chronic CB treatment has not been previously reported. Again, these results suggested that reductions in \( \beta_2 \)-AR density may not always be reflected in increased muscle mass.
O-Methyl VUF 8303 also caused a slight decrease in receptor density (≈30%) and an increase in the dissociation constant (≈80%) of β2-ARs found in the quadriceps, but no significant change in the weight of this muscle. This result was less dramatic than that observed for the CB treatment group, and suggested that this CB analogue was perhaps less able to cause dramatic changes in the properties of the β2-AR. However, in both treatment groups the lack of effect on muscle growth despite changes in receptor density again suggested that decreases in receptor density were not always associated with changes in muscle mass. The reason for the increase in K_D of the quadriceps β2-ARs for these two treatment groups was not clear from the present data.

It has been shown that the observed reduction in β2-AR density following chronic exposure to βAAs was due to receptor desensitisation and down-regulation.\textsuperscript{130,397} In the present study, the observed reduction in β2-AR density following chronic exposure to the βAA CB was consistent with previous similar studies\textsuperscript{26,381} and was, therefore, likely to have been due to receptor desensitization and down-regulation. Reductions in receptor density were also observed in the VUF 8303 and O-Methyl VUF 8303 treatment groups, and this was perhaps attributable to receptor desensitisation and down-regulation caused by chronic exposure of the β2-ARs to these compounds.

It has also been suggested, that this reduction in receptor density explains the observed reduction in the anabolic effects of βAAs with prolonged treatment.\textsuperscript{398} Therefore, the CB analogues may have had a CB-like growth promoting effect earlier in the treatment period that was reduced or lost over the full 22-day treatment period, due to an earlier reduction in receptor density. Such an effect might have been reflected in the 7-day weight gains at days 7, 14 and 21, since reductions in receptor density and increased weight gain after a 7-day CB treatment have been reported.\textsuperscript{26} However, consideration of 7-day weight changes showed no significant differences between any of the six treatment groups after 1, 2 or 3 weeks. The present study was designed to assess overall physiological effects of the analogues (at equimolar doses) over a treatment period previously shown to allow the growth-promoting effects of CB to be observed, rather than specific time-course or dose-response effects. Therefore, the present data did not provide conclusive evidence in relation to time-course or dose-response effects of the CB analogues, but provided a useful basis for future studies.

Alternatively, the anabolic effects (if any) of the analogues may have been observed after longer treatment periods, though this appears unlikely from the present data. If this was the case, anti-idiotypes which are the “internal image” of these haptenic analogues may also only mimic any anabolic effects the analogues may have after a similarly long treatment period. However, these anti-idiotypes may also be neutralised by the immune network system\textsuperscript{231} before any growth-
promoting effects occur, and hence prove ultimately impractical.

The present study also provided interesting additional data regarding the relationship of structure of βAAs and physiological function. The dramatic change in carcass composition of rats given CB (30% decrease in fat deposition, 20% increase in protein accretion) were not observed in any of the other treatment groups, and this strongly suggested that replacement of the aromatic ring amino group with any of the four moieties (hydroxy, methoxy, oxyacetic acid or 6-oxyhexanoic acid groups) of the four analogues resulted in almost complete loss of the energy-repartitioning effects of CB. It was, therefore, instructive to consider the structures of several other βAAs which have physiological properties similar to CB. Figure 3.6 shows the structures of several BAA growth promoting drugs.

![Chemical structures](image)

**Figure 3.6** Structures of some βAA growth promotants

One of the treatment compounds, VUF 8303, combined structural features of two known growth-promoting drugs which have demonstrated energy-repartitioning properties: RAC and CB (references previously cited). The physiological effects of RAC have been studied predominantly in swine, and it has been suggested that in this species the decrease in adipose tissue was due to the increased protein accretion rather than an actual decreased fat deposition, a so-called “dilution effect”. However, despite the structural similarity to both RAC and CB, there was no change in carcass composition in those animals treated with VUF 8303. This tended to suggest that the structural feature responsible for the energy-repartitioning properties of RAC may be the N-alkyl substituent. Chronic treatment of rats with fenoterol (Figure 3.6), a compound structurally similar to RAC, has led to significant increases in body protein, although reductions in body fat were not significantly different from controls. Furthermore, treatment of rats with the mixed (β₁/β₂) βAA, metaproterenol (Figure 3.6), has also reportedly led to increased weight of the gastrocnemius muscle as well as reductions in back and perirenal
Chapter 3 - Rodent Study

fat but without significantly greater weight gain. Characteristic repartitioning effects have also been observed in cattle treated with L-644,969 (Figure 3.6) which also has a large N-alkyl substituent. Therefore, the structural elements of βAAs responsible for their ability to alter carcass composition are not entirely clear, although the present study appeared to suggest that several structural features may be important.

Another compound, the des-4-amino analogue of CB (Figure 3.6), has also also been reported to cause a 43% increase in weight gain and a 22.6% reduction in fat pad weight in mice, although it was regarded as less potent than CB. These results, in addition to those of the present study, strongly suggested that where the para- substituent of a 3,5-dichlorophenylethanalamine was either hydrogen or an amine, energy-repartitioning properties were maintained whereas replacement with a hydroxyl, methoxy, oxyacetic acid or 6-oxyhexanoic acid group resulted in virtually complete loss of these properties.

This result was unexpected. In Chapter 1, the structural features necessary for the binding of the β-adrenergic agonist ISO to the β2-AR were outlined, as reported from the literature (refer section 1.2.2). The two aromatic ring hydroxyl groups (a structural feature shared with the endogenous adrenergic ligands ADR and NOR) are thought to interact, by hydrogen bonding, with two serine residues in the receptor protein (refer Figure 1.3 in section 1.2.2). Removal of one or both resulted in dramatically reduced ligand binding to the receptor. The β-agonist, CB, has been reported as a β2-selective agonist. Therefore, when the para- amino substituent of this compound was replaced by a hydroxyl group, a group shown to be necessary for the binding of another β-agonist to the same receptor, the almost complete loss of energy-repartitioning properties of this compound was somewhat unexpected, since the growth-promoting effects of βAAs are thought to be mediated by the β2-AR. However, from inspection of the structures of several βAAs (Figure 3.6) it would appear that compounds which do not contain the structural elements necessary for binding to the β2-AR still have energy-repartitioning properties. Indeed, isoprenaline itself does not have the physiological properties of CB, although it is known to be lipolytic.

The physiological properties of salbutamol (SALB; Figure 3.7), another β2-selective adrenergic agonist which has significant structural similarities to CB, have also been characterised, although the results were equivocal. Several studies in swine reported increased muscle mass but only the latter reported a decrease in fat. In contrast, dietary administration of SALB to young rats showed increases in muscle mass and protein content similar to those of CB. No effect on fat deposition was observed in these studies, even when the drug was delivered by continuous infusion, although fat content decreased in older animals in the latter study. An earlier study in rats treated perorally with SALB showed no significant increase in muscle mass or decrease in body fat. From these results it would appear that, at
least in young rats, SALB does not display the same energy-repartitioning properties as CB.

![SALB structure](image)

**Figure 3.7** Structure of the β₂-selective adrenergic agonist salbutamol (SALB)

This was possibly consistent with the relatively short half-life of SALB (3 to 6 hours\textsuperscript{405}), and therefore, administration by continuous infusion was equivalent to a “slow-release” of the drug. CB, by contrast, has an extremely long half-life (34 to 35 hours\textsuperscript{405}). The metabolism of SALB has been reported as being through sulfate-conjugate formation at the para-hydroxyl group\textsuperscript{406}. It was possible, therefore, that VUF 8303, which shared the para-hydroxyl structural moiety with SALB, had none of the energy-repartitioning properties of CB because it was more rapidly metabolised, similar to SALB. This also pointed to the long duration of action of CB as probably being important for its energy-repartitioning properties\textsuperscript{29,34}. In contrast, although VUF 8303 may have existed long enough to cause some increase in muscle mass, it may not have had a sufficiently long duration of action to achieve the dramatic alteration in carcass composition observed with CB.

The O-Methyl VUF 8303 compound, where the hydroxyl group was blocked from metabolism by sulfate formation, might also be expected to have a longer half-life than VUF 8303, and therefore have physiological effects approaching those of CB. However, the data from the present study demonstrated that this was not the case. This result suggested that steric factors may also have affected the ability of this compound to bind the β₂-AR. Indeed, the observation that neither the C2 Hapten or C6 Hapten, which contain bulkier phenoxy substituents but have no growth-promoting properties, could possibly support this. However, the presence of the carboxyl groups on both these compounds may also have led to relatively rapid metabolism, and this may have more readily explained the lack of effects of these compounds. The des-amino CB compound lacks a para-ring substituent, which could explain why it has significant energy-repartitioning effects, possibly due to lack of a metabolisable or bulky substituent in this position.

The hypothesis that a long N-alkyl substituent may be an important structural feature of βAA with energy-repartitioning effects (e.g. RAC; refer Figure 3.6) was supported by the present study, since although VUF 8303 combined structural features common to both CB and RAC, it exhibited none of the energy-repartitioning effects of either drug. Such a hypothesis has brought attention to the β₂-selective agonist, salmeterol (SALM), which is a bronchodilator
used in the treatment of asthma.\textsuperscript{407} The unique property of SALM is that although it has a longer onset of action, it has a very lengthy duration of action,\textsuperscript{408} considerably longer than that of SALB (also a bronchodilator used in the treatment of asthma), with which it shares structural similarities. The structure of this compound is given in Figure 3.8 below.

![Figure 3.8 Structure of the long-acting bronchodilator salmeterol (SALM)](image)

The combination of the long duration of action of SALM and superior potency at the $\beta_2$-AR as a bronchodilator,\textsuperscript{409} has led to its evaluation as an anabolic agent.\textsuperscript{410} Dietary administration led to increased weight gain and muscle hypertrophy (plantaris/gastrocnemius) at doses (2.4 mg/day) almost 25 times those of CB required for similar effects. A reduced dose (130 $\mu$g/day) of SALM administered via continuous infusion resulted in similar effects on weight gain and muscle mass. No effect of SALM on carcass composition was reported in either case. This data, together with previous work, suggested that a long duration of action may be a necessary but not sufficient characteristic for $\beta$AAs to exert growth-promoting and energy-repartitioning properties.

The ARs in rat adipocytes (brown and white) have been shown to be mainly $\beta_3$, but also $\beta_1$; further, the $\beta_3$-AR is thought to be the main receptor responsible for lipolysis.\textsuperscript{411} Activation of the $\beta_3$-AR in brown adipose tissue is also thought to be responsible for non-shivering thermogenesis, and has led to this receptor becoming the target for the development of anti-obesity drugs such as BRL 37344.\textsuperscript{412} Interestingly, evidence for the presence of the $\beta_3$-ARs in rodent hindlimb skeletal muscle has also been recently reported,\textsuperscript{413,414} although more recent reports have suggested this receptor is not functional.\textsuperscript{415} There is also some suggestion that muscle hypertrophic effects of CB could be mediated by this atypical AR, although conflicting results with the use of existing $\beta$-antagonists to inhibit these effects, coupled with the lack of a truly $\beta_3$-specific antagonist, have led to the conclusion that it is probably inappropriate to describe the CB receptor as a $\beta_3$-AR at this point.\textsuperscript{416} Consideration of the structure of the $\beta_3$-selective agonist BRL 37344 (Figure 3.9) demonstrates structural similarities with some of the energy-repartitioning $\beta$AAs discussed previously.
Figure 3.9 Structure of the β3-AR agonist BRL 37344

If the growth-promoting effects of CB do involve the β3-AR then this may impose further structural requirements on βAAs which give them their growth-promoting properties, structural requirements not met by the four CB analogues in the present study. Some studies have also suggested that the β3-AR is resistant to agonist-promoted down-regulation.\textsuperscript{161,417} If β3-ARs are involved in the observed physiological effects of CB, then a long duration of action may allow this drug to exert its dramatic growth-promoting effects by continual activation of a receptor apparently not subject to down-regulation. However, the present study did not provide any further evidence for this conclusion. Further work may better elucidate the involvement of the β3-AR in the mechanism of action of growth-promoting βAAs.

The results of the present study had important implications for the use of the haptenic analogues for the generation of anabolic antibodies. Replacement of the para- amino group of CB (refer Figure 3.1, where $Z = \text{NH}_2$) with any of the four structural features unique to the four CB analogues resulted in almost complete loss of the energy-repartitioning effects of CB, and suggested that this site may be important for these effects. Replacement with the least sterically intrusive group (hydrogen) has also been shown to maintain the energy-repartitioning effects of CB.\textsuperscript{9} Therefore, to construct protein conjugates by conjugation of haptens through this position may possibly lead to anti-idiotypes which bear a structural “internal image” similar to the four structural analogues of CB, in terms of a bulky structure in a position similar to that of the para- amino group of CB. Such anti-idiotypes may, therefore, not bear the energy-repartitioning “internal image” of CB.

However, the observation of some muscle hypertrophic effects with two of the CB analogues (VUF 8303 and O-Methyl VUF 8303) suggested that anti-idiotypes derived from the haptenic O-alkyl CB analogues (C2 and C6 Haptens) could perhaps exhibit some effects on muscle mass. This would require further investigation and, importantly, consideration of appropriate parameters with which to characterise any whole body effects the anti-idiotypes may exert. The present study provides the basis for this type of investigation.

The only conjugate reported to be effective in provoking anti-CB antibodies\textsuperscript{300} is that formed by conjugation of CB to a carrier protein through the important para- amino group (diazoisation chemistry). The results of the present study also suggested that use of this conjugate may not
lead to anti-idiotypes bearing the energy-repartitioning “internal image” of CB, despite structural similarities and the use of the actual drug to form the conjugate. Consideration in this laboratory (CSIRO DAP) of alternative sites of conjugation has lead to the synthesis of analogues of CB functionalised for conjugation through the \( N\text{-}tert\)-butyl group.\textsuperscript{301} Use of analogues of clenbuterol functionalised in this way (refer Conjugate B, Figure 1.7) may provide a more useful route toward the generation of anabolic antibodies, particularly in view of the rather bulky \( N\)-alkyl substituents of other known \( \beta \)AAs that have energy-repartitioning properties. The physiological study of such CB analogues, therefore, would appear to be a most interesting, and useful, investigation, the basis of which is provided by the present study.
3.5 Conclusion

The four analogues of CB were characterised physiologically in rats in comparison to both the parent drug, CB, and saline controls. Replacement of the para-amino substituent of CB with either a hydroxyl, methoxy, oxyacetic acid or 6-oxyhexanoic acid moiety resulted in virtually a complete loss of the energy-repartitioning properties of CB exemplified by the dramatic alteration of carcass composition (increased protein accretion, decreased fat deposition). Two treatment groups (VUF 8303 and O-Methyl VUF 8303) retained some skeletal muscle hypertrophic effects, although somewhat smaller than those of CB, and showed similar effects on β2-AR density in the SPG muscle bundle. Only VUF 8303 showed similar effects to CB on 22-day weight gain, although these effects were slightly less than the parent drug, as well as an increase in heart weight. Therefore, this study demonstrated that this para-amino group was important for the observed physiological effects of CB.

This study has important implications for the use of the haptenic analogues (C2 Hapten 5d, and C6 Hapten 5g), for the development of anti-idiotypic antibodies which may mimic the physiological effects of CB. Formation of required protein conjugates by conjugation through the para-amino site may result in production of idiotypic anti-CB antibodies which provoke anti-idiotypic antibodies that do not mimic the desired physiological effects of CB. Comparison of these four CB analogues and their physiological effects with other known βAA growth promotants also provided further data regarding the structural features necessary for the physiological effects of βAAs. Finally, the current protocol provided a useful animal model for the evaluation of other CB analogues of interest, as well as important parameters for the characterisation of anti-idiotypic antibodies which may bear the physiological “internal image” of CB.
CHAPTER 4 - PRODUCTION AND CHARACTERISATION OF ANTIBODIES TO O-ALKYL CLENBUTEROL ANALOGUES

4.1 Introduction

4.1.1 Aim

The aim of this section of the research was:

a) to investigate production of anti-CB Abs (polyclonally and monoclonally) using novel conjugates derived from the O-alkyl analogues, 5d and 5g.

b) to characterise the anti-CB Ab titre response of sheep to the novel conjugates and confirm Abs as anti-CB Abs by competitive binding assays.

c) to characterise the specificity of anti-CB Abs in competitive binding assays, by measurement of the cross-reactivity of the Abs with a panel of compounds that share structural similarities with CB.

d) to compare the use of different haptenic O-alkyl CB analogues and conjugation chemistries for the production of anti-CB Abs.

