MODEL STUDIES OF COENZYME B_{12}-DEPENDENT REACTIONS

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Declaration

The work presented in this thesis was carried out in the School of Chemistry, at Newcastle University, UK and at the Institute of Organic Synthesis and Photoreactivity (I.S.O.F.) - BioFreeRadicals, CNR, Bologna, Italy.

This work contains no material that has been accepted for the award of any other degree, diploma or any other qualification in any other Institution of University, and, to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference has been made in the next.

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Abstract

Coenzyme-B_{12} dependent glutamate mutase is a radical enzyme, which catalyses the isomerisation of (*S*)-glutamate to (*2S*,*3S*)-3-methylaspartate *via* enzyme-bound radical intermediates. This is the key step in the fermentation of glutamate to ammonia, carbon dioxide, acetate, butyrate and hydrogen by *Clostridium tetanomorphum*. It has been proposed that the mechanism of glutamate mutase involves removal of H_{si} from glutamate to give a 4-glutamyl radical that undergoes fragmentation to acrylate and a glycine radical. Recombination of these species leads to the 3-methylene-aspartate radical and hence (2*S*,3*S*)-3-methylaspartate.

Glutamate mutase has an active site containing three arginine residues that bind to the carboxylate groups of substrate, intermediates and product, the so-called 'arginine claw'. The model compound containing two guanidinium groups, 2,3-bis-guanidinomethyl-biphenyl dihydrochloride, has been synthesised in order to probe the function of this peculiar enzyme active site. NMR titration results showed that the receptor binds weakly to tetrabutylammonium glutarate in CD_3OD. To identify possible intermediates for the glutamate mutase catalysed reaction, a precursor for the product-related radical 3-methyleneaspartate has been designed and its synthesis has been explored.

Diol dehydratase from e.g. *Klebsiella oxytoca* is a radical enzyme that converts simple 1,2-diols into corresponding aldehydes and water. The enzyme requires adenosylcobalamin (coenzyme B_{12}) as cofactor and a metal cation (e.g. K^+). The mechanism of action of the dehydratase has previously been investigated by protein crystallography and *ab initio* molecular orbital calculations, aided by stereochemical and model studies. The 5'-deoxyadenosyl radical from homolysis of the coenzyme's Co-C bond abstracts a specific H atom from C-1 of diol substrate giving a substrate radical that rearranges to a product radical by 1,2-shift of hydroxyl from C-2 to C-1. It has been proposed that the rearrangement mechanism involves the action of acidic and basic residues in the protein, with the involvement of a bridged intermediate.

Ethanolamine ammonia-lyase (EAL) from e.g. *Clostridia* sp. converts ethanolamine into acetaldehyde and ammonia by a similar pathway.

Fluorine-substituted probes for coenzyme B_{12}-dependent enzymatic reactions: 3,3,3-trifluoropropane-1,2-diol for diol dehydratase and 2-amino-3,3,3-trifluoropropanol for EAL, have been synthesised. 3,3,3-Trifluoropropane-1,2-diol is a substrate for glycerol dehydratase (ca. 4 % of the activity of propane-1,2-diol). This result is surprisingly in the context of the proposed mechanism for diol dehydratase, although *ab initio* molecular orbital calculations (collaboration with Dr D. M. Smith, Zagreb) have indicated that CF_3 has a minimal effect on the stabilities of the proposed intermediates. A model study (performed in collaboration with Dr C. Chatgilialoglu, Bologna) showed that 1,1-difluoro-3-hydroxylpropanone was formed by continuous γ-irradiation of 3,3,3-trifluoropropane-1,2-diol. The expected 3,3,3-trifluoropropanal and 1,1,1-trifluoroacetone could not be determined from the ^1H NMR analysis of the irradiation mixuture. Propanal and acetone were formed by the continuous γ-irradiation of propane-1,2-diol (analysis by GC and GC-MS, and by formation of the corresponding 2,4-dinitrophenylhydrazones).

2-Amino-3,3,3-trifluoropropanol was found to be devoid of any activity against EAL according to a coupled assay with alcohol dehydrogenase (collaboration with Dr G. H. Reed, University of Wisconsin-Madison). EPR study (collaboration with Dr K. Warncke, Emory University) has concluded that the CF_3-substrate did not lead to the significant formation of a paramagnetic intermediate. This may be due to the CF_3 group reducing the basicity of the amino group and the acidity of the alcohol group and so that there is a significant diminished interaction with the active site in EAL. Or this may be caused by the significant steric effect introduced by the trifluoromethyl group (CF_3 group is between two and three times larger than CH_3).

List of abbreviations

AdoCbl	Adenosylcobalamin
ADP	Adenosine-5'-diphosphate
ACCN	1,1'-Azobis(cyclohexanecarbonitrile)
AIBN	2,2'-Azo-bis-isobutyronitrile
ATP	Adenosine-5'-triphosphate
BPO	Benzoyl peroxide
CNCbl	Cyanocobalamin
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DMB	5,6-Dimethylbenzimidazole
DNA	Deoxyribonucleic acid
2,4-DNP	2,4-Dinitrophenylhydrazine
EAL	Ethanolamine ammonia-lyase
EPR	Electron paramagnetic resonance
FTIR	Fourier transform infrared spectroscopy
GC	Gas chromatography
GCMS	Gas chromatography mass spectrometry
GM	Glutamate Mutase
HC	Hapocorrin
IF	Intrinsic factor
IR	Infrared spectrometry
MeCbl	Methylcobalamin
MeTHF	5-Methyltetrahydrofolic acid
MMA	Methylmalonic acid
2-MGM	2-Methyleneglutarate mutase
NBS	*N*-Bromosuccinimide
NMR	Nuclear magnetic resonance
HOCbl	Hydroxocobalamin

PA	Pernicious anaemia
PDB	Protein data bank
ppm	Parts per million
Petrol	Fraction of hexanes at 40-60 °C bp range
SAM	*S*-Adenosylmethionine
TC	Transcobalamin
TFA	Trifluoroacetic acid
THF	Tetrahydrofolate
THF	Tetrahydrofuran
TLC	Thin layer chromatography
UV	Ultraviolet
δ	Chemical shift
s	Singlet
d	Doublet
dd	Double doublet
t	Triplet
q	Quartet
m	Multiplet

Contents

Chapter 1

Introduction to Coenzyme B_{12}-dependent Cellular Reactions

1.1 INTRODUCTION TO VITAMIN B_{12}

Vitamin B_{12} was discovered because of its relationship to the disease pernicious anemia (PA). PA was a fatal illness before the 1920s. But this changed after Whipple suggested raw liver as a treatment. He found that ingesting large amounts of liver seemed to cure anemia from blood loss.[1] Minot and Murphy[2] then described the dramatic recovery of 45 patients suffering from PA after they consumed a special diet of lightly cooked liver. The three men shared the 1934 Nobel Prize in Medicine for the discovery of the cure of a previously fatal disease of unknown origin.

The active anti-PA factor was a mystery until 1948, when Folkers[3] and Smith[4] independently isolated a small quantity of a red crystalline compound from liver. After the substance was shown to lead directly to the recovery of PA patients it was named vitamin B_{12}

Vitamin B_{12} has the most complicated structure; the unique corrin ligand was only revealed by X-ray crystallography in full 10 years later its discovery. The structures of vitamin B_{12} and coenzyme B_{12} were established by X-ray crystallography in the laboratory of D.C. Hodgkin.[5] The structure of vitamin B_{12} is based on a corrin ring, which is a near planar, macrocyclic ring like the porphyrin system found in hemes, chlorophylls, and cytochromes. A cobalt atom lies at the center of corrin ring. The cobalt is in oxidation state +3,[6] four of the six coordination sites are provided by the corrin ring, and a fifth (α face, bottom site of the corrin ring) by a dimethylbenzimidazole group. The sixth coordination site (β face), the biological activity centre of vitamin B_{12}, is variable (**Figure 1**).