4.1.2 Background

The first step toward generation of anti-idiotypic antibodies, which may mimic the energy-repartitioning effects of clenbuterol (CB), would be production of idiotypic anti-clenbuterol antibodies (anti-CB Abs). Antibodies (Abs) against CB have previously been raised polyclonally, mainly in rabbits,\textsuperscript{300,303-308} using an immunoconjugate of CB with either Bovine Serum Albumin (BSA) or Human Serum Albumin (HSA). These conjugates were prepared using diazo chemistry,\textsuperscript{300} although this chemistry may lead to formation of N-nitroso compounds.\textsuperscript{309,310}

Consideration of conjugation chemistries other than CB diazotisation and linkage to the tyrosine residues of the carrier protein (refer Figure 1.9) led to the synthesis of O-alkyl CB analogues 5d and 5g and the preparation of hapten-protein conjugates from these analogues using carbodiimide-based chemistries (Chapter 2). These analogues were then characterised physiologically in comparison to CB using a rodent model (Chapter 3). The production and evaluation of anti-CB Abs raised against the analogue-protein conjugates thus provided an
immunological characterisation of the O-alkyl CB analogues. Ab titre and specificity were important elements of this characterisation,\textsuperscript{244,418} and allowed an assessment to be made regarding the possible use of anti-CB Abs (elicited by the novel O-alkyl analogue-protein conjugates) for the generation of anti-idiotype Abs that bear the “internal image” of CB.\textsuperscript{231}

The novel conjugates must be immunogenic; measurement of Ab titre over the immunisation period has been routinely used to characterise the magnitude of the immune response of an animal to a particular conjugate under investigation.\textsuperscript{419,420} Typically, Ab titre assays use a specific, constant amount of radiolabelled ligand added to a range of Ab dilutions, and after the antigen/antibody binding reaction has reached equilibrium, the titre is measured as the dilution at which half-maximum binding occurs.\textsuperscript{421,422} Abs produced using these novel conjugates must also be confirmed as anti-CB, as opposed to anti-hapten, since the conjugates were formed from structural analogues of CB. Abs that bind radiolabelled ligand are considered anti-ligand, since the radiolabel contains none of the elements of the hapten-protein bridge structure.\textsuperscript{423} Competitive displacement from Ab of radiolabel, as in Radioimmunoassay (RIA), or of microtitre plate coating conjugate (prepared similarly to the immunising conjugate on a different carrier protein), as in Enzyme-Linked Immunosorbent Assay (ELISA), by non-radiolabelled authentic ligand confirms that Abs are specific for the ligand.\textsuperscript{421}

Evaluation of specificity is an important aspect of Ab characterisation.\textsuperscript{422,423} The identification of compounds (other than the original ligand) which compete with radiolabelled ligand for antigen binding sites of Abs and displace the bound radiolabelled ligand from these sites, allows elucidation of structural elements recognised by these Abs. For example, anti-steroid Abs have been extensively studied in this way (for review, see\textsuperscript{313}). These kinds of competition studies allow determination of the immunological cross-reactivity of the Abs for these compounds, and therefore assist in the characterisation of Ab specificity. The structures of the non-radiolabelled ligands to be used in the competition studies are given in Figure 4.1 below.

Abs raised against the CB-diazo conjugate have been previously characterised in this way.\textsuperscript{300,303-308,313} However, the availability of the O-alkyl analogues of CB (Chapter 2) has allowed further characterisation of the specificity of Abs raised against the conjugates formed from either the C2 Hapten (5d) or the C6 Hapten (5g) (refer Chapter 2), as well as the CB-diazo conjugate, which all contain a CB-like epitope (i.e. either CB, or C2 Hapten or C6 Hapten analogues; refer Figure 1.9). Competitive binding studies may also allow further elucidation of the structural moiety of CB recognised by these Abs.
Conjugates were prepared from both the oxyacetic acid hapten (C2 Hapten, 5d) and the oxyhexanoic acid hapten (C6 Hapten, 5g), using ECDI or DCC (refer Chapter 2). Therefore, comparisons of the immune response to each conjugate, as well as the specificity of the Abs generated, would further characterise the haptenic O-alkyl analogues of CB and the method of conjugation. It has been suggested that an increased distance between hapten and carrier protein provides better presentation of the epitope of interest to the immune system, resulting in Abs with improved specificity; the optimum length is believed to be approximately four to six carbons.\(^{3,13}\) Hence, C6 Hapten 5g (refer Figure 4.1) would provide a longer “chemical bridge” between the carrier protein and the CB-like epitope, compared with the C2 Hapten 5d.

Two strategies for the production of anti-CB Abs were investigated: polyclonal Abs (sheep, mouse), and monoclonal Abs (mouse). Assessment of the immunogenicity of the analogue-protein conjugates, and comparison with the CB-diazo conjugate, was made by measurement of the polyclonal anti-CB Ab titre response in sheep. In concurrent experiments, production of monoclonal anti-CB Abs using the analogue-protein conjugates was also investigated, since individual monoclonal antibodies (mAbs) may potentially provide greater specificity than polyclonal Abs (pAbs). Highly specific monoclonal idiotypes may be useful in the generation of anti-idiotypes that bear the “internal image” of CB.
4.2 Materials and Methods

Suppliers of materials, equipment and animals are given in Appendix 1.

4.2.1 Polyclonal Antibodies to O-Alkyl Clenbuterol Analogues

The protocol for the production of polyclonal antisera was approved by CSIRO DAP Animal Care and Experimental Ethics Committee (Protocol No. 93022).

4.2.1.1 Experimental Animals

Mature Merino ewes (CSIRO DAP, Badgerys Creek Field Station), weighing ≈50 kg, were maintained in open paddocks with free access to food and water. Insufficient animals were available for evaluation of all conjugates synthesised (refer Chapter 2); therefore only analogue-protein conjugates made from BSA were used (a conjugate made with ethylenediamine-modified BSA was excluded, since a slightly different carrier protein was used).

4.2.1.2 Immunisation Regime

Sheep (n = 6 per conjugate) were immunised with one of five conjugates (Table 4.1):

Table 4.1 Immunisation conjugates derived from clenbuterol and O-alkyl clenbuterol analogues 5d (C2 Hapten) and 5g (C6 Hapten) (refer Chapter 2)

<table>
<thead>
<tr>
<th>Hapten</th>
<th>Conjugation Reagent</th>
<th>Carrier Protein</th>
<th>Conjugate Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2 Hapten</td>
<td>ECDI</td>
<td>BSA</td>
<td>C2/ECDI/BSA</td>
</tr>
<tr>
<td>C2 Hapten</td>
<td>DCC</td>
<td>BSA</td>
<td>C2/DCC/BSA</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>ECDI</td>
<td>BSA</td>
<td>C6/ECDI/BSA</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>DCC</td>
<td>BSA</td>
<td>C6/DCC/BSA</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>Diazotisation</td>
<td>HSA</td>
<td>CB/Diazo/HSA*</td>
</tr>
</tbody>
</table>

* conjugate synthesised by a method similar to

The required conjugate was dissolved in demineralised water (1.0 mg in 0.3 cm³) and mixed with an equal volume of an aqueous mixture of the cationic polyelectrolyte diethylaminoethyl dextran (DEAE-dextran; 20% w/v + 0.1% phenol, adjusted to pH 7.5 with Tris(hydroxymethyl)aminomethane base). This solution of conjugate (0.6 cm³) was then emulsified with a mineral oil/surfactant mixture (1.4 cm³) using an Ultra-Turrax probe for 1
min. This adjuvant system, comprising a water-in-oil emulsion of DEAE-dextran in a mineral oil, has been developed in this laboratory previously.424 The CB-Diazo-HSA conjugate was a kind donation from Dr. Robin Rigby (CSIRO DAP). Sheep were immunised i.m. (both hind legs) with these solutions of conjugates which were emulsified as above (1.0 mg conjugate in 2 cm³ final volume per sheep). Booster immunizations were given at 17 and 28 weeks post-primary immunisation. Sheep were bled from the jugular vein into heparin-containing tubes under vacuum at 0, 17, 18, 19, 21, 28, 30, 32 and 36 weeks. Blood samples were stored at 4 °C prior to centrifugation (20 min at 2 000 rpm, 4 °C), followed by plasma collection and storage (-20 °C).

Restrictions (AQIS) on the use of Freund's Adjuvant in other than a laboratory environment led to the use of the emulsified DEAE-dextran-mineral oil adjuvant above. This adjuvant has been shown to produce higher Ab titres in sheep than either DEAE-dextran or the mineral oil used separately, substances which individually are considered to be amongst the most potent adjuvants for production of antibodies to soluble immunoconjugates in ruminants.211

4.2.1.3 Polyclonal Antisera - Titre Determination

Solutions of NaH₂P₂O₇.2H₂O (7.8 g in 200 cm³ demineralised water; solution A) and Na₂HPO₄ (35.4 g in 1 dm³ demineralised water; solution B) were prepared. These solutions were then mixed (180 cm³ of solution A plus 800 cm³ of solution B), followed by adjustment to pH 7.4 which gave a 0.25 M stock phosphate buffer solution. A 50 mM solution of Phosphate-Buffered Saline (PBS) was prepared by dilution of this stock phosphate buffer solution (1:5 v/v in demineralised water) and addition of NaCl (0.9 g/dm³) to the diluted solution.

Phosphate-Sodium-Azide (PNA) buffer (0.1% w/v Na₂N₃ and 1% w/v gelatin (∼300 bloom) in 50 mM PBS, pH 7.4) was used for plasma dilutions. Charcoal solution was freshly prepared for each assay by addition of Dextran T70 (200 mg per 100 cm³) and charcoal (2.0 g per 100 cm³) to 50 mM phosphate buffer (1:5 v/v dilution of 0.25 M stock phosphate buffer solution in demineralised water, pH 7.4) and was stirred continuously at 4 °C overnight prior to use. The assay was performed in 12 x 75 mm borosilicate glass culture tubes, at 25 °C unless otherwise stated. Plasma samples from sheep immunised with the CB/Diazo/HSA conjugate (in a previous experiment in this laboratory) were used as reference samples.

Sheep plasma samples were diluted 1:10 in PNA, then serially diluted (1:1) ten times (200 mm³/tube). [³H]Clenbuterol ([³H]CB; specific activity: 16.8 Ci/mmol) in PNA was added to the plasma dilutions (20 000 cpm/cm³, 500 mm³/tube), followed by incubation overnight at 4
°C. Bound and free label were separated by addition of the homogeneous charcoal suspension (100 mm³/tube) and incubated for 20 min at 4 °C, prior to centrifugation (10 min, 2,400 rpm, 4 °C). Supernatants were transferred into vials, scintillant added (4 cm³/vial), vials capped then shaken, and stored in the dark for at least 60 min to allow dissipation of chemiluminescence. The radioactivity in each vial was counted in a beta counter (Beckman 1217, [³H] counting efficiency 56.5%) for 60 s per vial.

Non-Specific Binding (NSB) was determined from tubes containing no antisera and was subtracted from the observed counts of each tube. Inter-assay Coefficient of Variation (CV) was 11.0% and intra-assay CV was 12.7%, calculated from the reference samples. The percentage of Specific Binding (%SB) was calculated as the percentage of maximum binding. From dilution curves (plots of %SB vs log of dilution), the titre (dilution corresponding to 50% SB) was determined. Plasmas of three sheep (per group) with the highest titres were reassayed (duplicate) and aliquots (50 mm³) stored at -20 °C.

4.2.1.4 Polyclonal Antisera - Competition Studies

PNA buffer (0.1% w/v NaNO₃ and 1% w/v gelatin in 50 mM PBS, pH 7.4, or pH 6.5 plus 2 mg/cm³ ascorbic acid for the catecholamines isoprenaline, adrenaline and noradrenaline) was used for plasma dilutions, ligand solutions and radiolabel solution. Charcoal solution was freshly prepared for each assay by addition of Dextran T70 (200 mg per 100 cm³) and charcoal (2.0 g per 100 cm³) to 50 mM phosphate buffer (pH 7.4, or pH 6.5 plus 2 mg/cm³ ascorbic acid for the catecholamines) and was stirred continuously at 4 °C overnight prior to use. The assay was performed in 12 x 75 mm borosilicate glass culture tubes, at 25 °C unless otherwise stated. The non-radiolabelled ligands used in the competition assays were clenbuterol, VUF 8303 (5e), O-Methyl VUF 8303 (5e), C2 Hapten (5d) C6 Hapten (5g), salbutamol, sotalol, ICI 118 551, isoprenaline, adrenaline and noradrenaline, and their structures are given in Figure 4.1.

Fresh aliquots of sheep plasmas (plasmas with the highest titres in each group, three per group, total 15 samples) were diluted in PNA buffer to their corresponding titre dilution (determined in section 4.2.1.3) and added to assay tubes (200 mm³/tube, triplicate). A range of concentrations of the non-radiolabelled ligand were freshly prepared (final concentration range ≈ 50 or 250 nM to 40 pM), added to diluted antisera (100 mm³/tube) and incubated for 4 h at 25 °C.

[³H]CB (specific activity: 16.8 Ci/mmol) in PNA was then added (25,000 cpn/cm³, 400 mm³/tube), followed by incubation overnight at 4 °C. Bound and free label were separated by addition of the homogeneous charcoal suspension (100 mm³/tube), and incubated for 20 min at 4 °C, prior to centrifugation (10 min, 2,400 rpm, 4 °C). Supernatants were transferred into
vials, scintillant added (4 cm$^3$/vial), vials capped then shaken, and stored in the dark for at least 60 min to allow dissipation of chemiluminescence. The radioactivity in each vial was counted in a beta counter (Beckman 1217, [$^3$H] counting efficiency 56.5%) for 60 s per vial.

NSB was determined from tubes containing no antisera and subtracted from the observed counts of each tube. Inter-assay CV (based on comparison of Total Bindings) was 8.2% and the intra-assay CV was 5.1%. The %SB at each concentration of non-radiolabelled ligand was calculated as a percentage of the Total Binding of label by antisera in the absence of non-radiolabelled ligand. Displacement curves were generated by plotting %SB against the log of the concentration of non-radiolabelled ligand. The % Cross Reactivity (%CR) for each non-radiolabelled ligand for a given antisera was calculated from the displacement curves as below (CB = 100%):

\[
\text{% Cross Reactivity} = \frac{([\text{CB}] \text{ at 50% SB} + [\text{non-radiolabelled ligand}] \text{ at 50% SB})}{100}
\]

4.2.2 Monoclonal Antibodies to O-Alkyl Clenbuterol Analogues

The protocol for the production of murine-derived monoclonal hybridomas was approved by CSIRO DAP Animal Care and Experimental Ethics Committee (Protocol No. 90013).

4.2.2.1 Experimental Animals

Male and female BALB/c mice (eight to ten weeks old) were used for the production of mAb-secreting hybridomas. The animals had free access to food and water, \textit{ad libitum}, and were housed four per box with a 12:12 hr light:dark cycle. Lights were switched on at 0600 hr.

4.2.2.2 Immunisation Regime

BALB/c mice (n = 7 or 8 per conjugate) were immunised with one of the five conjugates (Table 4.2). The required conjugate was dissolved in saline (1 mg/cm$^3$), then emulsified with an equal volume of either Freund’s Complete Adjuvant (primary injection) or Freund’s Incomplete Adjuvant (boost injections) using a small Ultra-Turrax probe for 30 s. Mice were immunised i.p. with these solutions of conjugates which were emulsified as above (100 μg conjugate in 0.2 cm$^3$ per mouse). Booster immunisations were given at 4 and 8 weeks, and thereafter as required.
Table 4.2  Immunisation conjugates derived from O-alkyl clenbuterol analogues 5d (C2 Hapten) and 5g (C6 Hapten) (refer Chapter 2)

<table>
<thead>
<tr>
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</tr>
<tr>
<td>C6 Hapten</td>
<td>ECDI</td>
<td>BSA</td>
<td>C6/ECDI/BSA</td>
</tr>
<tr>
<td>C6 Hapten</td>
<td>DCC</td>
<td>BSA</td>
<td>C6/DCC/BSA</td>
</tr>
</tbody>
</table>

Mice were bled at 4, 6 and 10 weeks, and thereafter as required (two weeks after boost injections), by retroorbital venous puncture using disposable Pasteur pipettes. Blood samples (0.5-1 cm³) were collected in heparin-containing tubes (50 mm³, 250 units), centrifuged and stored (-20 °C). Samples were assayed for anti-hapten Abs by a screening ELISA (4 or 6 weeks), or for anti-CB Abs by a screening RIA using [³H]CB (10 weeks, and thereafter as required).