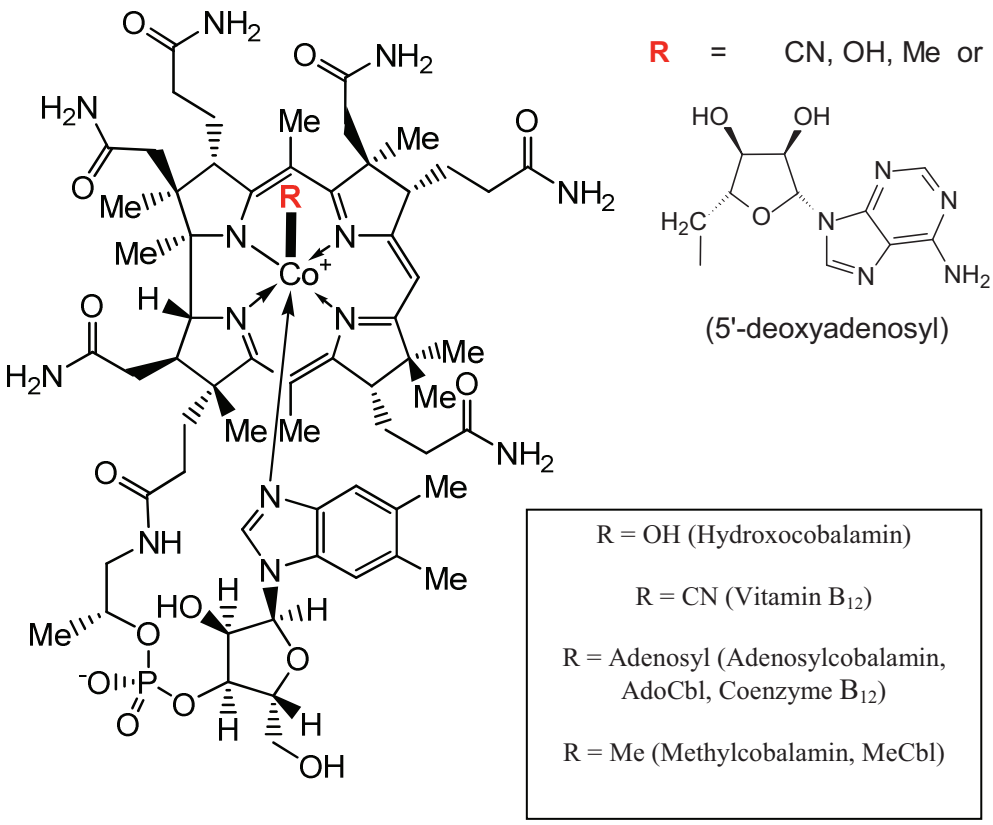


Figure 1: The chemical structure of vitamin B_{12} and derivatives

Vitamin B_{12} and its analogues are often called corrinoids while the forms of vitamin which contain the ribonucleotide α-D-ribofuranosyl-5, 6-dimethylbenzimidazole are also named cobalamins.

Vitamin B_{12} occurs naturally in several forms. Though the vitamin was isolated in the form of cyanocobalamin (CNCbl), vitamin B_{12} is biologically active in only three forms, adenosylcobalamin (AdoCbl), hydroxocobalamin (HOCbl) and methyl cobalamin (MeCbl). They can be found in the serum and tissues of man and higher animals. MeCbl is the preferred form for oral absorption because of its immediate activity. CNCbl and HOCbl must lose the cyanide or hydroxide moiety and add either a methyl or adenosyl group in order to be converted into either MeCbl or AdoCbl in

vivo. MeCbl or AdoCbl can be effective to cure pernicious anaemia. The structures of the most important cobalamins are shown in **Figure 1**.

1.2 VITAMIN B_{12} NUTRITION AND MEDICINAL USES

Humans and higher animals require vitamin B_{12} for only two enzymes: (*R*)-methymalonyl-CoA muatase (MCM; E.C.5.4.99.2) and methionine synthase (5-methyltetrahydrofolate, E.C.2.1.1.13). MCM catalyses the conversion of (*R*)-methylmalonyl-CoA to succinyl-CoA using adenosylcobalamin (AdoCbl) as a cofactor. In the cytosol, methylcobalamin (MeCbl) is synthesised through reduction and methylation of Cbl and is employed as a cofactor for methionine synthase in the conversion of homocysteine to methionine. These two enzymes have impact on DNA synthesis and regulation particularly, and on fatty acid synthesis and energy production as well.

Vitamin B_{12} is essential for rapid DNA production during cell division. This is especially important in tissues such as the bone marrow, where cells are dividing rapidly. Where there is a lack of B_{12}, the synthesis of DNA can be disrupted and abnormal cells called megaloblasts are produced during red blood cell formation. This can result in megaloblastic anaemia.[7, 8] B_{12} deficiency could also lead to damage in the nervous system by interrupting the maintenance of myelin. Despite the extensive animal-model research of B_{12} deficiency for more than 30 years, the specific role of vitamin B_{12} in maintaining normal myelination remains poorly understood.[9] Failure to convert methylmalonyl CoA into succinyl CoA results in a high level of methymalonic acid, which is a destabiliser of myelin.[9, 10]

Mammals are not able to synthesis cobalamins, so they must be supplied from the diet. B_{12} can be found primarily in meat, fish, eggs and dairy products. The daily requirement of vitamin B_{12} for the human body is low 1-2 μg.[7] Consequently, there is an elaborate mechanism for absorption, blood transportation and cellular uptake of dietary B_{12}. The absorption of vitamin B_{12} begins in the mouth where a small amount of unbound Cbl is absorbed through the mucosa membrane. Most of the vitamin B_{12} which is protein bound in food is digested in the stomach by proteolytic gastric enzymes, which require an acidic pH. Meanwhile hapotocorrin is secreted to bind to free Cbl and forms a hapotocorrin-bound Cbl. This can prevent free Cbl from breakdown in the low-pH environment of stomach. In the duodenum hapotocorrin-bound Cbl is digested by protease and Cbl becomes bound to intrinsic factor (IF), which is a protein synthesised by gastric parietal cells. The Cbl-IF complex is taken into the circulation in a complex with transcobalamin II (TC-II).[11]

After the complex TCII-Cbl has been transferred into the cell, Cbl is released into the cytoplasm. Subsquently, it is reduced from the 3+ to the 1+ oxidation state and converted to AdoCbl or MeCbl in the mitocondrion and cytoplasm, respectively. In cytoplasm, methionine synthase (synthase) catalyses the reaction, which converts homocysteine to methionine with 5-methyltetrahydrofolate as methyl group donor and MeCbl as a cofactor. AdoCbl is required for the activity of methylmalonyl-CoA mutase (mutase), which converts (*R*)-methylmalonyl CoA to succinyl CoA in the mitochondrion[12] (**Figure. 2**).

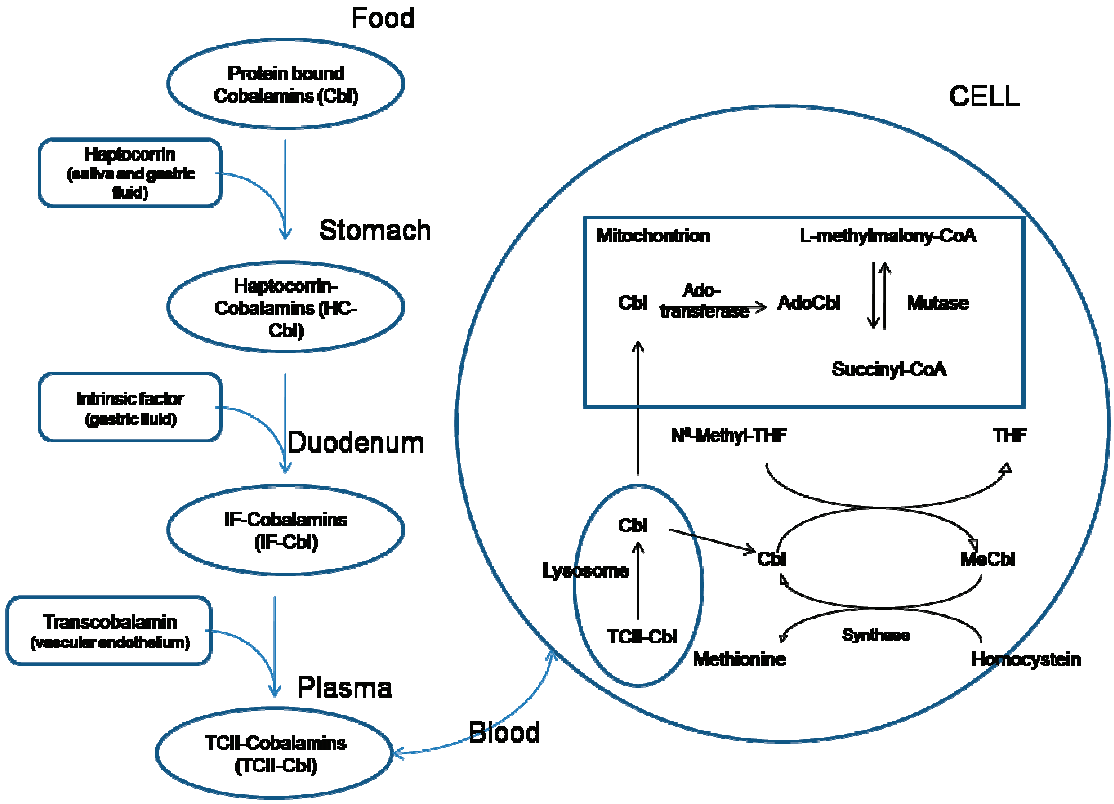


Figure 2: Cobalamin absorption and metabolic pathway. Cbl: cobalamin; HC: haptocorrin(transcobalamin I/III); IF: intrinsic factor; HC-Cbl: haptocorrin-bound cobalamin; IF-Cbl: intrinsic factor-bound cobalamin; TCII-Cbl: cobalamin bound to transcobalamin II.[13]

Vitamin B_{12} deficiency can be caused by failure in any of the steps of the elaborate mechanism of absorption, transport and synthesis of cobalamins. The total amount of vitamin B_{12} stored in body is about 2-5 mg in adults, which is normally maintained from the diet. Vitamin B_{12} deficiency can be treated with intramuscular injections or less well by oral intake of vitamin B_{12}. [14]

Hydroxocobalamin is not only used for B_{12} deficiency but also used as an acute treatment for cyanide poisoning in both Europe and United States. The mechanism of

action is that the hydroxide ligand of hydroxycobalamin is displaced by the toxic cyanide ion, resulting in the very stable harmless cyanocobalamin.