4.2.2.3  Cell Culture Conditions

Cells were cultured in vitro in either Dulbecco’s modification of Eagle’s Medium (DMEM; low glucose, contains 584 mg/dm³ L-glutamine and 110 mg/dm³ sodium pyruvate, without sodium bicarbonate) or Iscove’s modification of Dulbecco’s medium (ISC; contains 584 mg/dm³ L-glutamine, 25 mM N-2-hydroxethylpiperazine-N’-2-ethanesulfonic acid (HEPES), without albumin, transferrin, soybean lecithin or sodium bicarbonate) buffered with sodium bicarbonate at pH 7.4, according to the manufacturer’s instructions. Complete culture medium contained 1% v/v each of solutions of sodium pyruvate (100 mM), fungizone (250 µg/cm³) and non-essential amino acids (100x solution), penicillin (5 000 IU/cm³ demineralised water) and streptomycin (5 000 µg/cm³ demineralised water), and 2-mercaptoethanol (5 mM in demineralised water). Heat-inactivated (30 min at 60 °C prior to use) Foetal Calf Serum (FCS) was added to culture medium (20% v/v). Cells were cultured at 37 °C, 5% CO₂ and 95% humidity. Cell viability was determined by exclusion of Trypan Blue dye. BALB/c murine peritoneal macrophages, used as feeder cells in all cell culture work, were collected in incomplete ISC plus 1% penicillin/streptomycin and 1% fungizone (as above) when required and added to culture vessels at a rate of 2.5 x 10⁴ cells per cm³. Cells were cultured in quantity in 75 cm³ flat-bottomed culture flasks.
4.2.2.4 Cell Fusion Protocol

The following protocol was established in the laboratories of CSIRO DAP, based on previously reported protocols, with modifications as given below.\textsuperscript{425}

Immune mice were selected for cell fusion after at least 2 boosts, on the basis of a screening RIA (\(^3\)HJC) of antisera collected 2 weeks after the most recent boost. The mouse with the highest titre of anti-CB Abs was selected for the cell fusion experiment, and the fusion was generally performed within 10 days of this result. Three days prior to cell fusion, the selected mouse was injected i.p. with a solution of the immunising conjugate (1 mg per 2 cm\textsuperscript{3} PBS; 0.2 cm\textsuperscript{3} per mouse). This was repeated the next day, but not on the day before the cell fusion.

On the day of the fusion, the selected mouse was euthanized by cervical dislocation, the spleen removed and trimmed of fat, then placed into incomplete serum-free DMEM (ISF-DMEM, 15 cm\textsuperscript{3}). The entire spleen was then cut into pieces, which were broken up with a cell scraper and the debris allowed to settle (1 min). This mixture typically contained \(\approx\)150-200 \(\times\) 10\textsuperscript{6} cells. Viable splenocytes (50 \(\times\) 10\textsuperscript{6}) were then combined with Sp2/0 myeloma cells (30 \(\times\) 10\textsuperscript{6}), the suspension centrifuged (6 min, 8 000 rpm), and cells washed with ISF-DMEM (2 \(\times\) 50 cm\textsuperscript{3}) to remove FCS and HEPES.

Polyethylene glycol (PEG; 10 g) was dissolved in water (5 g). The fusion media was prepared by addition of this PEG solution (3.6 cm\textsuperscript{3}) to double strength ISF-DMEM (1.2 cm\textsuperscript{3}) and an aqueous solution of phytohemagglutinin P (1% w/v, 0.6 cm\textsuperscript{3}), and the entire solution filtered (0.45 \(\mu\)m) to give a final PEG concentration of 45%. The fusion media (0.8 cm\textsuperscript{3}) was added to the spleen/myeloma cell pellet with gentle agitation at room temperature over 1 min. This fusion mixture was incubated at 37 °C for 1 min, then gradually diluted with ISF-DMEM (1 cm\textsuperscript{3} over 1 min, then 20 cm\textsuperscript{3} over 5 min) with continual agitation. The mixture was then centrifuged (8 min, 6 000 rpm), washed with complete ISC (20% FCS, 40 cm\textsuperscript{3}) to remove PEG, the pellet resuspended in complete ISC (20% FCS, 12 cm\textsuperscript{3}) and then distributed (50 mm\textsuperscript{3}/well) into 10 flat-bottomed 24-well plates, containing 2 cm\textsuperscript{3}/well of complete ISC (20% FCS) supplemented with Hypoxanthine (0.1 mM final concentration), Aminopterin (0.5 \(\mu\)M final concentration) and Thymidine (0.1 \(\mu\)M final concentration) (HAT media).

Half the culture medium was replaced with fresh, HAT-supplemented complete ISC (20% FCS) after 7 days and with fresh complete ISC (20% FCS) supplemented with Hypoxanthine (0.1 mM final concentration) and Thymidine (HT media) after 14 days. Wells showing growth of hybridoma cell colonies between 14 and 21 days were identified. Supernatants from these wells were tested (neat only) for anti-hapten Abs by the antibody screening ELISA (see 4.2.2.4.1) from wells 90% confluent with hybridoma cells, and thereafter as required.
Wells with the highest absorbance by the antibody screening ELISA were cultured in new 24-well plates, containing 2 cm$^3$/well of fresh complete ISC (20% FCS), at a rate of 1,000 cells per well. Supernatant aliquots were screened (neat only) for anti-hapten Abs using the antibody screening ELISA when cells were 90% confluent (≈7-10 days). The well with the highest absorbance was then selected for a limiting dilution, where cells were plated out at a rate of 0.2 cells per well in fresh complete ISC (20% FCS). Screening of these wells for anti-hapten Abs using the same ELISA (neat supernatant only) led to selection of monoclonal hybridomas for large scale production of Abs, to further characterise the mAb. The mAbs from large scale cultures were assayed over a range of dilutions using the antibody screening ELISA.

4.2.2.5 Immunoassays

4.2.2.5.1 Antibody Screening ELISA

Figure 4.2 below illustrates schematically the principle of the detection of monoclonal anti-hapten Abs (derived from mice) using an ELISA. The microtitre plate coating conjugates were prepared similarly to the immunising conjugates used to raise anti-CB Abs, but with a different carrier protein. When the wells of the ELISA microtitre plates are then coated with these coating conjugates, it is expected that only those Abs specific for the CB-like epitope (which is contained in both the immunising and coating conjugates) will bind to this portion of the coating conjugate.

**Figure 4.2** Schematic diagram of the microtitre plate coating conjugates used in the screening ELISA (the linkage between the CB-like epitope and the carrier proteins of the
coating conjugates is expected to be identical to that found in the corresponding immunising conjugates formed using the same conjugation chemistry.

The anti-hapten Abs are then actually detected using a second Ab specific for mouse Abs (since the mAbs are derived from mice), which is conjugated to the enzyme horseradish peroxidase. After the addition of an appropriate substrate for this enzyme, color develops in the well which indicates the presence of anti-hapten Abs. Microtitre plates were coated with only one coating conjugate for any given ELISA. Figure 4.2 merely illustrates the types of coating conjugates used in these assays.

Microtiter plates (96 "U-shaped" wells) were washed by soaking each well in 250 mm$^3$ of ELISA Assay Solution (EAS, 0.2 g/dm$^3$ KH$_2$PO$_4$, 8 g/dm$^3$ NaCl, 0.2 g/dm$^3$ KCl, pH 7.4) containing azide (EAS-azide, 0.8 g NaN$_3$/dm$^3$ EAS) for a minimum of 60 min at room temperature (RT), then removed by aspiration. Each well then received a solution of the required coating conjugate (Table 4.3) in EAS-azide (100 μg/cm$^3$, 100 mm$^3$/well), the plates sealed and then stored (4 °C, minimum of 48 h).

Table 4.3 Antibody screening ELISA coating conjugates derived from clenbuterol and O-alkyl clenbuterol analogues 5d (C2 Hapten) and 5g (C6 Hapten) (refer Chapter 2)

<table>
<thead>
<tr>
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<th>Carrier Protein</th>
<th>Conjugate Abbreviation</th>
</tr>
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<tbody>
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<td>C2 Hapten</td>
<td>ECDI</td>
<td>Gelatin</td>
<td>C2/ECDI/Gel</td>
</tr>
<tr>
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<td>DCC</td>
<td>Gelatin</td>
<td>C2/DCC/Gel</td>
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<td>C6 Hapten</td>
<td>DCC</td>
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<td>C6/DCC/Gel</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>Diazotisation</td>
<td>Ovalbumin</td>
<td>CB/Diazo/OA*</td>
</tr>
</tbody>
</table>

* conjugate synthesised by a method similar to$^{300}$

Gel = Gelatin, OA = Ovalbumin

The coating solution was subsequently removed and plates washed with EAS-azide (3 x 250 mm$^3$/well). To each well, an EAS-Tween-OA blocking solution (0.5 cm$^3$ Tween 20 per dm$^3$ of EAS, pH 7.4, plus 0.5% w/v OA) was added (250 mm$^3$/well), and incubated for 1.5 h at RT to inhibit NSB. Non-immunised mouse plasma (negative control plasma, NCP) was diluted 1:2 500 in EAS-OA (EAS plus 0.5% w/v OA) and used to serially dilute plasma samples from immunised mice. After aspiration of the blocking solution, the murine plasma samples (serially diluted) or cell culture supernatants (neat or serially diluted) were added in duplicate to the plate (100 mm$^3$/well), covered, and incubated at RT for 1.5 h.
Wells were then aspirated and washed with EAS-Tween-OA (3 x 250 mm³/well, 3 min per wash), prior to addition of Rabbit Anti-Mouse immunoglobulin conjugated to Horseradish Peroxidase (RAM-HRP; diluted 1:1 000 v/v in EAS-Tween-OA), to each well (100 mm³/well) and incubated for a further 1.5 h at RT. Excess RAM-HRP was removed by aspiration and wells washed with EAS (4 x 250 mm³/well). Citrate buffer (2.10 g citric acid per 100 cm³ of 0.5 mM phosphate buffer, pH 4.9) was used to prepare the substrate solution (10 mm³ of H₂O₂ (30% w/v) per 10 cm³ citrate buffer plus 2 mg/cm³ o-phenylenediamine), which was added to the plates (100 mm³/well). The plates were then stored in the dark for exactly 6 min, and reaction terminated with NaN₃ solution (4.0 g/dm³ H₂O, 25 mm³/well). Plates were placed in an ELISA plate reader and specific absorbance at 450 nm calculated by subtraction of the average of the blank wells. Ab binding profiles (Specific Absorbance vs the log of the sample dilution) were plotted.

Ab classing (i.e. IgG, IgM or IgA) was determined in an identical assay using class-specific RAM-HRP. Culture supernatants were assayed neat, whilst the class-specific RAM-HRP was used at dilutions of 1:1 000, 1:2 000 and 1:4 000 (v/v).

4.2.2.5.2 Antibody Screening RIA

PNA buffer (0.1% w/v NaN₃ and 1% w/v gelatin (≈300 bloom) in 50 mM PBS, pH 7.4) was used for both plasma dilutions and the radiolabel solution. The charcoal solution was freshly prepared for each assay, and performed as described previously (section 4.2.1.3). Plasma from sheep immunised with the CB/Diazo/HSA conjugate (in a previous experiment in this laboratory) were used as reference samples.

Murine plasma samples were diluted 1:10, then serially diluted (1:1) ten times in PNA buffer (200 mm³/tube). Cell culture supernatants were assayed neat (200 mm³/tube). [³H]CB (specific activity: 16.8 Ci/mmol) in PNA was then added (20 000 cpm/cm³, 500 mm³/tube), followed by incubation overnight at 4 °C. Bound and free label were separated by addition of the homogeneous suspension of charcoal (100 mm³/tube) and incubated for 20 min at 4 °C, prior to centrifugation (10 min, 2400 rpm, 4 °C). Supernatants were transferred into vials, scintillant added (4 cm³/vial), vials capped then shaken, and stored in the dark for at least 60 min to allow dissipation of chemiluminescence. The radioactivity in each vial was counted in a beta counter (Beckman 1217, [³H] counting efficiency 56.5%) for 60 s per vial.

In a separate experiment, using only neat mAb cell culture supernatant, bound and free label were separated using Protein A-Sepharose beads. Briefly, supernatant (1 cm³/tube, duplicate) and [³H]CB in PBS-Tween (50 mM PBS plus 0.1% v/v Tween 20, 50 000 cpm/cm³, 200
mm³/tube) were incubated at RT for 2 h in small plastic centrifuge tubes (1.5 cm³). A solution of Protein A-Sepharose beads (0.5 g beads per 50 cm³ 50 mM PBS) plus Nonidet P-40 (1% v/v) was then added to each tube (200 mm³/tube) followed by incubation (30 min at 4 °C) with continual sample agitation, prior to centrifugation in a benchtop centrifuge (3 min) and washing with PBS-Tween (4 x 500 mm³/tube). A denaturing buffer (50 mM PBS plus 5% v/v 5 mM 2-mercaptoethanol) was then added (500 mm³/tube), the sample centrifuged and an aliquot of the supernatant carefully removed (400 mm³). This aliquot was added to the scintillation vial, and treated as described previously.

NSB for plasma samples was determined from tubes containing no antisera. NSB for culture supernatants was determined from tubes containing mAb-free culture media. In both cases, the NSB value was subtracted from the observed counts of corresponding tubes. Inter-assay CV was 11.0% and intra-assay CV was 12.7%, calculated from the reference samples. Dilution curves (plots of %SB vs log of dilution) were plotted.

4.2.2.5.3 Total IgG ELISA

Microtiter plates (96 “U-shaped” wells) were washed by soaking each well in 250 mm³ of EAS-azide for a minimum of 60 min, which was then removed by aspiration. Each well then received a solution of goat anti-(mouse IgG Fc fragment) (GAM) in EAS-azide (15 μg/cm³, 100 mm³/well), the plates were sealed and then stored (4 °C minimum of 96 h).

The coating solution was subsequently removed and plates washed once with EAS-azide (250 mm³/well). To each well, an EAS-azide-OA blocking solution (0.5% w/v OA, pH 7.4) was added (250 mm³/well), then incubated for 1.5 h at RT to inhibit NSB. After aspiration of the blocking solution, murine IgG standards, 2 μg/cm³ to 15 ng/cm³, or mAb culture supernatants, diluted 1:50 to 1:3 200, were added in duplicate to the plate (100 mm³/well), then covered, and incubated at RT for 1.5 h. Standards or samples were appropriately diluted with fresh culture medium identical to that of the mAb culture supernatant under test.

Wells were then aspirated and washed with EAS-OA (0.5% w/v OA, 3 x 250 mm³/well), prior to addition of Goat Anti-Mouse immunoglobulin conjugated to Horseradish Peroxidase (GAM-HRP; diluted 1:1 000 v/v in EAS-OA) to each well (100 mm³/well) and incubated for a further 1.5 h at RT. Excess GAM-HRP was removed by aspiration and wells washed with EAS (4 x 250 mm³/well). Substrate solution (10 mm³ of H₂O₂ per 10 cm³ citrate buffer plus 2 mg/cm³ o-phenylenediamine) was then added to the plates (100 mm³/well). The plates were then stored in the dark for exactly 6 min and reaction terminated with NaN₃ (4.0 g/dm³ H₂O, 25 mm³/well). Plates were placed in an ELISA plate reader and specific absorbance at 450 nm calculated by subtraction of the average of the blank wells.
A standard curve of specific absorbance against IgG concentration was constructed from those standards whose absorbance values were in the linear absorbance range according to Beer's Law (0.2-0.8 absorbance units). The total IgG concentration of mAb cell culture supernatants was determined from the mean of those dilutions found on the standard IgG curve. The inter-assay CV was 14.2% and the intra-assay CV was 14.0%.

4.2.2.6 Statistical Analysis of Results

Where appropriate, results were expressed as mean ± SEM.

4.3 Results

4.3.1 Polyclonal Antibody (Sheep) Characterisation

4.3.1.1 Antibody Titre

Figures 4.3 and 4.4 show the titre response curves of the four conjugates (derived from the haptenic O-alkyl CB analogues, 5d and 5g) over the immunisation period.

![Graph showing antibody titre over weeks](image)

**Figure 4.3** Titre response curve of sheep immunised with conjugates derived from C2 Hapten (5d) and the CB-Diazo conjugates (each data point represents the mean of six animals, except C2/ECDI/BSA where n=7)
Figure 4.4  Titre response curve of sheep immunised with conjugates derived from the C6 Hapten (5g), and the CB-Diazo conjugates (each data point represents the mean of six animals)

Group average anti-CB Ab titres (± SEMs) over the entire immunisation period are summarised in Table 4.4.