Cobalamins can be selectively modified at several functional sites due to its structural complexity without affecting its metabolic pathway in the human body. Therefore, these modified cobalamin analogues can be used in diagnosis and chemotherapy.[15,16]

1.3 B_{12} DEPENDENT ENZYMES

B_{12} dependent enzymes have been divided into three large classes on the basis of different types of biological reactions that they catalyse: isomerases, methyltransferases, and reductive dehalogenases. [17]

The isomerases are the largest subfamily of B_{12}-dependent enzymes found in bacteria. The only exception is methylmalonyl-CoA mutase, which is found in both bacteria and animals. The isomerases catalyse carbon skeleton rearrangements, that is 1,2 interchange between a variable substituent and a hydrogen atom on vicinal carbons. This type of enzyme is an AdoCbl-dependent enzyme. These enzymatic reactions are initiated by homolysis of the Co-C bond in AdoCbl.

The B_{12}-dependent methyltransferases catalyse the transfer of a methyl group from a methyl donor to a nucleophilic acceptor. This type of enzyme plays an important role in the anaerobic acetogenesis, methanogenesis and catabolism of acetic acid to methane and carbon dioxide.[18]

The B_{12}-dependent dehalogenases remove chloride ion from aliphatic or aromatic chlorinated organic compounds; such as chlorinated ethenes, chlorinated phenols, and polychlorinated biphenyls (PCBs), which are all in the 12 priority pollutant list of the USA Environmental Protection Agency.[19 20] The reductive dehalogenation was observed in a variety of anaerobes, including methanogenic and homoacetogenic bacteria; i.e. 3-chlorobenzoate reductive dehalogenase of *Desulfomonile tiedjei*[21] and *O*-chlorophenol reductive dehalogenases of *Desulfitobacteria.*[22] The role of B_{12} in the reductive dehalogenation is different from that of B_{12}-dependent isomerisation and methyltransfer.

Two mechanistic pathways have been proposed for B_{12}-dependent reductive dehalogenation (**Figure 3**). In Path A, an organo-cobalt adduct is formed, which undergoes subsequent β-elimination of the chlorine substituent. Then, homolysis of the Co-C bond gives an aryl radical, which is converted to aryl product *via* abstraction of a hydrogen atom. In Path B, the cobalamin serves as an electron donor. A radical anion intermediate is formed by a one-electron transfer from the cob(I)alamin. Then a chloro-aryl radical is produced as a result of protonation. Another electron transfer causes the cleavage of the chlorine anion and formation of product. Many questions regarding the dehalogenation mechanism remain unanswered as the B_{12} dependent dehalogenases have only been discovered in recent years.

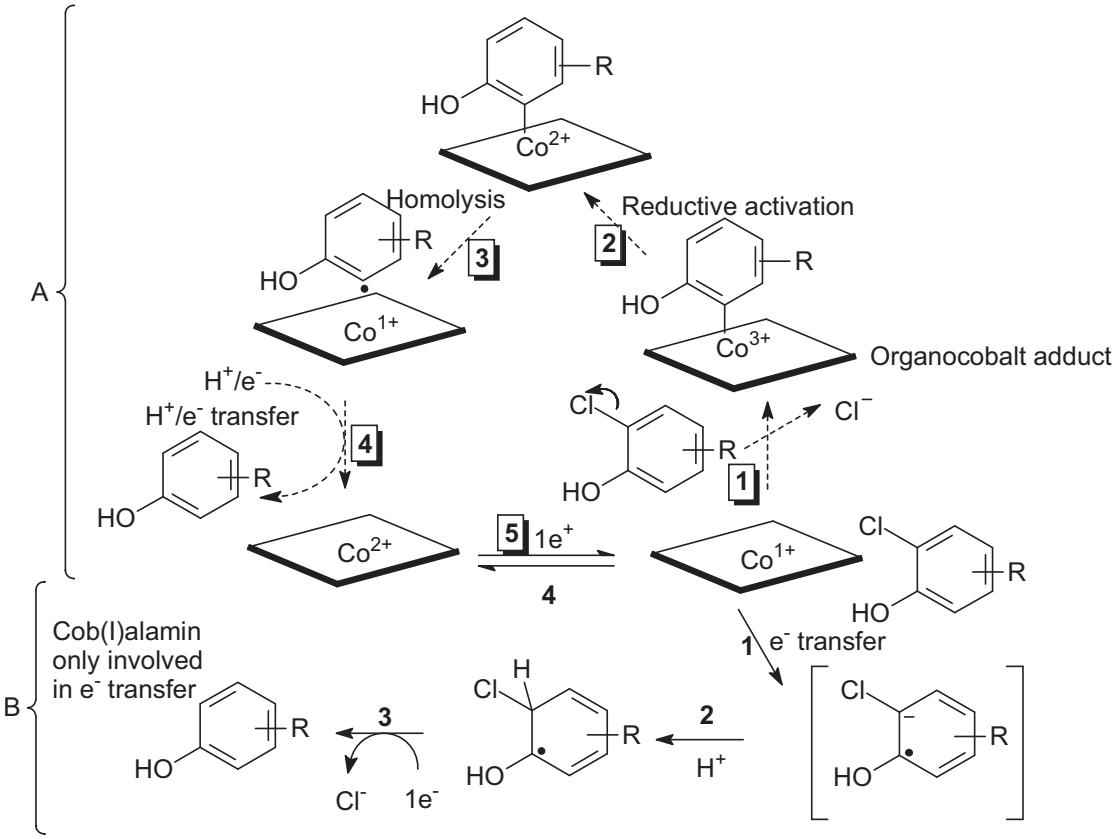


Figure 3: Proposed mechanistic pathways for B_{12}-dependent reductive dehalogenation.[23] The two pathways, A (dashed arrows) and B (solid arrows), are different in the roles of cobalamin.

The majority of methyltransferases have been found in bacteria, though *N*-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) and methylmalonyl-CoA mutase have been found in mammals. B_{12} dependent enzymes have been classified into two major sets, either methylcobalamin (MeCbl) or adenosylcobalamin (AdoCbl) dependent enzymes, according to the coenzyme involved.

Recent progress in the structural analysis of the enzymes involved in both coenzyme systems has led to a clearer understanding of both the chemical environment at the active site and the mode of B_{12} binding. (See section 1.6 and 1.7)

Natural cobalamins are known to exist in three different oxidation states: +3, +2 or +1[24, 25] (**Figure 4**). The cob(I)alamin is highly reactive, known as a 'supernucleophile'. It has been used in the synthesis of alkylcobalamins.[26] The catalytic activity of B_{12} dependent enzymes can be traced back to the reactivity of the B_{12}-derivatives with different oxidation states. In MeCbl dependent enzymatic reactions, the heterolytic formation/cleavage of the Co-C bond triggers the oxidation/reduction of cob(III)alamin and cob(I)alamin (formally a two electron reduction/oxidation of cobalt). It is represented by the reaction of cob(I)alamin with alkylating agents and by the nucleophile-induced demethylation of methyl-cob(III)alamin. This is particularly important in enzymatic methyl-transfer reactions. The coordination of Co-nucleotide ('base-on' mode) has a notable thermodynamic effect on the heterolytic reactions of methylcobalamin. [27] Occasionally, cob(I)alamin is oxidatively inactivated to cob(II)alamin, which requires reductive activation.

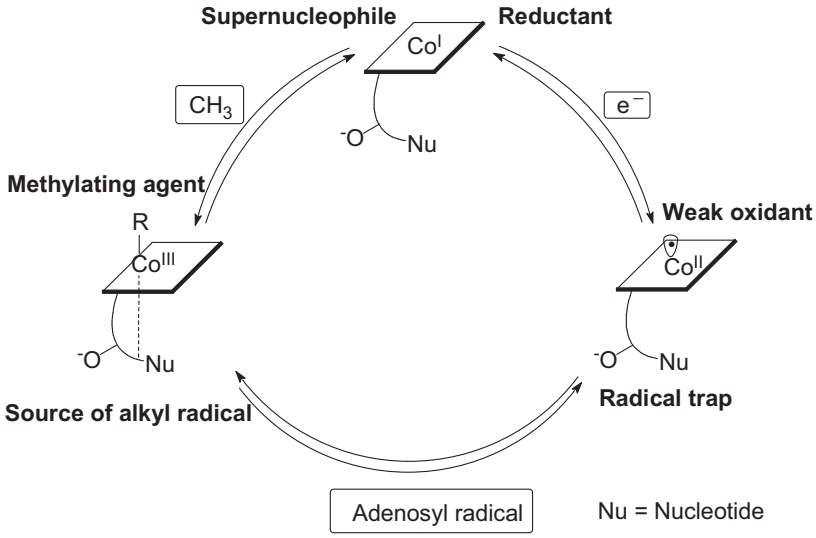


Figure 4: Reaction steps and reactivity relating to the oxidation state of cobalamins in B_{12}-dependent enzymes.[18]

The oxidative/reductive interconversion of cob(III)alamin and cob(II)alamin (formally a one-electron oxidation/reduction of the cobalt) is involved in AdoCbl-dependent enzymatic reactions. Homolysis of the Co-C bond generates the alkyl radical (i.e. 5'-adenosyl radical) and cob(II)alamin. Therefore one role of cobalamin is to act as a carrier of the highly reactive adenosyl radical, which is released after the binding of a substrate molecule to the enzyme, whereas cob(II)alamin acts as a radical trap, which will bind to the adenosyl radical after the catalytic cycle.Thus in AdoCbl-dependent enzymatic reactions the enzyme acts a reversible free radical reservoir.