Table 4.4  Group means* of anti-CB antibody titres from sheep immunised with either CB- or analogue-protein conjugates

<table>
<thead>
<tr>
<th>Week</th>
<th>Conjugate</th>
<th>C2/ECDI (n = 7)</th>
<th>C2/DCC (n = 6)</th>
<th>C6/ECDI (n = 6)</th>
<th>C6/DCC (n = 6)</th>
<th>CB/Diazo (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
<td>&lt; 70</td>
</tr>
<tr>
<td>17</td>
<td>492 ± 167</td>
<td>1758 ± 1423</td>
<td>312 ± 114</td>
<td>525 ± 298</td>
<td>514 ± 143</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>3175 ± 778</td>
<td>10366 ± 4599</td>
<td>2321 ± 694</td>
<td>2639 ± 866</td>
<td>4734 ± 1385</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>10115 ± 3048</td>
<td>18577 ± 8485</td>
<td>5555 ± 1869</td>
<td>6798 ± 1763</td>
<td>9508 ± 2257</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>10651 ± 4496</td>
<td>13126 ± 4658</td>
<td>7892 ± 3157</td>
<td>6663 ± 1985</td>
<td>8097 ± 1885</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>5743 ± 2991</td>
<td>5885 ± 2755</td>
<td>5207 ± 2604</td>
<td>2537 ± 1013</td>
<td>2286 ± 634</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>12234 ± 4422</td>
<td>24771 ± 11369</td>
<td>4608 ± 1950</td>
<td>8728 ± 2584</td>
<td>12558 ± 3808</td>
<td></td>
</tr>
<tr>
<td>32</td>
<td>9509 ± 3427</td>
<td>19105 ± 8706</td>
<td>4568 ± 2059</td>
<td>7375 ± 3242</td>
<td>6461 ± 1437</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>6794 ± 2476</td>
<td>13325 ± 5820</td>
<td>7420 ± 4669</td>
<td>6842 ± 3770</td>
<td>3276 ± 820</td>
<td></td>
</tr>
</tbody>
</table>

* Results expressed as Mean ± SEM

In all but one conjugate group (C6/ECDI/BSA) the highest Ab titre occurred two weeks post second boost (week 30). Therefore, plasma samples from this bleed (week 30) from the three sheep with the highest anti-CB Ab titres (from each conjugate group) were chosen and
reassayed in duplicate. The titres of these sheep are summarised in Table 4.5. Titres of these samples were the final dilutions of antisera subsequently used in the competition studies.

**Table 4.5** Anti-CB antibody titres at week 30 (2 weeks post second boost)* of sheep plasmas selected for competition studies

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Sheep No.</th>
<th>Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2/ECDI/BSA</td>
<td>547</td>
<td>30 137 ± 347</td>
</tr>
<tr>
<td></td>
<td>562</td>
<td>29 860 ± 2 060</td>
</tr>
<tr>
<td></td>
<td>624</td>
<td>17 147 ± 395</td>
</tr>
<tr>
<td>C2/DCC/BSA</td>
<td>439</td>
<td>20 146 ± 464</td>
</tr>
<tr>
<td></td>
<td>469</td>
<td>23 820 ± 2 730</td>
</tr>
<tr>
<td></td>
<td>485</td>
<td>99 951 ± 5 747</td>
</tr>
<tr>
<td>C6/ECDI/BSA</td>
<td>590</td>
<td>8 405 ± 387</td>
</tr>
<tr>
<td></td>
<td>608</td>
<td>14 770 ± 180</td>
</tr>
<tr>
<td></td>
<td>610</td>
<td>9 245 ± 849</td>
</tr>
<tr>
<td>C6/DCC/BSA</td>
<td>488</td>
<td>8 124 ± 467</td>
</tr>
<tr>
<td></td>
<td>552</td>
<td>16 185 ± 187</td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>22 341 ± 257</td>
</tr>
<tr>
<td>CB/Diazo/HSA</td>
<td>437</td>
<td>32 673 ± 753</td>
</tr>
<tr>
<td></td>
<td>451</td>
<td>21 505 ± 2 709</td>
</tr>
<tr>
<td></td>
<td>656</td>
<td>12 897 ± 1 037</td>
</tr>
</tbody>
</table>

* Results expressed as Mean ± SEM (n = 2)
4.3.1.2 Antibody Specificity

The pAbs (in sheep) raised against the five conjugates containing a CB-like epitope (either CB, C2 Hapten or C6 Hapten) were all found to bind to CB, since $[^3H]CB$ was displaced by non-radiolabelled CB, illustrated by the displacement curves below (Figures 4.5, 4.6 and 4.7). These curves were similar for Abs raised from all conjugates, except the C6/ECDI/BSA conjugate, where the curves were consistently displaced to the right, and reflected the increased amount of non-radiolabelled ligand required to displace 50% of the radiolabel ($[^3H]CB$).

**Figure 4.5** Displacement curves of polyclonal antibodies raised against C2 Hapten-protein conjugates in comparison with those raised against the CB-protein conjugate, where CB was the competing ligand

**Figure 4.6** Displacement curves of polyclonal antibodies raised against C6 Hapten-protein conjugates in comparison with those raised against the CB-protein conjugate, where CB was the competing ligand
Figure 4.7 Displacement curves of polyclonal antibodies from sheep plasmas with similar Ab titres, raised against either CB- or analogue-protein conjugates, where CB was the competing ligand.
Chapter 4 - Antibody Characterisation

The concentrations of various ligands required to displace 50% of the total amount of bound \(^{3}H\)CB (50% SB) from pAbs raised against the five immunising conjugates were calculated from ligand displacement curves. No displacement of radiolabel was observed for ISO, ADR, NOR, Sotalol and ICI 118551 at the highest ligand concentration (50 nM). Those concentrations (at 50% SB) of ligands which did displace \(^{3}H\)CB over the concentration range tested are summarised in Table 4.6.

**Table 4.6** Concentrations of various ligands* at 50% Specific Binding of \(^{3}H\)CB by polyclonal antibodies raised against either CB- or analogue-protein conjugates

<table>
<thead>
<tr>
<th>Sheep</th>
<th>CB (nM)</th>
<th>VUF 8303 (nM)</th>
<th>O-Methyl VUF (nM)</th>
<th>C2 Hapten (nM)</th>
<th>C6 Hapten (nM)</th>
<th>SALB (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>547</td>
<td>1.55 ± 0.10</td>
<td>5.10 ± 0.21</td>
<td>1.22 ± 0.01</td>
<td>1.61 ± 0.08</td>
<td>0.92 ± 0.03</td>
<td>59.8 ± 1.1</td>
</tr>
<tr>
<td>562</td>
<td>1.39 ± 0.07</td>
<td>7.18 ± 0.07</td>
<td>0.62 ± 0.01</td>
<td>1.22 ± 0.01</td>
<td>0.78 ± 0.02</td>
<td>110 ± 2</td>
</tr>
<tr>
<td>624</td>
<td>1.25 ± 0.03</td>
<td>5.03 ± 0.16</td>
<td>0.83 ± 0.01</td>
<td>1.21 ± 0.02</td>
<td>0.85 ± 0.01</td>
<td>39.4 ± 0.9</td>
</tr>
<tr>
<td>439</td>
<td>1.20 ± 0.02</td>
<td>3.59 ± 0.03</td>
<td>0.81 ± 0.02</td>
<td>1.06 ± 0.04</td>
<td>0.70 ± 0.03</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>469</td>
<td>1.24 ± 0.01</td>
<td>5.17 ± 0.15</td>
<td>0.68 ± 0.01</td>
<td>0.85 ± 0.01</td>
<td>0.71 ± 0.01</td>
<td>72.1 ± 1.9</td>
</tr>
<tr>
<td>485</td>
<td>1.13 ± 0.02</td>
<td>3.12 ± 0.06</td>
<td>0.84 ± 0.01</td>
<td>1.10 ± 0.02</td>
<td>1.01 ± 0.04</td>
<td>43.0 ± 0.4</td>
</tr>
<tr>
<td>590</td>
<td>1.49 ± 0.03</td>
<td>6.37 ± 0.27</td>
<td>0.96 ± 0.02</td>
<td>2.17 ± 0.03</td>
<td>0.91 ± 0.04</td>
<td>144 ± 3</td>
</tr>
<tr>
<td>608</td>
<td>1.81 ± 0.04</td>
<td>7.43 ± 0.22</td>
<td>1.20 ± 0.02</td>
<td>4.06 ± 0.04</td>
<td>1.10 ± 0.02</td>
<td>66.2 ± 1.8</td>
</tr>
<tr>
<td>610</td>
<td>2.15 ± 0.02</td>
<td>9.27 ± 0.26</td>
<td>1.72 ± 0.02</td>
<td>3.02 ± 0.04</td>
<td>1.77 ± 0.05</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>488</td>
<td>1.44 ± 0.02</td>
<td>7.58 ± 0.16</td>
<td>1.04 ± 0.02</td>
<td>2.27 ± 0.06</td>
<td>0.94 ± 0.01</td>
<td>246 ± 2</td>
</tr>
<tr>
<td>552</td>
<td>1.20 ± 0.03</td>
<td>6.58 ± 0.24</td>
<td>1.00 ± 0.03</td>
<td>1.63 ± 0.04</td>
<td>0.90 ± 0.01</td>
<td>106 ± 2</td>
</tr>
<tr>
<td>599</td>
<td>1.06 ± 0.04</td>
<td>8.84 ± 0.18</td>
<td>0.58 ± 0.01</td>
<td>2.67 ± 0.13</td>
<td>0.72 ± 0.02</td>
<td>169 ± 3</td>
</tr>
<tr>
<td>437</td>
<td>1.32 ± 0.03</td>
<td>4.46 ± 0.12</td>
<td>1.38 ± 0.03</td>
<td>1.44 ± 0.02</td>
<td>1.11 ± 0.03</td>
<td>37.8 ± 0.5</td>
</tr>
<tr>
<td>451</td>
<td>1.23 ± 0.03</td>
<td>3.91 ± 0.09</td>
<td>0.83 ± 0.02</td>
<td>1.13 ± 0.01</td>
<td>0.82 ± 0.02</td>
<td>26.6 ± 0.1</td>
</tr>
<tr>
<td>656</td>
<td>1.52 ± 0.02</td>
<td>3.88 ± 0.09</td>
<td>1.50 ± 0.01</td>
<td>2.90 ± 0.05</td>
<td>1.25 ± 0.04</td>
<td>73.6 ± 0.8</td>
</tr>
</tbody>
</table>

*Results expressed as Mean ± SEM (n=3)

CB = Clenbuterol,  SALB = Salbutamol

% Cross Reactivity (%CR) of each of the non-radiolabelled ligands which bound competitively to the pAbs was calculated from the ligand concentration at 50% SB. For each sheep, the %CR of CB was set at 100%. These results are summarised in Table 4.7.
## Table 4.7

% Cross Reactivity of various ligands with polyclonal antibodies raised against either CB- or analogue-protein conjugates (CB = 100% for each sheep)

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Sheep</th>
<th>Ligand VUF</th>
<th>O-Methyl C2 Hapten</th>
<th>C6 Hapten</th>
<th>SALB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8303 VUF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C2/ECDI/</td>
<td>547</td>
<td>30.4 127.3</td>
<td>96.1 168.4</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>562</td>
<td>19.3 224.8</td>
<td>113.7 178.8</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>624</td>
<td>24.7 150.7</td>
<td>102.9 146.2</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>C2/DCC/</td>
<td>439</td>
<td>33.5 149.0</td>
<td>113.2 170.6</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>469</td>
<td>23.9 183.2</td>
<td>145.6 174.9</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>485</td>
<td>36.2 135.0</td>
<td>102.9 112.0</td>
<td>2.6</td>
<td></td>
</tr>
<tr>
<td>C6/ECDI/</td>
<td>590</td>
<td>23.4 155.6</td>
<td>68.7 164.3</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>608</td>
<td>24.3 150.2</td>
<td>44.4 163.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>610</td>
<td>23.2 125.5</td>
<td>71.3 121.9</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>C6/DCC/</td>
<td>488</td>
<td>19.0 139.0</td>
<td>63.4 153.4</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>552</td>
<td>18.3 120.2</td>
<td>73.7 132.3</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>12.0 183.9</td>
<td>39.8 146.6</td>
<td>0.6</td>
<td></td>
</tr>
<tr>
<td>CB/Diazo/HSA</td>
<td>437</td>
<td>29.7 96.1</td>
<td>91.6 119.0</td>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>451</td>
<td>31.3 147.5</td>
<td>108.2 149.9</td>
<td>4.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>656</td>
<td>39.3 101.5</td>
<td>52.6 122.1</td>
<td>2.1</td>
<td></td>
</tr>
</tbody>
</table>

Figures 4.8A-4.9E shows representative examples of displacement curves these ligands.

### A

**Sheep No. 547 (C2/ECDI/BSA)**

![Displacement Curve](image)
Figure 4.8 Representative examples of displacement curves for CB and analogues from each conjugate group

The ligands which showed the highest %CR with the anti-CB Abs were C6 Hapten and O-Methyl VUF 8303. Representative examples of the displacement curves for these ligands from each conjugate group are given in Figures 4.9 and 4.10.

Figure 4.9 Displacement curves (non-radiolabelled C6 Hapten) of polyclonal antibodies raised in sheep against either CB- or analogue-protein conjugates
Figure 4.10 Displacement curves (non-radiolabelled O-Methyl VUF 8303) of polyclonal antibodies raised in sheep against either CB- or analogue-protein conjugates.

Scatchard plots\(^{431}\) were made from the non-radiolabelled CB competition data, and an example is given in Figure 4.11. Similar non-linear curves resulted from the non-radiolabelled CB competition data of other sheep plasmas, as well as from other competing non-radiolabelled ligands.

Figure 4.11 Scatchard plots for sheep #552 and #437

Competitive binding data was also analysed using the LIGAND computer program\(^{392}\) (MacLIGAND v4.93), which utilised non-linear least squares curve fitting techniques, however none of the competition data could be fitted to a single binding site model using this program.
4.3.2 Cell Fusion and Hybridoma Selection

Plasma collected from all mice two weeks post first boost vaccination showed the presence of anti-hapten Abs by the antibody screening ELISA (section 4.2.2.4.1). This was indicated by the presence of Abs which bound to the analogous gelatin-derived coating conjugate synthesised from the O-alkyl analogues 5d and 5g. Figure 4.12 is an example of the dilution curves obtained from these screening ELISAs, and shows an example from each of the four conjugate groups (two weeks post first boost).

![Graph](image)

**Figure 4.12** Dilution curve from the screening ELISA of plasma from mice 2 weeks after the first boost with the conjugate indicated (highest responders from each group only shown)

Plasma collected from all mice two weeks post second boost vaccination (and also two weeks post any boosts thereafter) showed the presence of Abs which bound [3H]CB by the antibody screening RIA (section 4.2.2.4.2). However, Ab titres were found to be very low (1:200 to 1:800).

Furthermore, dilution curves from the screening RIA were very shallow by comparison to the reference sample (as described previously). The mouse showing the greatest percentage of the Total Counts that were bound at a final plasma dilution of 1:70 (*i.e.* first of the serial dilution series) was selected for cell fusion experiments. Figure 4.13 is an example of the dilution curves observed in the mouse, and shows the highest responders for each of the four conjugates two weeks post second boost.
The spleen cells of mice selected by the screening RIA above were fused with Sp2/0 myeloma cells (as per section 4.2.2.4) within 10 days of the time the blood sample was taken. The results of the cell fusions performed are summarised in Table 4.8:

Table 4.8  Summary of cell fusions of splenocytes from mice immunised with conjugates derived from Haptens 5d and 5g

<table>
<thead>
<tr>
<th>Immunising Conjugate</th>
<th>Number of Fusions</th>
<th>Successful Fusions*</th>
<th>Fusion Efficiency (%) **</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2/ECDI/ED-BSA</td>
<td>2</td>
<td>1</td>
<td>6%</td>
</tr>
<tr>
<td>C2/ECDI/BSA</td>
<td>2</td>
<td>1</td>
<td>18%</td>
</tr>
<tr>
<td>C2/DCC/BSA</td>
<td>3</td>
<td>2</td>
<td>8%, 3%</td>
</tr>
<tr>
<td>C6/ECDI/BSA</td>
<td>1</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>C6/DCC/BSA</td>
<td>2</td>
<td>1</td>
<td>&lt;1%</td>
</tr>
</tbody>
</table>

* Unsuccessful fusions showed either no cell growth or were contaminated by bacteria
** Fusion Efficiency (%) = percentage of wells that contained growing hybridoma colonies

The successful fusion from mice immunised with the C2/ECDI/BSA conjugate (fusion efficiency 18%) provided polyclonal colonies that grew slowly. Attempts to culture positive polyclonal colonies (selected by highest absorbance in a screening ELISA) from this fusion were unsuccessful, and therefore no further work was done.
Monoclonal IgG Ab-secreting hybridomas (TB1-TB12) were obtained from the C2/DCC/BSA conjugate (first fusion, fusion efficiency 8%) by a limiting dilution of the polyclonal well with the highest absorbance (by the antibody screening ELISA) from the 1000 cells per well dilution (refer section 4.2.2.4). A subsequent fusion using an alternative source of FCS in the culture medium, was less successful (fusion efficiency 3%). However, no further work was done from this cell fusion due to the low fusion efficiency.

Some repeat cell fusions also involved experiments utilising two separate aliquots of murine splenocytes to improve the number of wells containing growing colonies of hybridomas. However, no improvement in the number of wells that contained growing hybridoma colonies was obtained from these experiments. Fusion efficiencies for the cell fusions resulting from C6 hapten-derived conjugates were very low, and therefore no further work was performed with these cells.

4.3.3 Monoclonal Antibody Characterisation

Previous cell fusions in this laboratory (CSIRO DAP) led to a monoclonal hybridoma (PD4), derived from a mouse immunised with the CB/Diazo/HSA conjugate (Table 4.1, refer section 4.2.1.2), that secreted CB-specific mAb. This cell line was kindly donated by Dr V.H. Huynh and Mr P. Donnelly. The PD4 monoclonal was grown in both complete ISC plus 20% FCS or complete ISC (SF), alongside monoclones TB1-TB12, cultured similarly. The culture supernatants from these clones were collected and used to characterise the mAb secreted by each of the monoclonal cell lines.
4.3.3.1 Total IgG Concentrations in Culture Supernatant

The Total IgG concentrations in culture supernatants of monoclones PD4 and TB1-TB12 grown in complete ISC (with or without 20% FCS) are summarised in Table 4.9. The IgG levels from all monoclones were within the range of 10-100 μg/cm³, a normal concentration expected from classical in vitro cell culture techniques. However, IgG concentrations arising from clones TB2 and TB11 cultured in serum-free culture medium were below 10 μg/cm³.

Table 4.9  Total IgG* concentration of culture supernatants of monoclones TB1-TB12

<table>
<thead>
<tr>
<th>mAb</th>
<th>Complete ISC Culture Medium</th>
<th>20% FCS (IgG, μg/cm³)</th>
<th>Serum-Free (SF) (IgG, μg/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PD4</td>
<td></td>
<td>19.3 ± 1.5</td>
<td>25.3 ± 1.7</td>
</tr>
<tr>
<td>TB1</td>
<td></td>
<td>35.3 ± 4.3</td>
<td>26.9 ± 2.5</td>
</tr>
<tr>
<td>TB2</td>
<td></td>
<td>14.5 ± 1.2</td>
<td>9.6 ± 0.8</td>
</tr>
<tr>
<td>TB3</td>
<td></td>
<td>32.3 ± 6.2</td>
<td>26.3 ± 1.4</td>
</tr>
<tr>
<td>TB4</td>
<td></td>
<td>21.8 ± 1.4</td>
<td>22.9 ± 2.7</td>
</tr>
<tr>
<td>TB5</td>
<td></td>
<td>16.2 ± 1.6</td>
<td>11.5 ± 0.6</td>
</tr>
<tr>
<td>TB6</td>
<td></td>
<td>32.1 ± 3.7</td>
<td>23.4 ± 2.3</td>
</tr>
<tr>
<td>TB7</td>
<td></td>
<td>27.4 ± 2.6</td>
<td>15.9 ± 1.4</td>
</tr>
<tr>
<td>TB8</td>
<td></td>
<td>27.5 ± 2.7</td>
<td>19.7 ± 1.0</td>
</tr>
<tr>
<td>TB9</td>
<td></td>
<td>28.5 ± 2.8</td>
<td>21.5 ± 2.2</td>
</tr>
<tr>
<td>TB10</td>
<td></td>
<td>16.9 ± 1.8</td>
<td>16.4 ± 0.5</td>
</tr>
<tr>
<td>TB11</td>
<td></td>
<td>14.4 ± 0.7</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>TB12</td>
<td></td>
<td>26.8 ± 2.2</td>
<td>21.1 ± 1.6</td>
</tr>
</tbody>
</table>

* Results expressed as Mean ± SEM
4.3.3.2 Specificity of mAbs by ELISA

Two coating conjugates that contained a CB-like epitope (CB/Diazo/OA and C2/DCC/Gel) were recognised by the anti-CB mAb PD4 in the screening ELISA. The dilution curves for the serum-free PD4 mAb from the screening ELISA using these two plate-coating conjugates were plotted in Figure 4.14. Similar results were observed for samples grown in complete ISC (20% FCS).

![Dilution curves of PD4 mAb supernatants in the screening ELISA using two different plate-coating conjugates](image)

**Figure 4.14** Dilution curves of PD4 mAb supernatants in the screening ELISA using two different plate-coating conjugates

Similar assays of mAbs TB1-TB12 showed that none of the Ab present in the culture supernatants of any of these monoclones recognised either of the plate-coating conjugates. Figures 4.15 and 4.16 show representative examples of dilution curves from the assays of supernatant samples of clones grown in complete ISC culture media (SF and 20% FCS). Similar dilution curves were observed for all other TB monoclones, in both types of culture media and with both plate-coating conjugates.
Figure 4.15 Dilution curves of PD4 and TB4 mAb supernatants (SF) in the screening ELISA using the CB/Diazo/OA as plate-coating conjugate

Figure 4.16 Dilution curves of PD4 and TB3 mAb supernatants (20% FCS) in the screening ELISA using the C2/DCC/Gel as plate-coating conjugate

4.3.3.3 Specificity of mAbs by RIA

TB1-TB12 and PD4 mAbs were assayed (neat, charcoal radiolabel separation) for their ability to bind [3H]CB in the screening RIA. None of the TB1-TB12 mAbs were deemed specific for [3H]CB using this protocol. Figures 4.17 and 4.18 show representative examples from the assays of supernatant samples of clones grown in complete ISC culture media (SF and 20% FCS). Binding of [3H]CB is expressed in these Figures as the percentage of the available counts (% Total Counts) at equivalent levels of IgG.
Figure 4.17 Level of binding (% of Total Counts) by mAb in serum-free culture medium of monoclones TB1, TB4, TB6, TB12 and PD4

Figure 4.18 Level of binding (% of Total Counts) by mAb in 20% FCS culture medium of monoclones TB2, TB3, TB8, TB9 and PD4

TB9 mAb (SF) was also assayed using Protein A-Sepharose to separate bound from free label. In this case, the percentage of Total Counts specifically bound by this mAb was 0.02% (indistinguishable from the culture medium blank), whereas PD4 mAb bound 12.4% of the Total Counts.
4.4 Discussion

The purpose of this section of the research was to characterise immunologically the $O$-alkyl analogues of CB, through an evaluation of Abs raised against protein conjugates derived from analogues 5d (C2 Hapten) and 5g (C6 Hapten) (Chapter 2). Two methods for the production of these Abs, polyclonal (pAbs) and monoclonal (mAbs), were investigated. Polyclonal anti-CB Abs were produced in sheep using the novel $O$-alkyl analogue-protein conjugates, but attempts to produce murine-derived monoclonal anti-CB Abs from these conjugates were unsuccessful.

Sheep showed a typical pattern of Ab response to immunisation with the analogue-protein or CB-protein conjugates. Anti-CB Ab titre was highest after the second boost immunisation, in all groups except the C6/ECDI/BSA conjugate group. Titre levels of sheep in this group were comparable to the C6/DCC/BSA group. However, when titres of all other groups four weeks post boost (1st or 2nd) were decreasing, Ab titres in the C6/ECDI/BSA group were still greater at this point than at any previous point in the immunisation regime of this conjugate. The reason for the apparently slower immune response to the C6/ECDI/BSA conjugate was unclear from the present data, although it may have been indicative of poorer immunogenicity of this conjugate, since the highest titre levels for this group tended to be slightly lower than the highest titres observed in the other conjugate groups.

Titres of anti-CB Abs from C2 Hapten-derived conjugates were comparable to those of the CB/Diazo conjugate, and generally higher than those from C6 Hapten-derived conjugates. Furthermore, higher Ab titres generally persisted longer in sheep immunised with the novel conjugates than with the CB/Diazo conjugate, and Ab titres of sheep from the CB/Diazo group were lower than any other group at 28 and 36 weeks (except for C6/DCC at second boost). This may have indicated that the C2 Hapten- and C6 Hapten-protein conjugates were better immunogens than the CB-protein conjugate. Indeed, these conjugates tended to produce group mean anti-CB Ab titres slightly higher (though statistically insignificant) than the CB/Diazo group at 28 and 36 weeks. Considerable between-animal variation in Ab titre was observed (CV range $\approx$50-120%). These variations were to be expected since no two animals will respond similarly to a given immunogen.$^{427}$

Some reports have suggested that lower Ab titres result from immunisation with conjugates which have a lower hapten density,$^{428}$ although others have suggested a limit to hapten density beyond which no further advantage in Ab titre was gained.$^{429}$ The highest anti-CB Ab titre obtained from immunisations with the analogue- or CB-protein conjugates was $\approx 1:100$ 000 (C2/DCC/BSA), and could have been due to a higher hapten density for this conjugate. However, no conclusion relevant to either of the foregoing viewpoints was possible from the
present data since hapten densities were not determined, and the high titres were not consistently replicated within this conjugate group.

Specificity of pAbs for CB was confirmed by displacement of $[^3]$H]CB with non-radiolabelled CB (Figures 4.5, 4.6 and 4.7, section 4.3.1.2). However, pAbs from two of the three sheep immunised with the C6/ECDI/BSA conjugate required CB concentrations (at 50% SB) that were higher than for pAbs from any other conjugate group (including C6/DCC/BSA). The results suggested that these Abs may have had a slightly lower affinity for CB. The pAbs raised against the two C2 Hapten-derived conjugates (ECDI or DCC) also tended to show similar differences, albeit less marked (slightly lower CB concentrations at 50% SB for DCC conjugate group). These observations perhaps indicated that DCC-mediated hapten conjugation produced pAbs of greater affinity for CB, in comparison to ECDI-mediated hapten coupling. This was consistent with conjugation of the C2 and C6 Haptens via activated NHS esters (formed using DCC) being designed to more efficiently and specifically couple these analogues to the carrier protein.

CB concentrations at 50% SB for pAbs elicited by the CB/Diazo/HSA conjugate tended to be similar to those of the C2/ECDI/BSA conjugate group, and greater than either of the DCC conjugates. From the present data, it appeared that anti-CB Abs produced from the DCC conjugates tended to have a greater affinity for CB than anti-CB Abs from the other conjugates, because lower concentrations of authentic CB were required to displace 50% of the radiolabelled CB. However, this difference was not significant in comparison to the previously reported conjugate (CB/Diazo).

Competitive binding studies with non-radiolabelled CB (or any other non-radiolabelled ligand which displaces the radiolabel) may be used to quantify the affinity of pAbs for CB (or any other non-radiolabelled ligand), through calculation of the affinity constant ($K_a$). However, the heterogeneous nature of pAbs results in a $K_a$ value that is an average of the affinities of all Abs present which bind radiolabel.\textsuperscript{421,422} In addition, considerable Ab titre variation between animals, even within a single conjugate group, results in differing concentrations of Ab from plasma to plasma in each competition assay. Furthermore, each animal will have a unique immunological response to a given antigen, reflected not only in the affinity but in the specificity for particular epitopes.\textsuperscript{313} In contrast, a similar characterisation of mAbs allows accurate determination of $K_a$ (for a given non-radiolabelled ligand) as well as specificity, since there is a homogeneous population of ligand binding sites, at equal Ab concentrations.

Approximations of $K_a$ can be made from the ligand displacement curves,\textsuperscript{430} where $K_a = 1/[$Ligand$]$ at 50% SB. Alternatively, Scatchard plots\textsuperscript{431} of the ratio of Bound/Free ligand against concentration of Bound ligand allow estimations of $K_a$ from the slope of the resulting
curve (slope = -$K_a$). Scatchard plots also provide information regarding the nature of the ligand binding. When the type of binding site is homogeneous, the curve is linear and an accurate determination of $K_a$ can be made from the plot. However, a heterogeneous population of binding sites gives rise to a more complex situation, reflected by non-linear Scatchard plots.\textsuperscript{432,433} An example of a Scatchard plot from the non-radiolabelled CB competition data is given in Figure 4.11 (refer section 4.3.1.2). Such a non-linear curve tended to suggest a heterogeneous population of binding sites, as would be expected from pAbs. However, the precise nature of these binding sites would require an increased number of data points to better define the Scatchard plot.

Characterisation of pAb specificity was required to assess the utility of the analogue-protein conjugates in the generation of anti-idiotypic Abs that mimic the physiological properties of CB. Determination of a Rank Order of Affinity of the panel of non-radiolabelled ligands for pAbs from several animals from each group would provide a characterisation of specificity. The Rank Order of Affinity may be constructed from $K_a$ values of the pAbs for each ligand, but was more conveniently obtained by comparison of % Cross Reactivities (%CR). This approach circumvents the complex analysis of non-linear Scatchard plots obtained from the competition data. Estimates of $K_a$ from the ligand concentrations at 50% SB were of the order of $10^8$ to $10^9$ M$^{-1}$, which was in reasonable agreement with the general $K_a$ range of antibodies\textsuperscript{421} of $\approx10^9$ to $10^{11}$ M$^{-1}$. The Rank Orders of Affinity are given in Table 4.10 and the structures of the ligands in that table are reproduced below in Figure 4.19.

![Figure 4.19 Structures of Clenbuterol (CB), Salbutamol (SALB), VUF 8303, O-Methyl VUF 8303, C2 Hapten and C6 Hapten](image-url)
Table 4.10 summarises the results of the competition studies for the ligands which showed significant cross-reactivity, by ranking them according to their %CR.

Table 4.10  Rank Order of Affinity of various ligands* for the polyclonal antibodies raised against either CB- or analogue-protein conjugates

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>Sheep</th>
<th>Order of Affinity of Competing Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2/ECDI/BSA</td>
<td>547</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>562</td>
<td>O-Me &gt; C6 &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>624</td>
<td>O-Me &gt; C6 &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td>C2/DCC/BSA</td>
<td>439</td>
<td>C6 &gt; O-Me &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>469</td>
<td>O-Me &gt; C6 &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>485</td>
<td>O-Me &gt; C6 &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td>C6/ECDI/BSA</td>
<td>590</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>608</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>610</td>
<td>O-Me &gt; C6 &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td>C6/DCC/BSA</td>
<td>488</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>552</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>599</td>
<td>O-Me &gt; C6 &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td>CB/Diaz/HSA</td>
<td>437</td>
<td>C6 &gt; CB &gt; O-Me &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>451</td>
<td>C6 &gt; O-Me &gt; C2 &gt; CB &gt; VUF &gt; SALB</td>
</tr>
<tr>
<td></td>
<td>656</td>
<td>C6 &gt; O-Me &gt; CB &gt; C2 &gt; VUF &gt; SALB</td>
</tr>
</tbody>
</table>

* Ligands: CB = Clenbuterol, VUF = VUF 8303 (5e), O-Me = O-Methyl VUF 8303 (5c).
C2 = C2 Hapten (5d), C6 = C6 Hapten (5g), SALB = Salbutamol

None of the pAbs from any conjugate groups recognised any of the catecholamines (ISO, ADR, NOR), a nonselective β-antagonist (sotalol) or a β2-selective antagonist (ICI 118 551), since these ligands (for structures refer Figure 4.19) did not displace Ab-bound [3H]CB, even at the highest ligand concentrations (≈50 nM). Therefore, the pAbs did not bind any of the adrenergic ligands mentioned above which bind to the β2-AR (either agonist or antagonist). This suggested that the polyclonal anti-CB Abs, produced in response to the immunising conjugates, were not β2-AR-like.

VUF 8303 (%CR 20-40%) was less well recognised than the other CB analogues. It has been reported that 96% of VUF 8303, at pH 7.5, exists as the zwitterion,⁴³⁴ probably due to increased acidity resulting from the ortho- dichloro substitution; the pK value⁴³¹ of 2,6-dichlorophenol is ≈6.8, compared with that of phenol, pK ≈10. Since the conjugates did not contain a charged moiety at this position, Abs to these conjugates were probably less able to
recognise VUF 8303 present as the phenoxide anion at assay pH 7.4, despite the remaining structural similarities. Further, proton and carbon-13 NMR data of VUF 8303 (Chapter 2) tended to suggest an electrostatic surface different to that of the other CB analogues (due to steric inhibition of resonance; refer 2.2.5), which was consistent with the lower %CR of this compound.

By comparison, CB, which has a 4-amino rather than a 4-hydroxyl ring substitution, was recognised much better than VUF 8303 (refer Figure 4.19) by all pAbs, yet could be potentially charged (protonated amine) at the same position. However, the unprotonated aromatic amine was more likely to be favoured, due to resonance stabilization by the aromatic ring and the greater basicity of the N-tert-butyramino group (secondary aliphatic amine). Indeed, 99.5% of CB is present as the cation at pH 7.5, probably protonated on the aliphatic amine. Thus, the CB aromatic amine may have existed largely uncharged, and therefore been better recognised by pAbs generated here than VUF 8303.