1.4 METHYLCOBALAMIN DEPENDENT ENZYMES

Methyltransferases catalyse a methyl transfer reaction using either vitamin B_{12} or the CFeSP (corrinoid iron-sulfur protein)[28] as a cofactor. Methylcobalamin is the vitamin B_{12} derivative involved in the reaction. Methylcobalamin-dependent enzymes exist in many bacteria and animals. Anaerobic acetogenesis, [29] methanogenesis [30] and catabolism of acetic acid to methane and carbon dioxide all depend on B_{12}-dependent methyl transfer reactions.

Methylcobalamin transfers its methyl group from donor to acceptor. Thiols are the methyl group acceptors in most cases of methylcobalamin dependent enzymatic reactions. The substrates are the methyl group donors, which are variable in different organisms, i.e. methanol, aromatic methyl esters, methylamines and methyltetrahydropterins (such as N^5-methyltetrahydrofolate). (**Figure 5**)

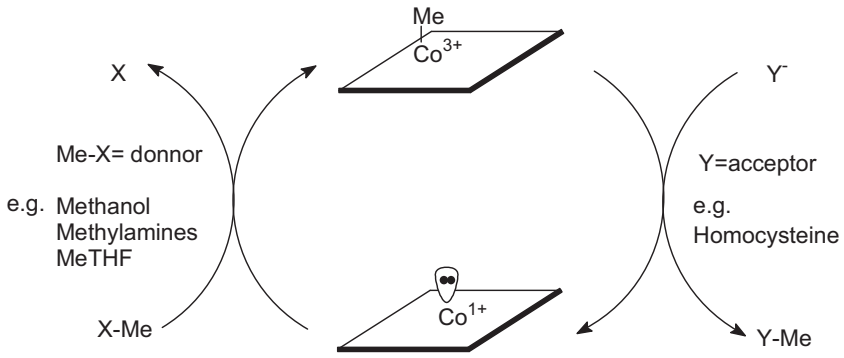
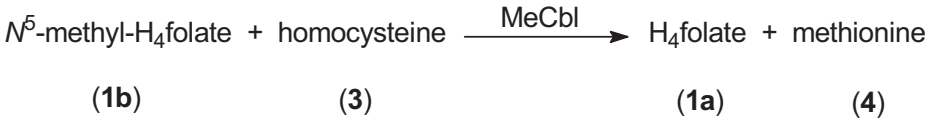


Figure 5: Catalytic cycle of methylcobalamin-dependent enzymatic reactions

1.4.1 Methionine Synthase

Methionine synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13) catalyses the transfer of a methyl group from N^5-methyltetrahydrofolate (**1b**) to homocysteine (**3**), forming tetrahydrofolate (**1a**) and methionine (**4**).[31, 32](**Figure 6**) Low activity of methionine synthase in humans could result in megaloblastic anemia, and eventually cause the subacute combined degeneration of the spinal cord.[33]



Scheme 1: Methylcobalamin-dependent methionine synthase reaction.

The overall catalytic mechanism involves two successive methyl transfer reactions from methyltetrahydrofolate to cob(I)alamin and from MeCbl to homocysteine. In the methylcobalamin-dependent methionine synthase reaction, cobalt-carbon σ-bond heterolytic cleavage and formation are important and the 'supernucleophilic' cob(I)alamin is an intermediate that can remove a methyl group from a substrate of relatively low reactivity, such as N^5-methyltetrahydrofolate [34] **(1b)** and N^5-methyltetrahydromethanopterin[35] (**2b**). (**Scheme 1**)

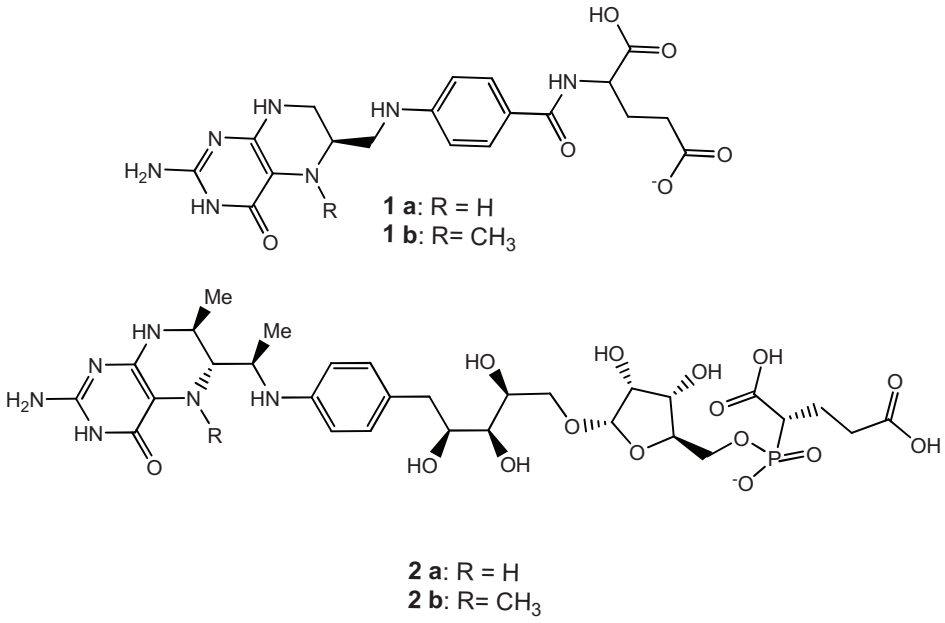


Figure 6: Structure of: **(1a)** = tetrahydrofolate, **(1b)** = N^5-methyltetrahydrofolate, **(2a)** = tetrahydromethanopterin, **(2b)** = N^5-methyltetrahydromethanopterin.

The structure of methionine synthase (136 kDa) was reported by Drennan *et al*.[34] The conformational structure remains unknown. But, it has been proposed on the basis of proteolytic studies that methionine synthase is a modular enzyme constructed of 1227 amino acids, which are divided into four functional modules; each module has a specific function in the overall catalytic cycle. The individual domain structures can be found in the Protein Data Bank. (PDB ID: 1Q8J, 1BMT, 1K7Y)

N^5-Methyltetrahydrofolate, cobalamin, homocysteine, and *S*-adenosylmethionine (SAM) bind to separate domains.[36] The cobalamin domain in its different oxidation states interacts specifically with each of the other domains: the form with the N^5-methyltetrahydrofolate domain, the cob(II)alamin form with the SAM binding domain,

and the methylcobalamin form with the homocysteine binding domain, which correspond to the three reactions in **Figure 7**.

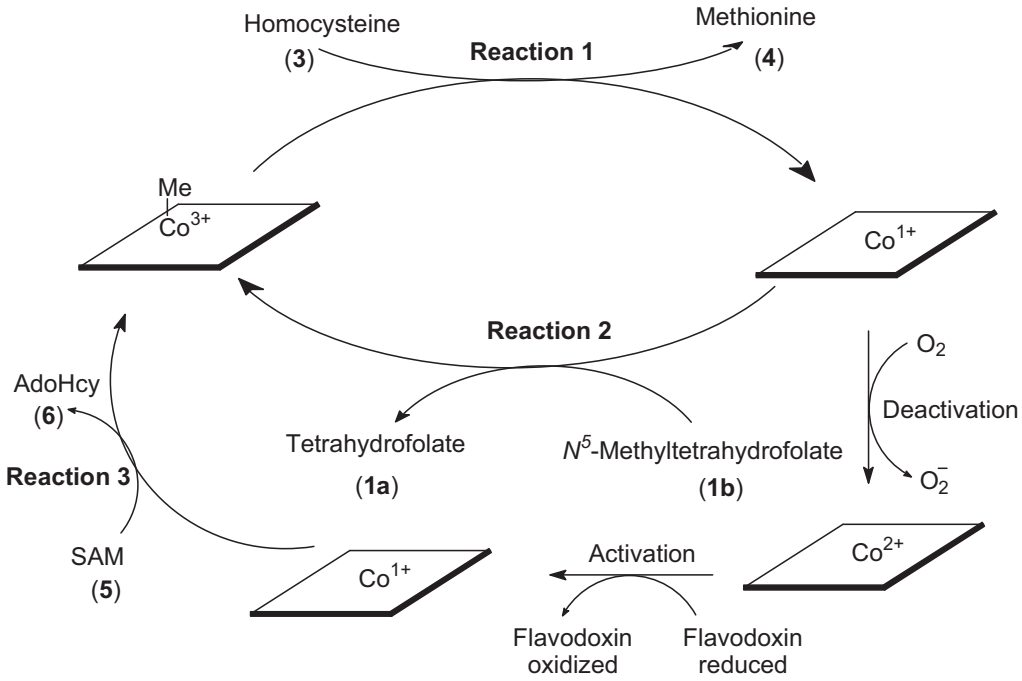


Figure 7: The complete catalytic cycle and the deactivation/activation of the coenzyme in methionine synthase