SALB (%CR ≈ 1-4; refer Figure 4.19), a β₂-selective bronchodilator, was even less well recognised than VUF 8303. The absence of the dichloro ring substitution, rather than deprotonation of the phenolic hydroxyl group, may have contributed to this phenomenon since there was roughly an order of magnitude difference in the %CR. Previously reported %CRs of SALB with pAbs raised against the CB/Diazo conjugate vary considerably: 2.7%, 3.3%, 3.05 7.8%, 12% and 26.3%. The present data compared favourably with such studies, and suggested a possibly greater specificity of these pAbs for the dichlorinated ring moiety than anti-CB Abs previously reported, although the analogue-protein conjugates showed no advantage over the CB-protein conjugate in this regard. In contrast, an anti-SALB mAb with 75% CR for CB would appear to be less specific for the dichlorinated aromatic ring moiety.

CB and the O-alkyl analogues (O-Methyl VUF 8303, C2 and C6 Haptens; refer Figure 4.19) demonstrated an ability to inhibit binding of [3H]CB to pAbs elicited from all conjugates, (%CR ≈100-200%). This suggested that the O-alkyl CB analogues were recognised at least as well as CB, if not better. An Analysis of Variance (ANOVA) of %CRs showed no significant differences between CB and the O-alkyl analogues (O-Methyl VUF 8303, C2 and C6 Haptens) for the pAbs within a conjugate group, nor between any of the conjugate groups for a given ligand. However, some insignificant differences were noted between conjugate groups and between ligands, and these are discussed below.

The pAbs to the CB/Diazo conjugate showed considerable between-animal variation with respect to the Rank Order of Affinity (Table 4.10). The %CR of these pAbs with C6 Hapten tended to be slightly less than for groups immunised with the novel conjugates, but still greater than any other ligand within the CB/Diazo conjugate group. However, it appeared from the
data that pAbs from the CB/Diazo conjugate tended to distinguish less well between the remaining three ligands (CB, O-Methyl VUF 8303, C2 Hapten), compared with other conjugate groups.

Both the C2/ECDI/BSA and C2/DCC/BSA conjugates elicited pAbs with similar Rank Order of Affinity (O-Me VUF ≈ C6 Hapten > CB ≈ C2 Hapten). However, pAbs from the C2/DCC/BSA group showed a range of %CRs for C2 Hapten slightly higher than the range of %CRs for CB, which suggested that these Abs tended to recognise C2 Hapten slightly better than CB. The reason for this difference may have been due to the method of conjugation (ECDI vs DCC; refer Chapter 2) i.e. the C2 Hapten epitope was perhaps presented more efficiently to the immune system in the case of the DCC conjugate than the ECDI conjugate. However, it was unclear from the present data why this should have been the case, since both conjugates had, theoretically, the same bridge structure between hapten and carrier protein (refer Figure 1.9).

The C6 Hapten conjugates elicited pAbs which showed slightly different Rank Orders of Affinity compared to those generated by the C2 Hapten conjugates. In both C6 Hapten conjugate groups the Rank Order of Affinity (O-Me VUF ≈ C6 Hapten > CB > C2 Hapten) suggested that pAbs from the C6 Hapten conjugates tended to distinguish between CB and C2 Hapten better than pAbs raised against the C2 Hapten conjugates. The %CRs of C2 Hapten with pAbs elicited by the C6 Hapten conjugates were in the range 40-70%, compared with 100-140% with pAbs elicited by the C2 Hapten conjugates. This result suggested, not surprisingly, that pAbs to the C6 Hapten conjugates may have recognised the hydrophobic “spacer” (the five methylene groups between the dichlorinated ring and the carboxylic acid) of the C6 Hapten from which they were formed, better than the much shorter analogous structure (one methylene group) in the C2 Hapten (refer Figure 1.9).

This C6 Hapten was designed to present the CB-like portion of the molecule to the immune system better than the C2 Hapten or CB, by increasing the distance of the epitope from the carrier protein through the use of a spacer.\textsuperscript{313} Better recognition of the epitope may generate more CB-specific Abs. However, pAbs elicited using the C6 Hapten did not have a specificity for CB significantly greater than those derived from any other conjugate. Indeed, some pAbs from the C6/ECDI/BSA conjugate group required markedly greater CB concentrations at 50% SB (discussed previously). These pAbs also tended to recognise the change in hapten spacer length (recall lower %CR with C2 Hapten, compared with pAbs produced from C2 Hapten conjugates), which suggested that the spacer may have been part of the epitope recognised. Furthermore, titres of anti-CB Abs from the C6 Hapten conjugates tended to be lower than the C2 Hapten conjugates. Therefore, the C6 Hapten conjugates did not appear to offer any significant advantages in either specificity or Ab titre in comparison with either the C2 Hapten
Chapter 4 - Antibody Characterisation

or CB/Diazo conjugates.

Of all the ligands evaluated, C6 Hapten and O-Methyl VUF 8303 (refer Figure 4.19) tended to be the best recognised ligands (highest %CR; refer Table 4.7) by pAbs from all conjugate groups. The lowest concentrations of either ligand at 50% SB were observed for pAbs raised against the C2/DCC conjugate (refer Table 4.6), which suggested these Abs had the greatest affinity for these two ligands. However, the reason for the better recognition of C6 Hapten and O-Methyl VUF 8303 by pAbs from the C2/DCC conjugate group was unclear from the present data, although it may have indicated a better presentation of the CB-like epitope of this conjugate. Furthermore, the trend for better recognition of C6 Hapten by all pAbs was consistent with previous reports, where anti-hapten Abs had a greater affinity for hapten modified by attachment of long chain bridging groups than for the original hapten (for review see436). Hence, pAbs to the C2 Hapten-derived conjugate showed a greater %CR with C6 Hapten, than with C2 Hapten.

C6 Hapten and O-Methyl VUF 8303 also tended to be better recognised by pAbs to the C2 Hapten or C6 Hapten conjugates than to the CB/Diazo conjugate; this result was probably consistent with the different conjugation chemistries. CB, conjugated via diazotisation, results in a diazo linkage in the ortho- position of tyrosine residues, whereas the O-alkyl analogues of CB, conjugated using carbodiimide-based chemistries, results in an amide bond. This difference in the linkages between CB or the analogues to a carrier protein is illustrated in Figure 4.2 (refer section 4.2.2.5.1). Thus, pAbs to the analogue-protein conjugates tended to recognise ligands which were more like the conjugate against which they were generated.

In contrast, pAbs to the analogue-protein conjugates tended to be less specific for C2 Hapten (which also contained the O-alkyl moiety) than for C6 Hapten or O-Methyl VUF 8303 (refer Figure 4.19 for structures). This may have been due to the carboxylate anion (deprotonated carboxyl group of the C2 Hapten at the assay pH), which may have tended to reduce the hydrophobic nature of the C2 Hapten adjacent to the ring (compare with lower %CR of VUF 8303 present as the phenoxide anion). Conversely, the carboxylate anion of the C6 Hapten molecule would be further removed from the dichlorinated ring. Hence, the compound may have been more hydrophobic in nature nearer the ring, similar to the actual analogue-protein immunising conjugate, and so was better recognised by these pAbs. O-Methyl VUF 8303 is hydrophobic in this region and contained no ionic moieties near the ether linkage, and would appear likely to be most conjugate-like of all the O-alkyl CB analogues. Hence, it was one of the two ligands for which pAbs tended to be most specific.

When there is at least an order of magnitude difference in the concentrations of different ligands required to achieve 50% SB, the Abs can usually be regarded as having significantly greater
specificity for the ligand with the lower concentration.\textsuperscript{313,437} Therefore, none of the pAbs evaluated were able to distinguish immunologically between CB, VUF 8303, O-Methyl VUF 8303, C2 Hapten and C6 Hapten. The %CR results as well as the similar shaped displacement curves across all conjugates strongly suggested that the common epitope recognised by all pAbs was the 1-(3,5-dichlorophenyl)-2-(tert-butylamino) ethanol structural moiety, illustrated in Figure 4.20. This was consistent with the identical position of hapten conjugation of the CB/Diazo and novel conjugates. In contrast, phenylethanolamines that lacked this structural moiety (ISO, ADR, NOR, sotalol) were not recognised by pAbs elicited by any conjugate.

\begin{center}
\includegraphics[width=0.8\textwidth]{figure420.png}
\end{center}

**Figure 4.20** Schematic diagram of the common epitope of the CB conjugates recognised by the polyclonal antibodies

The lower %CR of VUF 8303 tended to suggest that the pAbs may have recognised a neutral rather than a charged substituent between the two ring chlorines. However, pAbs from all conjugate groups were unable to significantly distinguish between different neutral substituents in this position. Further, the %CR range of VUF 8303 (20-40\%) was still much higher than that of SALB (1-4\%), which did not have the dichloro ring substitution pattern, consistent with the dichlorinated phenylethanolamine moiety being the epitope to which anti-CB Abs were raised. Thus, with respect to epitope recognition, pAbs to the novel conjugates were very similar to those produced by the CB/Diazo conjugate.

Concurrent investigations into production of anti-CB mAbs using the novel conjugates were unsuccessful. This conclusion was established by standard ELISA (two coating conjugates bearing a CB-like epitope) and RIA (\textsuperscript{3}H_CB) procedures of cell culture supernatants. Insufficient quantities of supernatant were available during initial monoclonal isolation for these immunochemical characterisations, hence the selected cell lines were grown in quantity to produce sufficient mAb. Cells were also grown in serum-free (SF) culture medium during the logarithmic growth phase to give SF supernatant containing only mAbs. These samples were thus free from any interfering effects that the foetal calf serum (FCS) may have caused during immunoassay procedures.

The ELISA plate-coating conjugates were prepared using similar chemistries to those of the
immunising conjugates (carbodiimides or diazotisation), but with different carrier proteins (refer Figure 4.2). Hence, the two plate-coating conjugates (C2/DCC/Gelatin and CB/Diazo/OA) should only detect Abs specific for “CB-like” hapten. This was confirmed using an anti-CB mAb previously produced in this laboratory (PD4). PD4 bound both plate-coating conjugates, and when serially diluted, gave a characteristic sigmoidal Ab dilution curve (refer section 4.3.3.2). However, it was not possible from these experiments to conclude whether PD4 was more specific for one coating conjugate than the other, since the amount of hapten incorporated into each coating conjugate was not known. The use of a coating conjugate formed using a different chemistry to that of the immunising conjugate should lead to detection of only hapten specific Ab, because the potential for detection of bridge-specific Abs has been removed.

None of the twelve IgG-secreting monoclonal hybridomas (TB1-TB12, derived from a mouse immunised with C2/DCC/BSA) recognised the CB-like epitopes of the two different coating conjugates at identical Ab concentrations to that of PD4. Further, no specific binding of [3H]CB by TB1-TB12 in the Ab screening RIA was detected. By contrast, the anti-CB mAb, PD4, did bind the CB-like epitopes of both coating conjugates in the ELISA, and [3H]CB in the Ab screening RIA, at concentrations of IgG identical to those of mAbs TB1-TB12. The possibility that charcoal separation of bound from free radiolabel perhaps masked the presence of putative anti-CB mAb amongst TB1-TB12, albeit of lower affinity for the radiolabel, was investigated using Protein A-Sepharose as a gentler separation method. In this modified screening RIA, TB9 showed no specific binding of [3H]CB, whereas PD4 did. Therefore, it was concluded that the TB1-TB12 monoclones secreted mAbs that were not specific for CB.

The Ab screening ELISA was used to determine positive wells, at both the polyclonal and monoclonal stages, by the assay of neat culture supernatants (20% FCS). ELISA results from the TB mAbs (C2/DCC/Gelatin plate-coating conjugate) gave SF supernatant Ab dilution curves much shallower than those obtained from 20% FCS supernatant samples. Furthermore, specific absorbances between 1-1.5 were observed for neat samples with 20% FCS, whereas neat SF samples showed specific absorbances of ≈ 0.2. These results tended to suggest that the FCS in the culture medium may have led to a “false positive” in the Ab screening ELISA and although the specific absorbance was much greater than the culture medium blank (uncorrected absorbance ≈ 0.4), this was rapidly reduced as the sample was diluted.

By comparison, anti-CB mAb PD4 (20% FCS) showed specific absorbances greater than 2 at a 1:8 dilution (C2/DCC/Gelatin plate-coating conjugate), or a 1:128 dilution (CB/Diazo/OA plate-coating conjugate). Therefore, selection of both polyclonal and monoclonal wells as positive only on the basis of the highest absorbance in an ELISA of neat culture supernatant appeared to be misleading. Indeed, dilutions of 1:4 have been suggested previously for selection of
mAbs. These results were consistent with the need to characterise any putative positive polyclonal or monoclonal wells by an Ab dilution curve, and confirm the presence of specific Abs in the culture supernatants. The results showed that no CB-specific mAbs were isolated, and no further work on these monoclones was warranted.

The failure to generate monoclonal anti-CB Abs using the analogue-protein conjugates was probably due to several factors. Of these, low fusion efficiency was probably the dominant factor, since only very low numbers of viable hybridomas were produced from cell fusion experiments. To improve the cell fusion process, several fusion experiments were repeated using an alternative source of FCS, as well as simultaneous fusions of separate aliquots of mouse spleen cells from a single mouse. However, the number of wells containing growing colonies of hybridomas was not improved, and the results suggested that further optimisation of the fusion conditions was required.

Low Ab titres of anti-CB pAbs in the immunised mice used for cell fusion experiments may also have contributed to the failure to generate anti-CB mAbs from the analogue-protein conjugates. The low titres observed (less than 1:1,000, in the screening RIA using [3H]CB, immediately prior to fusion) and the shallow Ab dilution curves, tended to suggest that there were only a low number of B-cells which secreted anti-CB Abs. The results from Abs produced in sheep showed that the C2 Hapten-derived conjugates had similar immunogenicity to that of the CB/Diazo conjugate. This result tended to reinforce the conclusion that the fusion conditions possibly required further optimisation, since an anti-CB mAb (PD4) was previously raised in this laboratory using the CB/Diazo conjugate. However, a further evaluation of the immunisation regime would also be warranted, with an increased time interval between primary and first boost immunisations to improve the Ab titre. In summary, low Ab titre coupled with low fusion efficiencies probably explains the failure to successfully isolate any monoclones secreting CB-specific mAb from the spleen cell fusions of these mice.

The dilution of positive polyclonal wells at the rate of 1,000 cells/well prior to a limiting dilution was included in the protocol to enhance the probability of isolating any positive clones present, which may have been overgrown by surrounding clones with specificities directed toward other epitopes. Further, the possibility of the presence of mycoplasma which may have affected the viability of the cells and the hybridomas produced from the fusion was also considered. However, no mycoplasma was found in either the FCS or the Sp2/0 myeloma cells used. Together, these observations reinforce the need for a further optimisation of the fusion conditions, before any re-evaluation of the analogue-protein conjugates for mAb production can be made. However, such work was beyond the scope of the present research.

The O-alkyl CB analogues (C2 Hapten and C6 Hapten) allowed conjugation of a CB-like
hapten using a conjugation chemistry other than diazotisation. The present study has shown that conjugates so formed elicit a polyclonal anti-CB Ab response in sheep which was at least the equivalent of the CB/Diazo conjugate, in terms of titre and specificity. Therefore, these analogues may also have utility in other areas of the research directed towards the development of an anti-idiotypic "internal image" of CB. For example, there has been some suggestion that, for the detection of Abs to small molecular mass haptens by ELISA, it is preferable to use a coating conjugate where the hapten has been conjugated, not only to a carrier protein different to that of the immunising conjugate, but also using a different conjugation chemistry.\textsuperscript{438} The carbodiimide conjugation of the \textit{O}-alkyl analogues could be used in this way. Purification of anti-CB idiotypes (elicited by the CB/Diazo conjugate) through affinity chromatography columns constructed with an \textit{O}-alkyl analogue may be another use of these analogues, so that the isolation of anti-CB Abs which recognise the bridge structure between the hapten and the carrier protein may be minimised.

An \textit{O}-alkyl analogue-enzyme conjugate could also be used in an ELISA for the detection of CB-like anti-idiotypie (by competition with anti-idiotypie for anti-CB Ab). CB-conjugated enzymes (formed using diazo chemistry) have been previously reported,\textsuperscript{300,303,308} as well as a SALB-biocytin conjugate,\textsuperscript{306} for detection of CB residues in biological samples by enzyme immunoassay. Since anti-CB Abs to the novel conjugates recognise CB at least as well as the CB/Diazo conjugate, they could also be used in radio or enzyme immunoassay for the detection of CB residues in biological samples. Anti-CB Abs produced from the CB/Diazo conjugate have been previously used in this way.\textsuperscript{300,303,304,306-308}
4.5 Conclusion

Polyclonal anti-CB Abs were raised in sheep by immunisation with the novel conjugates formed from the \( O \)-alkyl CB analogues 5d and 5g. Anti-CB Ab titres that resulted from these immunogenic conjugates were comparable with those of the only reported CB conjugate (CB/Diazo/HSA), under an identical immunisation regime and adjuvant. Abs to the analogue-protein conjugates tended to remain at higher levels for longer than the CB/Diazo conjugate, and Ab titres from the C2 Hapten conjugates tended to be higher than those from the C6 Hapten. The highest anti-CB Ab titre (\( \approx 1:100\,000 \)) was obtained with the C2/DCC/BSA conjugate. Displacement of \([3H]CB\) with authentic CB confirmed that pAbs from all conjugate groups recognised CB.