In the catalytic cycle (**Figure 7**), a methyl group is transferred from the methylcobalamin to homocysteine (**3**), producing methionine (**4**) and cob(I)alamin. The cob(I)alamin is methylated by N^5-methyltetrahydrofolate (**1a**).[37] During the catalytic cycle, the cob(I)alamin form is occasionally converted to cob(II)alamin in the presence of oxygen, which occurs once in every 100-2000 turnovers.[38] The cob(II)alamin is catalytically inactive in the reaction. So, the enzyme is reactivated by reductive methylation of the cob(II)alamin form where SAM (**5**) binds to the C-

terminal domain as the methyl donor. In the bacterial systems, the reductant (reduced flavodoxin) is comprise of two flavoproteins, NADPH-flavodoxin(or ferredoxin) oxidoreductase [39] and flavodoxin,[32] which transfer the electron from NADPH to methionine synthase. Recent investigation of the reactivation reaction showed that the inactive cob(II)alamin form is firstly reduced to cob(I)alamin by an electron from flavodoxin and its then methylated by SAM. Flavodoxin is lacking in mammals and the physiological reducing mechanism was completely unknown until recently. A dual-flavoprotein family member, methionine synthase reductase, is the reductant for the reactivation of cob(II)alamin.[40]

Methionine synthase from *Escherichia coli* is the most extensively studied B_{12}-dependent methyltransferase. The X-ray crystal structure of the cobalamin binding domain led to the discovery of the 'base-off/His-on' binding mode.[34] It revealed that the dimethylbenzimidazole (Dmb) side chain of the methylcobalamin is displaced from the cobalt by a histidine residue from the protein as the axial ligand in the cobalt complex. As a result of this displacement, the stability and reactivity of cobalamin derivatives is directly controlled by the protein rather than by a substituent of the cofactor. Model studies have shown that in free alkyl cobalamins, the cobalt-carbon bond is stabilised by the basic lower ligand, which increases the electron density on the cobalt. This ligand acts the homolytic cleavage to form cob(II)alamin and heterolytic cleavage to form cob(I)alamin. So the 'base off/His on' mode is believed to modulate the reactivity and stability of the cobalamins and facilitate the heterolytic cleavage of the cobalt-carbon bond.

In the 'base off/His on' mode, the key His759 residue is found in the consensus motif (D*X***H***XX*G), where **H** represents the lower axial histidine ligand. The structure study[34] revealed that it is a set of hydrogen-bonded residues, His759-Asp757-Ser810, named 'ligand triad' that transfers a proton from solvent to His759 and ensures that His759 is positioned to the corrin ring. Therefore, a catalytic quartet is formed by the His759-Asp757-Ser810 of the enzyme and the cobalt of cobalamin. It facilitates the methylation and demethylation by transferring protons in and out of the said region. In this mechanism, Asp757 and His759 share a single proton, with one negative charge delocalised across the two residues. Ser810 provides an essential connection between bulk solvent and the two residues. (**Figure. 8**)

This 'base-off/His-on' mode is also conserved in all AdoCbl-dependent mutases (e.g. methylmalonyl-CoA mutase and glutamate mutase), which also features the consensus motif (D*X***H***XX*G). [18, 41]

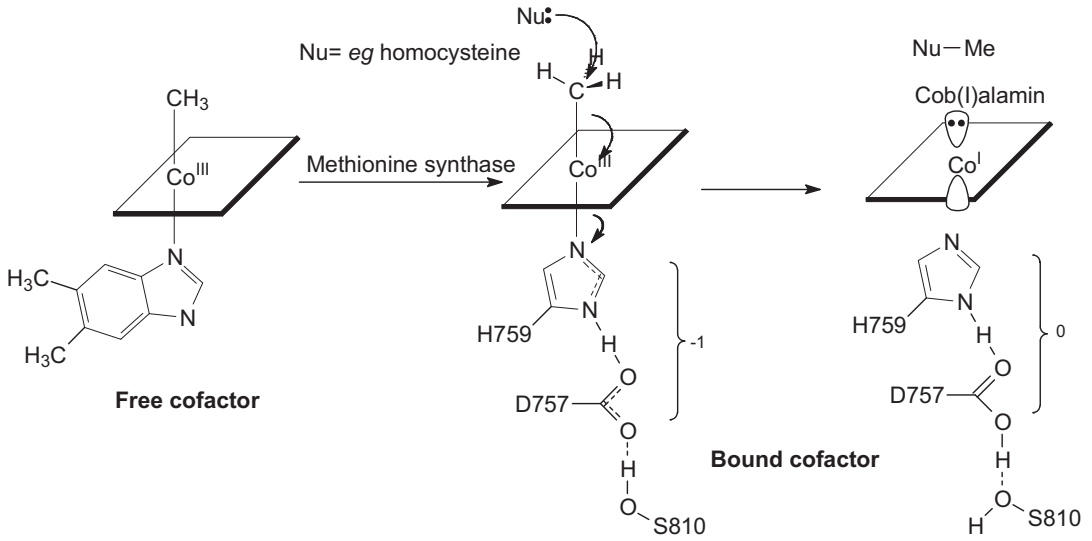


Figure 8: Displacement of dimethylbenzimidazole by His759-Asp757-Ser810, named 'ligand triad', in MeCbl on binding to methionine synthase and heterolysis of proteien-bound cofactor leading to cob(I)alamin. Charges have been assigned assuming a partial imidazolate charater for H759.[42]

1.5 ADENOSYLCOBALAMIN DEPENDENT ENZYMES

At least ten biological reactions are catalysed by AdoCbl dependent enzymes, using a variety of substrates (**Table 1**). A general rearrangement of the type shown in **Figure 9** can be seen in all cases, where X is an electron withdrawing group.

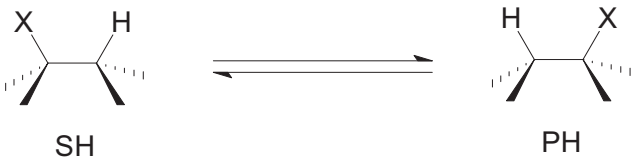


Figure 9: General type of rearrangement catalysed by AdoCbl dependent enzymes, where X is an electron withdrawing group.

AdoCbl-dependent enzymes catalyse the stereospecific 1, 2-shift of the X group in the substrate molecule SH, exchanging places with an adjacent hydrogen atom, to form the product PH. In most cases this step is reversible and leads to an equilibration between SH and PH. Some AdoCbl dependent 'eliminase' enzymes promote the loss of water or ammonia from the rearranged product: an irreversible process shown schematically in **Figure 10**.

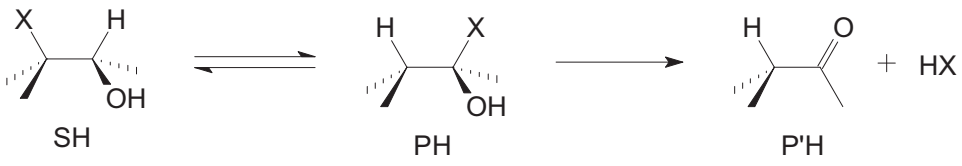


Figure 10: AdoCbl catalysed rearrangement of a substrate molecule (SH) into a product molecule (PH) followed by the subsequent dehydration to give P'H (X=OH or NH_2)

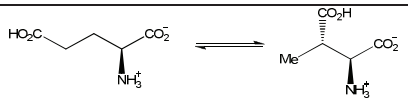
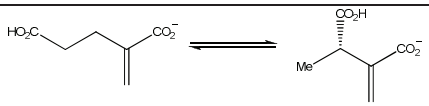
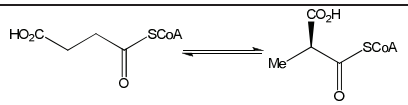
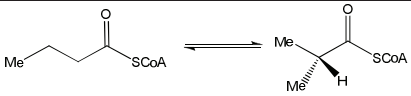
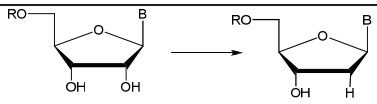
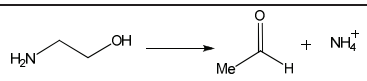
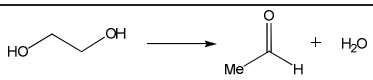
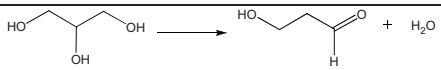
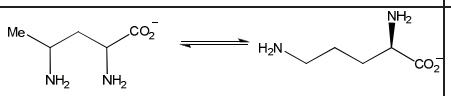
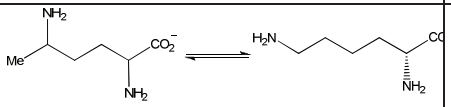
CLASS	ENZYME	REACTION	GROUP X
Class I enzymes **Carbon skeleton mutases**	Glutamate mutase		-CH(NH_2)CO_2H
	2-Methyleneglutarate mutase		-C(=CH_2)CO_2H
	Methylmalonyl-CoA mutase		-COSCoA
	Isobutyryl-CoA mutase		-COSCoA
Class II enzymes **Eliminases**	Ribonucleotide reductase		None
	Ethanolamine ammonia lyase		-NH_2
	1,2-Diol dehydrase		-OH
	Glycerol dehydrase		-OH
Class III enzymes **Aminomutases**	D-α-Ornithine mutase		-NH_2
	D-α-Lysine mutase		-NH_2

Table 1: Enzymatic reactions catalysed by AdoCbl

These AdoCbl-dependent reactions can be divided into three classes on the basis of the specifics of the migrating group and the receiving carbon.[43, 44] Class I enzymes are carbon skeleton mutases, which are reversible reactions; Class II enzymes are eliminases including lyases (such as dehydratases and deaminases) and ribonucleotide reductase. This type of enzyme catalyses the 1,2 shift of the H atom with an OH or NH_2 group resulting elimination of water or ammonia and the irreversible formation of the corresponding aldehyde. Class III enzymes are amino mutases which catalyse the reversible 1,2 shift of a H atom with a NH_2 group.