Competition studies with a variety of phenylethanolamine analogues indicated that the common CB-like epitope recognised by Abs raised against the analogue-protein conjugates, as well as the CB-protein conjugate, was the 1-(3,5-dichlorophenyl)-2-(\textit{tert}-butylamino)ethanol moiety. Indeed, Abs raised against all the immunising conjugates tended to better recognise ligands with a neutral (non-ionic) substituent that is \textit{ortho} to both ring chlorines. However, these pAbs were unable to distinguish immunologically between CB and the \( O \)-alkyl analogues of CB (synthesised in Chapter 2). The pAbs did not recognise the adrenergic ligands, which indicated that the anti-CB pAbs produced were probably not like the \( \beta_2 \)-AR in terms of ligand specificity. Overall, pAbs to either the reported CB/Diazo conjugate or the C2 Hapten or C6 Hapten conjugates were very similar with regard to specificity, as well as Ab titre response.

Attempts to raise monoclonal anti-CB Abs using the C2 Hapten or C6 Hapten conjugates were unsuccessful. This was due in large part to poor fusion efficiency and this, coupled with relatively low Ab titre levels in the mice prior to fusion, was likely to have diminished the probability of isolating a positive monoclonal. It was also noted that the 20% FCS culture media gave a “false positive” in the screening ELISA, which reinforced the need to conveniently characterise putative positive hybridoma colonies at the earliest possible stage by an ELISA using a serial dilution of culture supernatant.
CHAPTER 5 - GENERAL DISCUSSION

5.1 Discussion

Research in this laboratory into the generation of an anti-idiotypic antibody mimicking the in vivo energy-repartitioning effects of clenbuterol (CB), has led to the evaluation of a variety of functionalised analogues of CB suitable for chemical conjugation to a carrier protein. The present research has focussed on two novel O-alkyl analogues suitable for protein conjugation (5d and 5g) and two model compounds (5c and 5e); 5e was previously unreported. These four compounds were synthesised and structure confirmed by various spectroscopic methods. The in vivo physiological effects of these compounds were characterised in rats, to elucidate the impact of structure on function. The structural specificity of antibodies raised using protein conjugates of the haptenic analogues were also characterised. Together, these investigations provided useful data for the evaluation of the potential use of these analogues in the generation of anti-idiotypic antibodies which may bear the “internal image” of CB.

Target compounds (haptens 5d and 5g and model compounds 5c and 5e; refer Figure 1.8 for structures) were synthesised according to a synthetic scheme (refer Chapter 2, section 2.2.1) similar to that reported for CB, with modifications to accommodate key structural differences. Aromatic ring chlorines were more conveniently included in the starting material, thus eliminating synthetic chlorination. The key starting material (3,5-dichloro-4-hydroxyacetophenone; DCHA) was synthesised in two steps\cite{337} in overall yield of 68%. Introduction of the required O-alkyl substituent onto the phenolic oxygen of DCHA was via a modified Williamson ether synthesis (alkyl halide plus DMF/K₂CO₃), in 55-95% yield. This reaction also demonstrated a successful application of the DMF/K₂CO₃ reaction conditions to the alkylation of an ortho- dichlorinated phenol; there has been some suggestion previously that 2,6-dichlorophenol may be difficult to alkylate.\cite{331} α-Bromination of the acetyl group was achieved under conditions similar to those of CB, in 75-85% yield, with some indication by RP HPLC analysis of minor amounts of reaction by-product (possibly α,α-dibromination).

Nucleophilic substitution of the various phenacyl bromides with the tert-butyramino moiety was achieved using the secondary N-benzyl-tert-butyramine in 30-70% yield, rather than tert-butyramine as in the CB synthesis. Reaction with tert-butyramine gave only a mixture of products by RP HPLC analysis. Use of the secondary N-benzyl-tert-butyramine, rather than primary amine, confirmed the superiority of this alternative as demonstrated by similar syntheses previously reported.\cite{317,320,323,324} Removal of excess amine was achieved by solubility differences of amine and product in aqueous acid, necessitating a sufficiently hydrophobic O-alkyl substituent. Hence, in the synthesis of C2 Hapten 5d, benzyl 2-
bromoacetate was used for O-alkylation, rather than methyl 2-bromoacetate. Catalytic
debenzylolation gave secondary aminoketone intermediates, and borohydride reduction of these
ketones gave the target compounds, in a total of five synthetic steps from DCHA, with an
overall yield of 5.5%-27.8%. These results were in reasonable agreement with the reported
overall yield\(^1\) of CB of 18%.

The infra-red, nuclear magnetic resonance (proton, carbon) and mass (low and high resolution)
spectral data of the target compounds and their synthetic intermediates were consistent with
proposed chemical structures. In the proton and carbon-13 NMR spectra, identical protons and
carbons in analogous intermediates were in good agreement, as were characteristic functional
group bands in the infra-red. Similar fragmentation patterns were observed for analogous
intermediates in the mass spectra. Proton-carbon correlation NMR experiments provided
further data for a more definite assignment of proton and carbon NMR spectra of the tertiary \(N\)-
benzyl amine intermediate 3c. Typical patterns for non-equivalent methylene protons adjacent
to an asymmetric centre were observed in the case of the target compounds and \(N\)-benzyl
tertiary amines. However the present data did not provide a definitive assignment of these
geminal protons; further NMR spectroscopic investigations may help elucidate the present data.
Overall, 15 previously unreported compounds (12 intermediates and 3 target compounds,
excluding phenacyl bromide intermediates) were synthesised and fully characterised.

The *in vivo* physiological effects of the four \(O\)-alkyl CB analogues were characterised in rats,
following a 22-day treatment period. This study showed that when the aromatic ring amino
group was substituted with a hydroxy, methoxy, oxyacetic acid or 6-oxyhexanoic acid moiety
(refer Figure 3.1), the energy-repartitioning effects of CB were significantly diminished. VUF
8303 (5e) showed significantly greater weight gain and increased weight of the soleus muscle,
effects similar to CB although of slightly less magnitude. No other analogues showed any
significant increase in the weights of various hindlimb skeletal muscles. The percentage carcass
fat and protein of rats given the four analogues were not significantly different from saline-
treated animals. This result tended to suggest that weight gain was not always related to
changes in the fat/protein ratio as previously reported.\(^{26}\) VUF 8303 and \(O\)-Methyl VUF 8303
(5e) showed a reduction in \(\beta_2\)-AR density (SPG muscle bundle) which was less than that
observed with CB, although this appeared unrelated to changes in muscle mass.

These results also suggested structural features that may be required by phenylethanolamine
\(\beta\)AAs to elicit growth-promoting effects. In the present study, VUF 8303 (5e) combined
structural features common to both CB and RAC (refer Figure 1.2), and yet did not exhibit
similar physiological effects. The aromatic amine group of CB (and perhaps by analogy CIM;
refer Figure 1.2) may be important for energy-repartitioning effects, whilst the structural feature
of RAC important for similar effects may be the large \(N\)-alkyl substituent. Indeed, the \(\beta\)AA
growth promotant L-644,969 (refer Figure 1.2) contains both an aromatic amino group and a large $N$-alkyl substituent. Further, there has been some suggestion that more potent $\beta_2$-agonists may be formed by inclusion of larger, non-polar substituents on the aliphatic amine.\textsuperscript{440} The influence of aromatic ring substituents on $\beta_2$-AR binding may also have been previously underestimated.\textsuperscript{441}

In contrast, metaproterenol (Figure 3.5) contains neither an aromatic amine nor a large $N$-alkyl substituent, but is known to exert energy-repartitioning effects.\textsuperscript{400} However, investigations of the physiological effects of the $\beta_2$-agonist SALB (Figure 3.6) have been equivocal (refer section 3.4), and it would appear that SALB may not have the same dramatic effects on carcass composition as CB. Consideration of the large number of studies of $\beta$AAs appears to suggest a reasonably complex mechanism or series of mechanisms of action not yet completely understood (refer section 1.2.3). The present study of $O$-alkyl CB analogues did not elucidate this mechanism of action, but suggested structural moieties of CB that may be required for energy-repartitioning. Indeed, structural requirements thought to be required for $\beta_2$-receptor binding (refer section 1.2.2) may only partially explain the observed physiological effects of $\beta$AAs (refer section 1.2.1), and the influence of structure on biological half-life or drug distribution amongst various tissues may also be important.

Haptenic analogues 5d and 5g were coupled to BSA, using two different carbodiimide reagents, to form protein conjugates for production of anti-CB Ab1. Measurement of Ab titre confirmed the immunogenicity of the protein conjugates and allowed comparison to the diazo conjugate previously reported.\textsuperscript{300} In the present study, the novel conjugates produced polyclonal anti-CB Abs in sheep in quantities equivalent to the diazo conjugate over the immunisation period. Anti-CB Ab titres tended to remain higher for longer periods of time after boost with the novel conjugates than with the diazo conjugate. The specificity of these Abs was also characterised. A characterisation of the specificity of anti-hapten Ab1 is thought to be necessary\textsuperscript{442} prior to production of Ab2 that are the internal image of the hapten using anti-hapten Ab1. The common structural feature of CB recognised by pAbs raised against both the novel and diazo conjugates was the 1-(3,5-dichlorophenyl)-2-(tert-butylamino)ethanol moiety (refer Figure 4.20). These Abs appeared to be similarly specific for authentic CB.

The selection of Ab1 which are specific for that part or parts of a hapten which activate(s) a cellular receptor is implicit in the approach whereby anti-hapten Ab1 is used to generate Ab2; ideally the putative active site(s) of the hapten should remain unaltered during conjugation.\textsuperscript{244} Characterisation of Ab specificity in the present study suggested that Abs formed in response to either the diazo or novel conjugates were unable to differentiate between compounds with different aromatic ring substitutions between the two chlorines. By comparison, the difference in the nature of this substitution could be distinguished \textit{physiologically}, as shown from the rat
study of the four analogues and CB. Together, these results tended to suggest that these anti-CB Abs may be inappropriate for production of Ab2β with energy-repartitioning effects since these pAbs were equally specific for CB as for the apparently biologically inactive analogues. Hence, conjugation of CB or O-alkyl analogues 5d and 5g through the site which appears important for the energy-repartitioning effects of CB may be unsuitable for the generation of Ab2β which mimic these same effects.

It has been suggested that use of hapten analogues for the generation of Ab2β may result in Ab1 which recognise structural elements common to both biologically active and inactive analogues alike, and hence be unsuitable for the generation\textsuperscript{244} of Ab2β. The results of the present study were consistent with this notion. Given that the aromatic amino group of CB is important for the observed growth-promoting effects and that other energy-repartitioning βAAAs also have large N-alkyl substituents, the use of analogues of CB functionalised for conjugation through the tert-butylamino group may be more appropriate (refer Conjugate B in Figure 1.7, section 1.3.3). These analogues have been synthesized\textsuperscript{301} and have been under investigation in this laboratory. Protein conjugates of these alternative analogues may present, unaltered to the immune system, the apparently important aromatic portion of the CB molecule, for production of anti-CB Ab1. These antibodies could conceivably differentiate the nature of the aromatic substituents, and Ab2β generated from them may mimic, at least structurally, this portion of the molecule.

The mechanism whereby CB and other βAA growth promotants exert their physiological effects is not entirely understood (refer section 1.2.3). Increased protein accretion has been attributed to both an increased protein synthesis and a decreased rate of degradation; decreased adipose deposition has also been ascribed to reductions in lipogenesis and increased lipolysis. Indeed, assumptions that a common mechanism of action could be ascribed to these effects, or to all βAAAs, have been questioned.\textsuperscript{3,395} Investigations of endocrine status of βAA-treated animals have reported mixed conclusions (refer section 1.2.1) and even the involvement of calcium-dependent proteases has received some attention (refer section 1.2.3). Agonist binding to the β2-AR activates adenyl cyclase, resulting in increased intracellular cAMP and activation of a cAMP-dependent hormone-sensitive lipase. CB is a β2-selective adrenoceptor agonist, albeit with an intrinsic activity less than 1.0. Hence, the physiological effects of CB may be mediated in this way, at least in part. However, other β2-AR agonists (bronchodilator drugs e.g. salbutamol, terbutaline) do not appear to also elicit the dramatic changes in carcass composition observed with certain βAAAs (CB, CIM, RAC and L-644,969; refer Figure 3.6).

Brown adipose tissue has been identified as an important site of non-shivering thermogenesis. The stimulation of brown adipose tissue leads to increased oxygen consumption and heat production as energy is dissipated without production of ATP.\textsuperscript{87} Increased oxygen
consumption, increased body temperature and a dramatic repartitioning of energy away from fat deposition toward protein accretion have been observed following CB treatment (refer section 1.2.1). Brown adipose contains predominantly the atypical or β3-AR, identified as the predominant β-adrenergic mediator of lipolysis.416 Hence, CB may perhaps exert a dual β2/β3 action, which may partially explain the dramatic carcass effects observed. However, some caution has been suggested in identification of the β3-AR as the CB receptor in the absence of a β3-specific antagonist.416 The β3-AR has also been identified in rat skeletal muscle.413,414

Therefore, if β3-ARs mediate, in part, the energy-repartitioning effects of CB, the O-alkyl analogues may have lacked important structural features for β3-AR binding and activation; hence no energy-repartitioning effects were observed for these compounds. However, the binding pocket of the β3-AR remains undefined, although this receptor has 63% homology with the β2-AR in the transmembrane domains.159 The present data did not conclusively elucidate structural features of ligand binding to this receptor, although they did suggest that the aromatic amine group of CB may have been important in this regard, assuming CB is a β3-AR agonist. This hypothesis is also consistent with the observation that not all β2-AR agonists are growth promotants. Interestingly, in the case of VUF 8303, despite replacement of this aromatic amine with a hydroxyl group (a group apparently important for the binding of isoprenaline to the β2-AR, through hydrogen-bonding to receptor serine residues; refer Figure 1.3, section 1.2.2), this compound did not elicit any change in carcass composition. A β3-AR specific antagonist would appear able to greatly assist in the determination of the adrenergic receptor through which the effects of CB are mediated. However, a β3-specific antagonist has yet to be reported. Investigations into the synthesis of β3-AR specific antagonists, and structure-activity relationships for the β3-AR are currently under investigation.443,444

The β3-AR is apparently resistant to desensitisation and down-regulation unlike the β1- or β2-ARs (refer section 1.2.2). The β3-AR lacks specific serine residues targeted by intracellular kinases involved in receptor phosphorylation, and subsequent receptor desensitization and down-regulation (refer section 1.2.2). Activation by CB (which has a long biological half-life) of a receptor (β3-AR) which not only mediates lipolysis, but effectively cannot be “turned off”, may explain the significant repartitioning of energy from fat deposition toward protein accretion. The O-alkyl analogues 5e and 5c and CB were all observed to decrease the β2-AR receptor density in hindlimb skeletal muscle, but only CB altered carcass composition. At present, a similar characterisation of the β3-AR has not been reported, since a β3-specific radiolabelled antagonist is unavailable, although changes in receptor density of a receptor apparently resistant to desensitisation (β3-AR) may be difficult to quantitate. There has also been some suggestion that attenuation of the effects subsequent to agonist binding to the β3-AR could involve a post-receptor process.445
Anti-hapten Ab1 which are receptor-like with regard to specificity for receptor ligands are considered the most likely candidates for production of Ab2 that bear the internal image of the original hapten\footnote{Refer section 1.3.2.} Structural mimicry of hapten binding to receptor by Ab2β would appear to be necessary as a prerequisite for functional mimicry of hapten-initiated post-receptor events. Such a notion assumes structural features of Ab2β are primary determinants of functional mimicry. In contrast, antibodies to the β-agonist alprenolol generated Ab2s with both antagonistic and agonistic properties \textit{in vitro}.\footnote{In the present study, anti-CB Ab1 raised against both the diazo and novel conjugates did not recognise any of the adrenergic ligands (ADR, NOR, ISO). This suggested that Ab1 specificity was unlike that of the \( \beta_2 \)-AR. However, if the effects of CB are mediated by other biochemical events in addition to receptor binding and activation (for example, inhibition of the calpain proteases, or binding to the \( \beta_3 \)-AR), then further structural parameters may influence selection of those Ab1 most likely to generate functional mimics.}

The physiological study in rats also suggested that a bulky substituent (\textit{e.g.} the methoxy, oxyacetic acid or 6-oxyhexanoic acid moieties) between the two aromatic chlorines may have negated the energy-repartitioning effects of CB. This was consistent with the report that des-amino CB (where hydrogen replaces the aromatic amino group) has energy-repartitioning effects,\footnote{In the present study, anti-CB Ab1 raised against both the diazo and novel conjugates did not recognise any of the adrenergic ligands (ADR, NOR, ISO). This suggested that Ab1 specificity was unlike that of the \( \beta_2 \)-AR. However, if the effects of CB are mediated by other biochemical events in addition to receptor binding and activation (for example, inhibition of the calpain proteases, or binding to the \( \beta_3 \)-AR), then further structural parameters may influence selection of those Ab1 most likely to generate functional mimics.} whilst the \( O \)-alkyl analogues did not. Therefore, conjugation at this position may generate Ab1 which recognise a somewhat bulky substituent in this position, by virtue of the bulky structure of the hapten-protein conjugate, and hence generate Ab2β which structurally mimic a similarly bulky feature. \( O \)-Methyl VUF 8303 (5c), the simplest \( O \)-alkyl analogue that was most conjugate like (no ionisable group, other than the aliphatic amine), elicited no growth-promoting effects like CB, and (together with the C6 Hapten) was the ligand for which anti-CB Ab1 appeared most specific. These results suggested a further limitation of these Ab1 to generate Ab2β which functionally mimic CB. Therefore, screening Ab1 to determine that portion of the CB molecule recognised by these Abs was important, since the Ab1 paratope would appear to influence the Ab2β paratope, the structure responsible for immunological mimicry.