Depending on the binding structure of enzymes and the AdoCbl form, these three classes can be grouped into two categories.[11] The cobalamin exists in the base-on (Dmb-on) or base-off (Dmb-off) conformations with different B_{12} dependent enzymes. The Class I and Class III enzymes (mutases) bind AdoCbl in base-off form, with the dimethylbenzimidazol(Dmb) replaced by the imidazole of a histidine residue, known as 'base-off/His-on'. All enzymes feature the consensus sequence (D*X*H*XX*G). The 'ligand triad' in the sequence of His-Asp-Ser was first discovered for methylcobalamin in the B_{12} denpendent methionine synthase. The Class II enzymes (eliminases) all bind AdoCbl in base-on form with the Dmb group coordinated to cobalt in the lower axial position.

AdoCbl (**7**) has been associated with free radical biochemistry for more than 30 years. Unlike the methylcobalamin dependent enzymes, it is now widely accepted that AdoCbl dependent enzymes operate *via* homolytic rather than heterolytic cleavage of the Co-C σ-bond. Homolytic cleavage of this relatively weak, long bond (130 kJ mol^{-1},

~2 Å),[45] leads to formation of cob(II)alamin (**9**) and a highly activated organic radical, which is localised on the methylene group of 5'-deoxyadenosine (**8**).(**Figure 11**)

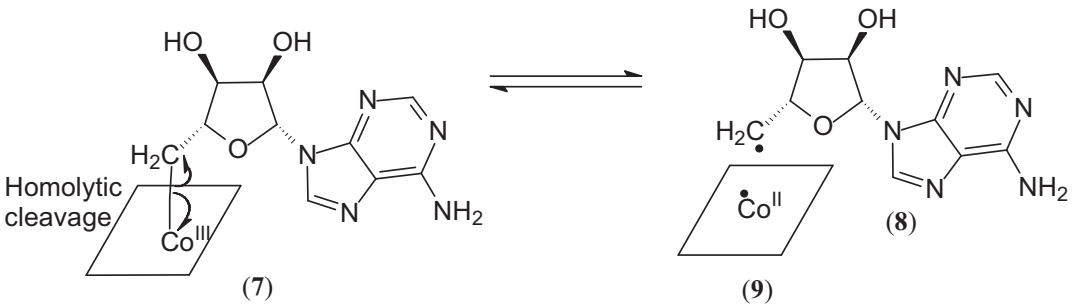


Figure 11: Homolytic cleavage of the cobalt-carbon σ-bond of AdoCbl (**7**) leading to cob(II)alamin (**9**) and 5'-deoxyadenosyl radical (**8**).

The pressure for Co-C σ bond homolysis must derive primarily from the protein, by specific interactions with the periphery amide side chains of the corrin ring and from the influence of the imidazole *trans* to the adenosyl group.[46, 47]

Therefore, activation of the AdoCbl is known as the first step in the catalytic cycle of the adenosylcobalmin dependent reactions. The Co-C σ-bond is cleaved, forming a 5'-deoxyadenosyl radical and cob(II)alamin. The adenosyl radical subsequently abstracts a specific hydrogen atom from a substrate molecule to give a substrate-derived radical (S˙) and 5'-deoxyadenosine. The next step is the rearrangement between the substrate-derived radical (S˙) and product-related radical (P˙). But how does this isomerisation occur? This is the key question that many groups in many countries are trying to probe. The product related radical (P˙) abstracts the hydrogen atom from the methyl group of 5'-deoxyadenosine forming a product molecule and the adenosyl radical. Product release occurs where the coenzyme B_{12} is regenerated

by the combination of the 5'-deoxyadenosyl radical and the cob(II)alamin species, (**Figure 12**). It is considered to be the final step of one catalytic cycle.

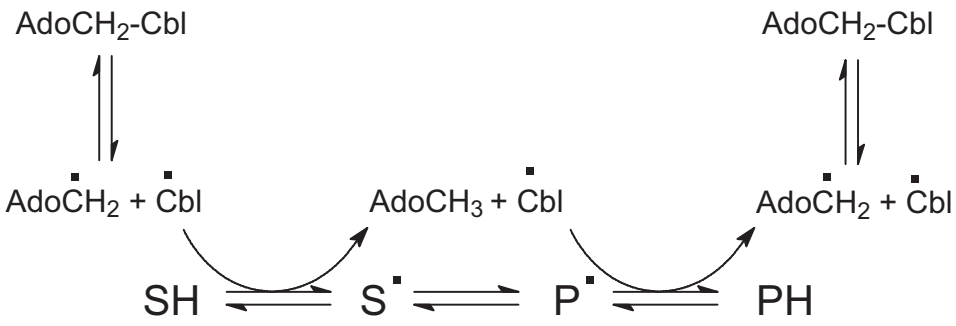


Figure 12: 'Minimal mechanism' for coenzyme B_{12}-dependent enzymatic rearrangements ($AdoCH_2$-Cbl = adenosylcobalamin (coenzyme B_{12}), $AdoCH_2^{\bullet}$ = 5'-deoxyadenosyl radical, $AdoCH_3$ = 5'-deoxyadenosine, $Cbl^{\bullet}$ = cob(II)alamin; SH = substrate, PH = product, $S^{\bullet}$ = substrate-derived radical, $P^{\bullet}$ = product-related radical).

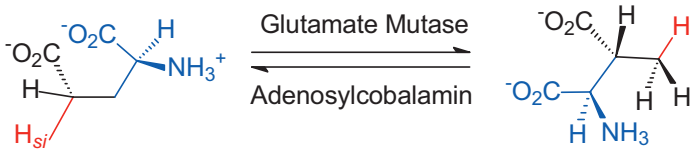
Experiments with tritium-labelling substrate in the AdoCbl-dependent diol dehydratase carried out by Frey *et al.*[48] indicated the intermolecular transfer of the migrating hydrogen atom between substrate/product and cofactor. Furthermore, the cofactor servers as a hydrogen carrier which transfers the hydrogen atom from substrate to product. Experiments carried out by Zagalak *et al.*[49] proved that a product radical will acquire either the hydrogen from its substrate precursor, or one hydrogen atom of the cofactor from another substrate precursor.

X-ray crystal structures showed that in the Class II enzymes (eliminases, e.g. diol dehydratase), intermediate radicals are generated about 10 Å away from the cobalt atom in cob(II)alamin Therefore, cob(II) alamin is proposed to act as a 'spectator' in

the catalysis. The fact that some Class II enzymes use a radical generator other than adenosylcobalmin (i.e. *S*-adenosylmethionine) supported this premise. In the crystal structure of glutamate mutase the 5'-deoxyadenosyl radical remains within 3-4 Å of the cobalt atom with the intermediate radicals approximately 6 Å away. It is suggested that cob(II)alamin acts as a 'conductor' which stabilises the highly reactive methylene radical. This distance also avoids the possibility of the covalent bond formation between intermediate and cob(II)alamin.[50] Recently it has been proposed that the cob(II)alamin reduce the transition-state energy leading to the intermediate radical.[51]

1.6 GLUTAMATE MUTASE

The coenzyme-B_{12} dependent glutamate mutase catalyses the reversible isomerisation of (*S*)-glutamate (**10**) to (2*S*,3*S*)-3-methylaspartate (**11**). [52] (**Scheme 2**) In this reaction, the H_{si} at C-4 and the glycyl moiety of glutamate are interchanged. The glycyl group migrates with retention of configuration at C-2, whereas C-4 undergoes inversion of configuration. The resulting methyl group of 3-methylaspartate becomes 'racemic'. This enzyme is extremely specific for (*S*)-glutamate (**10**) and (2*S*,3*S*)-3-methylaspartate (**11**), it did not show any activity with other substrates.[53]