The effects of BAA growth-promoting drugs are usually observed in response to chronic treatment, although attenuation follows drug removal.\footnote{This tends to suggest that Ab2β which physiologically mimic these drugs may need to exert a similarly chronic mode of action. The nature of the antibody response, in light of the idiotypic network theory,\footnote{Previous reports have suggested neutralisation of Ab2β by Ab3 (anti-anti-idiotypes); in fact the Ab2 response was completely lost despite subsequent boost immunisations.\footnote{Thus, the Ab2β response may be unable to mimic the chronic BAA treatment required for energy-repartitioning effects. This could represent a possible biological barrier to the immunological mimicry of whole body}}
physiological effects of βAA treatment. In any event, a growth-promoting vaccine which requires a large number of immunisations to sustain sufficient levels of Abβ may be considered commercially disadvantageous.

Recently, functional immunological mimicry of the in vivo whole body effects of porcine growth hormone by Abβ in rats has been reported and patented. These reports suggested that Abβ, characterised as “internal images” by various immunochemical and receptor assays, caused significant increases in body weight and changes in carcass composition in response to Ab1 immunisation. However, these effects were monitored over a period of 5-7 days (growth promoting effects of βAAs usually observed after at least 7 days) in response to daily Ab1 immunisation. Although effects on carcass composition due to Abβ were reported the evidence was anecdotal as no data was reported in the Australian patent document, nor in a subsequent paper. The nature of receptor binding of growth hormone is also of interest. The growth hormone receptor is a single-pass receptor protein (one transmembrane domain) and the hormone binds to the extracellular domain. Growth hormone is also a peptide molecule, considerably larger than CB (molecular mass 22 000 vs. 277 Da). These factors may positively influence the ability of the Abβ to mimic in vivo effects of growth hormone, since the receptor binding site is easily accessible and the Abβ protein is mimicking a large protein hormone.

By comparison, Abβ functional mimicry of CB presumably requires binding at the ligand binding pocket which, in the case of the β2-AR (refer Figure 1.3), is believed to be buried ≈11Å into the core of the receptor protein bundle (refer section 1.2.2). This kind of receptor binding by an Ab may be difficult given the size of the Ab (molecular mass ≈1.50 kDa); a flatter, rather than a protuberant, paratopic topology may also present similar difficulties (refer section 1.3.2). By comparison, binding by Abβ to the growth hormone receptor may be somewhat easier with a single pass cell surface receptor and an extracellular hormone-binding domain.
5.2 Conclusion

The present research characterised four O-alkyl analogues (VUF 8303 (5e), O-Methyl VUF 8303 (5c), C2 Hapten (5d) and C6 Hapten (5g)) of the energy-repartitioning β-agonist CB chemically, physiologically and immunologically. It was proposed that these analogues may be effective structural and functional congeners of CB, and hence could be used to generate anti-idiotypic antibodies which mimic the in vivo energy-repartitioning effects of CB. The results of this research suggested that despite structural similarities, these analogues lacked functional similarity to CB and hence would be unsuitable for the production of the anti-idiotypic antibodies required.

The previously unreported O-alkyl analogues of CB were synthesised and fully characterised to confirm structure. The analogues were then characterised in vivo in a rodent model, which suggested that the aromatic amino moiety of CB may have been important for the energy-repartitioning effects. The reasons for this result were rationalised by consideration of the interaction of the compounds with β-ARs. The novel analogues suitable for conjugation (5d and 5g) were used to form protein conjugates and were able to elicit CB-specific Abs. The characterisation of the specificity of anti-CB Abs elicited by these conjugates suggested that these anti-CB Abs were unable to distinguish the nature of the aromatic substitution between the aromatic chlorines. Therefore, it would appear that these O-alkyl analogues may be unsuitable for the generation of antibodies which mimic the growth-promoting effects of CB, since the antibodies to protein conjugates formed from them were unable to distinguish between the biologically active (with respect to energy-repartitioning effects) compound (CB) and the inactive O-alkyl CB analogues. Indeed, alternative sites of conjugation on the CB molecule may be more suitable for generation of anti-idiotypic antibodies which mimic the energy-repartitioning effects of CB.
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APPENDIX 1 - MATERIALS AND EQUIPMENT

Chapter 2: Organic Synthesis

Material (Supplier)

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECDI; Sigma Chemical Co., St Louis, MO, USA)
2,6-Dichlorophenol (Aldrich Chemical Co., Milwaukee, WIS, USA)
Acetic acid (HOAc; Ajax Chemicals, Sydney, Australia)
Acetic anhydride (Ajax Chemicals, Sydney, Australia)
Acetonitrile (Rhone Poulenc, Melbourne, Australia)
Aluminium trichloride (AlCl₃; Aldrich Chemical Co., Milwaukee, WIS, USA)
Ammonia (aqueous, NH₃; Ajax Chemicals, Sydney, Australia)
Benzene (Ajax Chemicals, Sydney, Australia)
Benzyl 2-bromoacetate (Aldrich Chemical Co., Milwaukee, WIS, USA)
Benzyl chloride (Ajax Chemicals, Sydney, Australia)
Bovine Serum Albumin (BSA; CSL, Melbourne, Australia)
Bromine (BDH Chemicals, Melbourne, Australia)
Chloroform (CHCl₃; Ajax Chemicals, Sydney, Australia)
Diethyl ether (Et₂O; Ajax Chemicals, Sydney, Australia)
Ethanol (EtOH; Ajax Chemicals, Sydney, Australia)
Ethyl 6-bromohexanoate (Aldrich Chemical Co., Milwaukee, WIS, USA)
Ethyl acetate (EtOAc; Ajax Chemicals, Sydney, Australia)
Ethynenediamine-modified BSA (ED-BSA; gift from Dr. Robin Rigby)
Gelatin (Bloom 300; Sigma Chemical Co., St Louis, MO, USA)
Hydrochloric acid (12 M; Ajax Chemicals, Sydney, Australia)
K₂CO₃ (Ajax Chemicals, Sydney, Australia)
Methyl 2-bromoacetate (Aldrich Chemical Co., Milwaukee, WIS, USA)
Methyl iodide (Ajax Chemicals, Sydney, Australia)
Methylene chloride (CH₂Cl₂; Ajax Chemicals, Sydney, Australia)
N,N-Dimethylformamide (DMF; Ajax Chemicals, Sydney, Australia)
N,N'-Dicyclohexylcarbodiimide (DCC; Sigma Chemical Co., St Louis, MO, USA)
N-Benzyl-tert-butylamine (Aldrich Chemical Co., Milwaukee, WIS, USA)
N-Hexane (Ajax Chemicals, Sydney, Australia)
N-Hydroxy succinimide (NHS; Sigma Chemical Co., St Louis, MO, USA)
Na₂SO₄ (Ajax Chemicals, Sydney, Australia)
Palladium (5%) on charcoal (5% Pd/C; Aldrich Chemical Co., Milwaukee, WIS, USA)
Phosphate buffer salts (Ajax Chemicals, Sydney, Australia)
Pyridine (Ajax Chemicals, Sydney, Australia)
Sodium borohydride (NaBH₄; BDH Chemicals, Melbourne, Australia)
Sodium cyanoborohydride (NaBH₃CN; BDH Chemicals, Melbourne, Australia)
Sodium hydroxide (NaOH; Ajax Chemicals, Sydney, Australia)
Sodium iodide (NaI; Ajax Chemicals, Sydney, Australia)
*tert*-Butyl 2-chloroacetate (Aldrich Chemical Co., Milwaukee, WIS, USA)
*tert*-Butylamine (Sigma Chemical Co., St Louis, MO, USA)
Tetrahydrofuran (THF; Ajax Chemicals, Sydney, Australia)
Triethylamine (Ajax Chemicals, Sydney, Australia)

Reversed Phase HPLC (RP HPLC) was carried out on a Waters Millipore system (USA) as below. Retention times (tᵣ) are given for the conditions described.

Column: 10 cm x 5 mm (id) Novapak C18 cartridge (4 µm), with a Guardpak, in a Radial Compression Module (RCM-100)
Flow Rate: 1 cm³ per min
Buffers: A = 50 mM Triethylaminephosphate (TEAP) pH 3
B = 80% Acetonitrile, 20% A
Detection: variable wavelength UV spectrophotometer
Chapter 3: Rodent Study

Material or Equipment (Supplier)

(-)-Propranolol (Sigma Chemical Co., St Louis, MO, USA)
[^125]I-(±)Iodocyanopindolol ([^125]I-CYP; Du Pont Company, Wilmington, DE, USA)
A[^14] mono[^125]Iiodotyrosyl human insulin (Amersham, North Ryde, NSW, Australia)
ABBOTT Quickstart™ Glucose assay and ABBOTT Quickstart™ VP System (Abbott Laboratories, Abbott Park, Illinois, USA)
Blender for carcass homogenisation (Braun AG, Frankfurt, West Germany)
Bovine serum albumin (BSA insulin-free, Sigma, St Louis, MO)
Histidine (Calbiochem Corp., La Jolla, CA, USA)
Iodoacetamide (Sigma Chemical Co., St Louis, MO, USA)
KCl (BDH Merck Pty Ltd, Melbourne, Australia)
Kjltc Auto 1030 Analyzer (Tecator, Hognas, Sweden)
LIGAND computer program (MacLIGAND v4.93)
MgCl₂ (BDH Merck Pty Ltd, Melbourne, Australia)
NaCl (BDH Merck Pty Ltd, Melbourne, Australia)
NaHCO₃ (BDH Merck Pty Ltd, Melbourne, Australia)
NEFA colorimetric assay kit (Wako Pure Chemical Industries, Osaka, Japan)
Porcine insulin (NOVO Research Institute, Bagsvaerd, Denmark)
Protein colorimetric assay kit (Bio-Rad Laboratories, Hercules, California, USA)
Sheep anti-guinea pig immunoglobulin G (Silenius, Melbourne, Victoria, Australia)
Statistics packages (Statview SE ± graphics, Abacus Concepts/Brainpower Inc, CA, USA, and Genstat 5, Statistics Dept., Harpenden, Lawes Agricultural Trust, UK)
Sucrose (BDH Merck Pty Ltd, Melbourne, Australia)
Trizma (Tris(hydroxymethyl)aminomethane base; Sigma Chemical Co., St Louis, MO, USA)
Ultra-Turrax probe (Janke and Kunkel, GmbH & Co., Germany)
Whatman GF/B Glass Microfilters (25 mm diameter; Whatman International Ltd, Maidstone, England)
Wistar rats (female; University of New South Wales, Specific Pathogen Free Facility, Little Bay, NSW, Australia)
Chapter 4: Antibody Production and Characterisation

Material or Equipment (Supplier)

2-Mercaptoethanol (Ajax Chemicals, Sydney, Australia)
24-Well plates (flat-bottomed; Costar, Denmark)
Adrenaline (Sigma Chemicals Co.)
Ascorbic acid (Sigma Chemical Co., St Louis, MO, USA)
BALB/c mice (Male and female; CSIRO DAP, Prospect, Australia)
Benchtop centrifuge Z230M (BHG Hermele, John Morris Scientific, Sydney, Australia)
Borosilicate glass culture tubes (Borex, Chase Instruments, Norcross, GA, USA)
Charcoal (Ajax Chemicals, Sydney, Australia)
Citric acid (Ajax Chemicals, Sydney, Australia)
Clenbuterol (gift from J.J. Finnerty)
Dextran T70 (Pharmacia Fine Chemicals, Uppsala, Sweden)
Diethylaminoethyl dextran (DEAE-dextran; Pharmacia, Uppsala, Sweden)
Dulbecco's modification of Eagle's Medium (DMEM; Gibco BRL, Melbourne, Australia)
Eppendorf plastic centrifuge tubes (1.5 cm³; Netheler-Hinz-GmbH, Hamburg, Germany)
Flat-bottomed culture flasks (75 cm³; Costar, Denmark)
Foetal Calf Serum (FCS; MultiSer™, Cytosystems, Sydney, Australia, or ICN Biomedicals Inc., Costa Mesa, CA, USA, screened for mycoplasma and bovine adventitious agents)
Freund's Complete Adjuvant (Sigma Chemical Co., St Louis, MO, USA)
Freund's Incomplete Adjuvant (Sigma Chemical Co., St Louis, MO, USA)
Fungizone (MultiCel™, Cytosystems, Sydney, Australia)
Gelatin (≈300 bloom, Sigma Chemical Co., St Louis, MO, USA)
Goat Anti-(Mouse IgG Fc fragment) (GAM; Immunopure®, Pierce, Rockford, IL, USA)
Goat Anti-Mouse immunoglobulin conjugated to Horseradish Peroxidase (GAM-HRP; Cappel, Organon Tenika Corp, West Chester, PA, USA)
H₂O₂ (BDH Chemicals, Melbourne, Australia)
Heparin (The Boots Co., Sydney, Australia)
Hypoxanthine-Aminopterin-Thymidine supplement (HAT; Sigma Chemical Co., St Louis, MO, USA)
Hypoxanthine-Thymidine supplement (HT; Sigma Chemical Co., St Louis, MO, USA)
ICI 118 551 (Cambridge Research Biochemicals)
IgG, IgM or IgA class-specific RAM-HRP (ICN Biomedicals Inc., Costa Mesa, CA, USA)
Iscove’s modification of Dulbecco’s medium (ISC; Flow Laboratories, McLean, VA, USA)
Isoprenaline (Sigma Chemicals Co.)
Mature Merino ewes (CSIRO DAP, Badgerys Creek Field Station)
Microtiter plates with 96 “U-shaped” wells (Dynatech Laboratories, Virginia, USA)
Murine IgG standards (Sigma Chemical Co., St Louis, MO, USA)
NaNO₃ (Ajax Chemicals, Sydney, Australia)
Non-essential amino acids (100x solution; MultiCel™, Cytosystems, Sydney, Australia)
Nonidet P-40 (Sigma Chemical Co., St Louis, MO, USA)
Noradrenaline (Sigma Chemicals Co.)
o-Phenylenediamine (Sigma Chemical Co., St Louis, MO, USA)
Ovalbumin (OA; Grade V, minimum 98% (agarose electrophoresis); Sigma Chemical Co., St Louis, MO, USA)
Penicillin (Sigma Chemical Co., St Louis, MO, USA)
Phytohemagglutinin P (Bacto Laboratories Pty Ltd, Sydney, Australia)
Polyethylene glycol (PEG-1500, average molecular weight 1 400-1 600, Koch-Light Ltd, Suffolk, England)
Protein A-Sepharose beads (CL-4B; Pharmacia Fine Chemicals, Uppsala, Sweden)
Rabbit Anti-Mouse immunoglobulin conjugated to Horseradish Peroxidase (RAM-HRP; Dako-immunoglobulins, Denmark)
Salbutamol (Sigma Chemicals Co.)
Scintillant (Optiphase Highsafe III, Australian Chromatography, Sydney, Australia)
Scintillant vials (Pony, Packard, Meriden, CT, USA)
Sodium pyruvate (MultiCel™, Cytosystems, Sydney, Australia)
Sotalol (gift from Astra Pharmaceuticals, Sydney, Australia)
Streptomycin (Sigma Chemical Co., St Louis, MO, USA)
Tween 20 (polyoxyethylene sorbitan monolaurate; Sigma Chemical Co., St Louis, MO, USA)
Ultra-Turrax probe (Janke and Kunkel, GmbH & Co., Germany)
Vacutainer (heparin-containing tubes under vacuum for blood collection; Becton Dickinson Co., Rutherford, NJ, USA)
[^3H]Clenbuterol ([^3H]CB; Amersham, North Ryde, NSW, Australia)