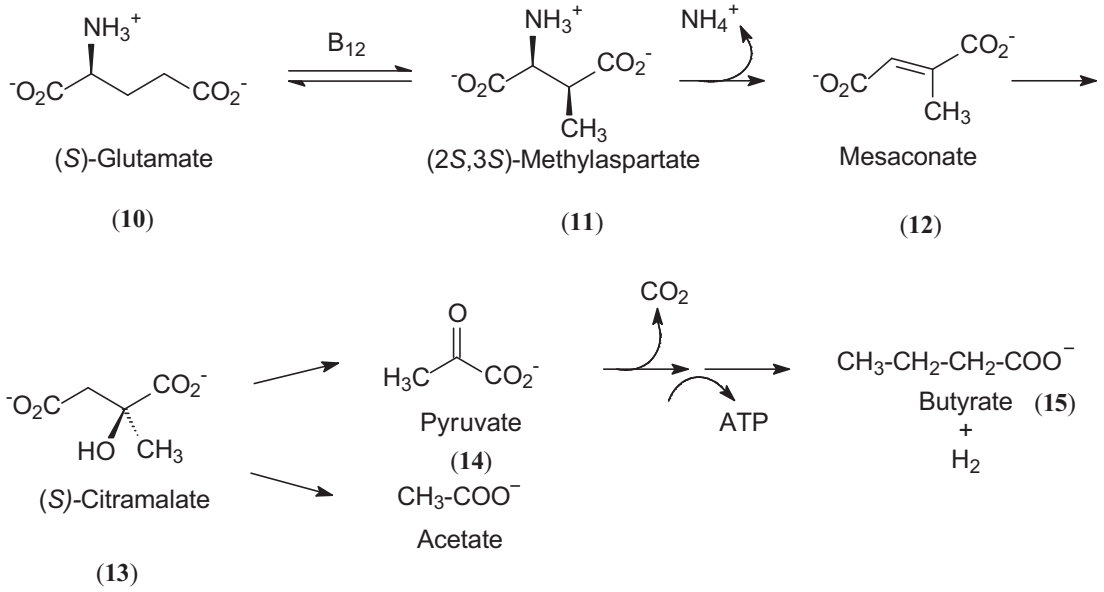


(**10**) (**11**)

Scheme 2: Reaction catalysed by glutamate mutase

Glutamate mutase (EC 5.4.99.1) was first discovered in *Clostridium tetanomorphum* by Barker. [54] It was the first discovered coenzyme B_{12} dependent mutase. More recently, glutamate mutase was purified from the closely related organism *Clostridium cochlearium*. It can be found in various clostridia and many other anaerobic bacteria. In these microorganisms, the mutase is involved in the first step of fermentation of glutamate to ammonia, CO_2, acetate, butyrate and molecular hydrogen. (**Scheme 3**)[55]

Glutamate mutase is the first enzyme in this fermentation pathway, which converts (*S)*-glutamate (**10**) to (*2S,3S)*-methylaspartate (**11**). Then the β-hydrogen becomes acidic enough to enable the elimination of ammonia to yield mesaconate (**12**). The addition of water to the alkene in mesaconate results in (*S)*-citramalate (**13**), which is cleaved to pyruvate (**14**) and acetate by citramalate lyase. Whereas acetate is released, pyruvate is converted to butyrate (**15**) and hydrogen by formation of CO_2 and ATP from ADP.

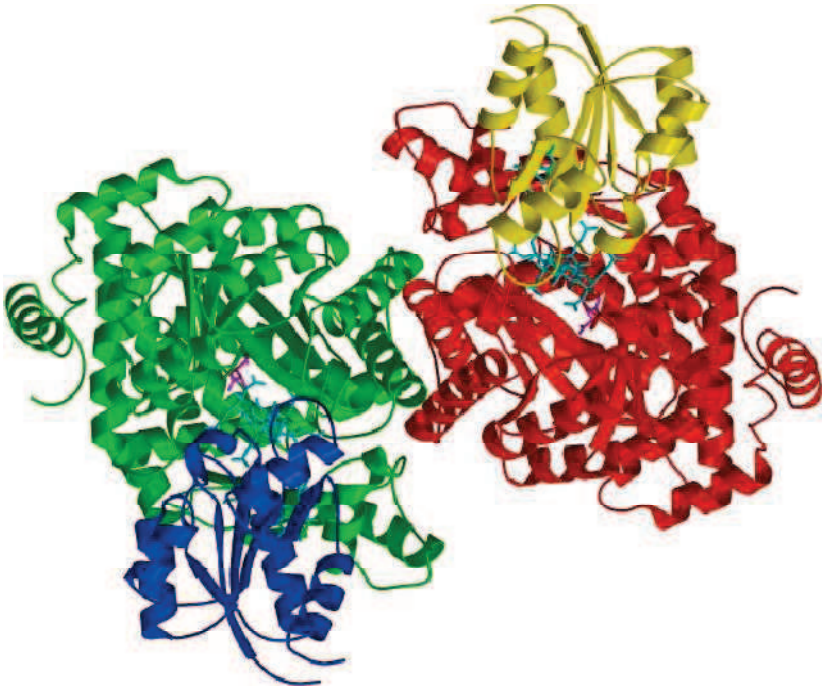


Scheme 3: Fermentation of glutamate *via* 3-methylaspartate to ammonia, carbon dioxide, acetate, butyrate and hydrogen by *C. tetanomorphum* and *C. cochlearium*.[53]

1.6.1 Crystal structure of glutamate mutase

The crystal structure of glutamate mutase from *C. cochlearium* was initially determined by using inactive enzyme reconstituted with cyanocobalamin and methylcobalamin.[56] The enzyme exists in the crystal as a heterotetramer consisting of

two protein components; E, a homodimer (ε_2, 2 × 53.5 Kda) and S, a monomer (σ, 14.8 Kda). Incubation of the purified recombinant components E and 2S with AdoCbl results in the formation of the active enzyme (ε_2, 2σ).[57] The σ subunit acts as the B_{12} binding unit and ε as the catalytic unit. The cofactor is bound at the interface of the two subunits. (**Figure 13**) The σ subunit recognises the upper face of AdoCbl and contains the substrate-binding site, and the ε subunit is a conserved cobalamin-binding domain that interacts with the lower face of the coenzyme, called 'base off' mode.



$\varepsilon_2\sigma_2$ + 2 AdoCbl

Figure 13: Crystal structure of glutamate mutase from *Clostridium Cochlearium*. ε subunits are shown in green and red, σ subunits are shown in blue and yellow. The cofactor and substrate are shown in light blue and purple, respectively.

According to the research of Kratky's group, the substrate was held in the active site by three arginines and one glutamate of the glutamate mutase. (**Figure 14**) Marsh's experiments[58] indicate that the Glu171 acts as a general base which deprotonates the amino group of the substrate during the catalysis. It also shows no effect on the stability of the substrate/product radical themselves. So the active site of glutamate mutase contains the interaction of three arginines (R66, R100 and R149) from the enzyme and two carboxylic acid groups of substrate, the so called 'arginine claw'. The crystal structure also confirmed the distance ca. 6.5 Å derived from the simulation of the EPR spectra of Co(II)alamin and the substrate radical[59](**Figure 15**).

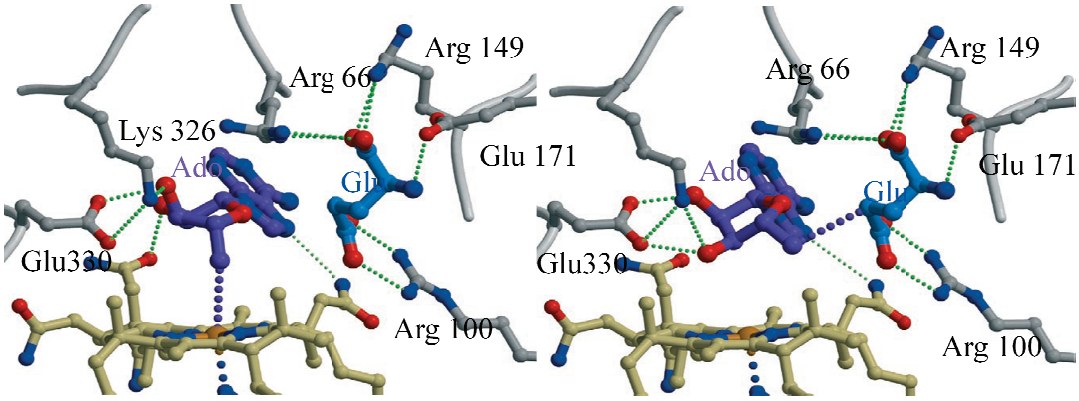


Figure 14: Stereoview of the protein's arginine claw; the illustration alongside shows the adenosyl radical released from coenzyme B_{12} attacking a substrate molecule.[57]

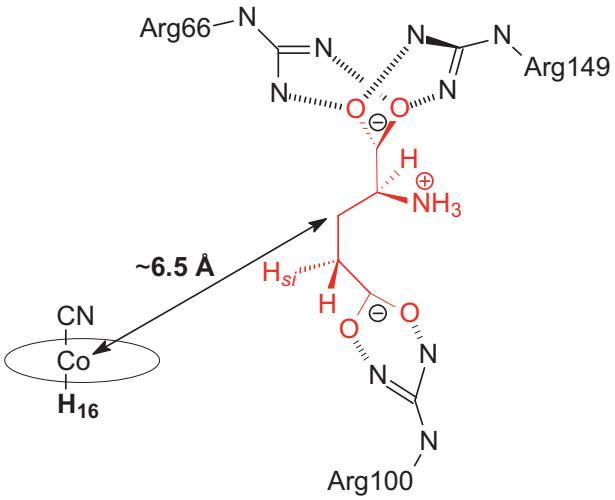


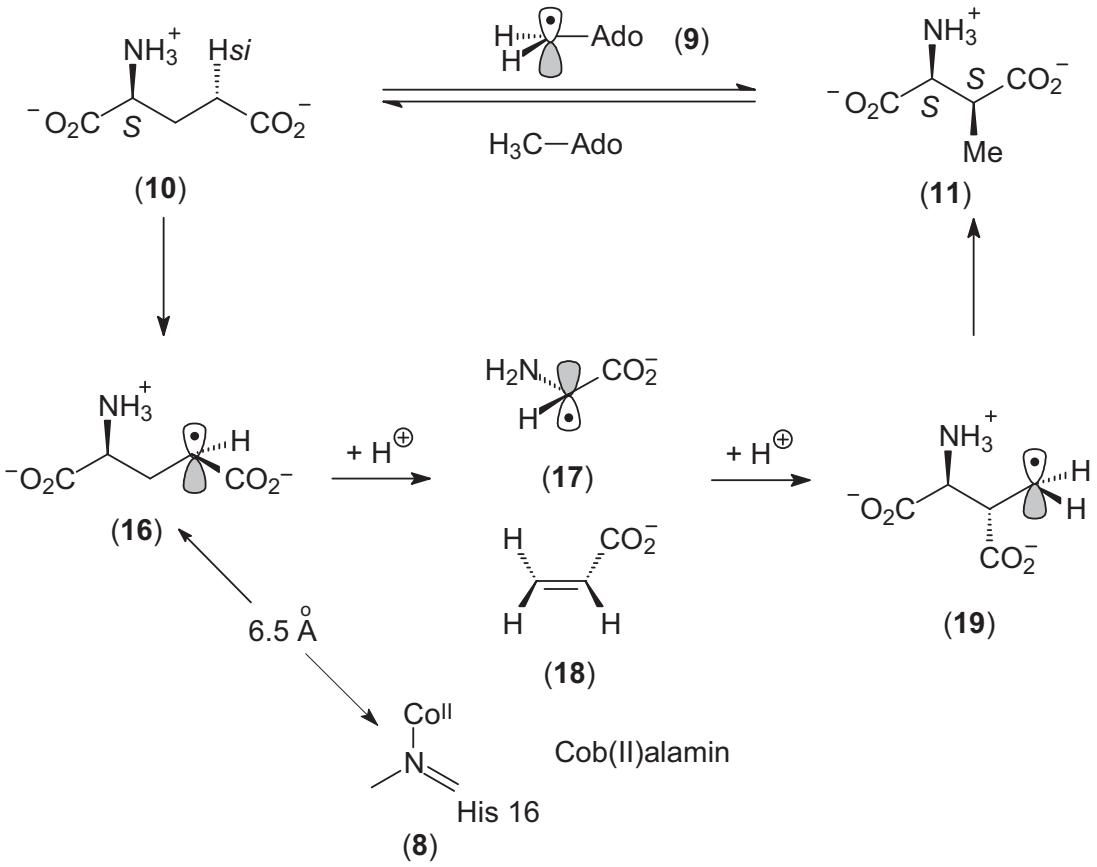
Figure 15: Schematic representation of the active site portion of glutamate mutase with cyanocobalamin as cofactor and glutamate as sustrate showing the 'arginine claw' and the 'base off' mode of the B_{12}.

1.6.2 Mechanism of action of glutamate mutase

According to the generally accepted mechanism, the initial step is the homolysis of the carbon-cobalt bond of the coenzyme B_{12}, due to the enzyme-coenzyme binding the substrate as occurs in all other AdoCbl-dependent enzymatic reactions, by which the 5'-deoxyadenosyl radical (**9**) and cob(II)alamin (**8**) is formed. The 5'-deoxyadenosyl radical abstracts a specific hydrogen atom from a substrate molecule to form a substrate-derived radical, which rearranges to a product-related radical.

A cyclic intramolecular pathway is chemically implausible for this reaction, while a fragmentation/recombination route is feasible.[60] During the catalysis of glutamate mutase the initially formed 4-glutamyl radical (**16**), which was identified by EPR spectra,[59] fragments into acrylate (**18**) and 2-glycinyl radical (**17**), which recombine to 3-methylaspartate radical (**19**). Final redonation of hydrogen from 5'-methyl group of

5'-deoxyadenosine yields the product and recycles the initial radical. (**Scheme 4**) This idea is supported by the synergistic inhibition of this enzyme by glycine and acrylate. Furthermore, the characteristic EPR signal at g = 2.1 is only induced by both glycine and acrylate.[60] Recently, rapid quench flow studies have provided evidence for the formation of a glycyl radical and acrylate at kinetically competent rates, although the amount of these species detected was very low.[61] This experiment supports the 'fragmentation-recombination' mechanism.



Scheme 4: Fragmentation mechanism proposed for glutamate mutase reaction.

1.6.3 Active site and radical rearrangement.

Ab initio molecular orbital calculations predict that the fragmentation barrier for a substrate with neutral substate (carboxylate protonated and amino group unprotonated) is significantly lower than for one in which the glutamyl radical is either deprotonated or protonated.[62] The calculations also indicate that the fragmentation-recombination mechanism is carried out by glutamate mutase controlling the protonation state of the migrating glycine through appropriate proton transfer in the active site, i.e., by (partially) deprotonating the NH_3^+ group and (partially) protonating the COO^- substituent. This proved that the arginine claw (interaction between three arginine residues and the two carboxylic acid groups) and the hydrogen bound between E171 and amino group of the substrate play an important role in the fragmentation-recombination mechanism.

In the proposed mechanism, a key question is how glutamate mutase stabilises the highly reactive radical substrates and intermediates and directs the fragmentation of the substrate radical and the combination of the intermediates towards the product radical. As discussed above, the relative positions of the two carboxylate groups must change during the rearrangement of glutamate to 3-methylaspartate. It is proposed that E171 in the active site of glutamate mutase is a general base which deprotonates the amino group to stabilise the radical intermediate, and the arginine claw adjusts the structural modification of substrates. Deprotonation of the amino group by E171 is expected to facilitate the formation of glycine radical intermediate during the proposed mechanism of rearrangement.[58] Protonation of the amino group might be

expected to initiate the homolysis of the Co-C bond and the formation of glutamyl radical.

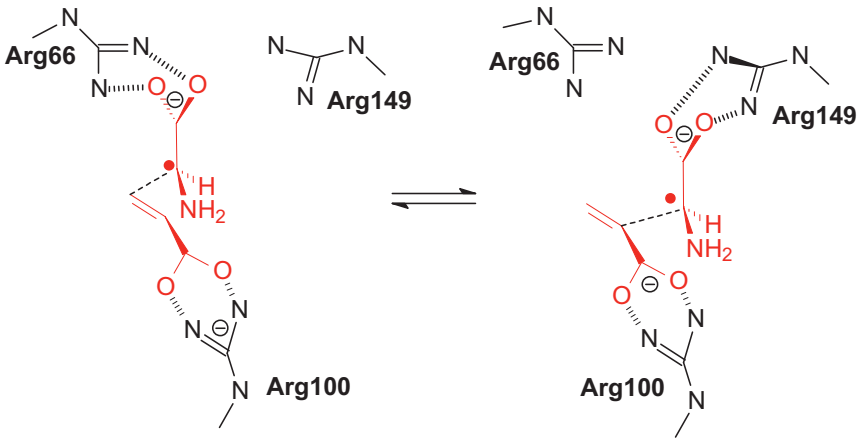


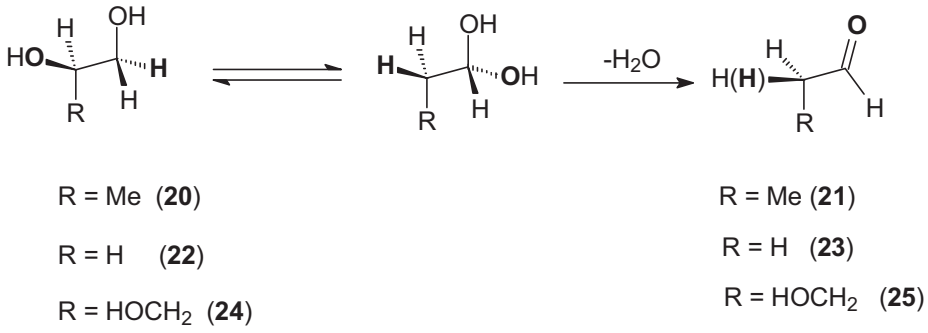
Figure 16: Using the 'arginine claw' to 'handover' glycyl radical from C-3 to C-2 of acrylate.

One arginine (R100) in the active site anchors the C-5 carboxylate of glutamate (or C-4 carboxylate of 3-methylaspartate) to hold the acrylate intermediate. The handover of migrating glycine radical by the other two arginines (R66 and R149) enables the shift of glycinyl moiety from C-3 to C-2 of the acrylate.(**Figure 16**) After formation of the 3-methylaspartyl radical, protonation of the amino group by E171 stabilises the product radical. The arginine claw holds the product radical to abstract a hydrogen atom from 5'-deoxyadenosine intermediate.

1.7 GLYCEROL AND DIOL DEHYDRATASE

Diol dehydratase and glycerol dehydratase have been identified as exclusively coenzyme B_{12} dependent enzymes until recently. The only exception for diol dehydratase has not been well characterised. EPR study of the B_{12} independent diol dehydratase from membranes of *Clostridium glycolicum* with propanediol indicated that a stable organic radical formed.[63] Raynaud *et al.*[64] reported the molecular characterisation of B_{12}-independent glycerol dehydratase from *Clostridium butyricum*. It is a glycyl radical enzyme activated by *S*-adenosylmethionine (SAM). The B_{12}-independent glycerol and diol dehydratase will not be discussed any further in this work.

The coenzyme B_{12}-dependent glycerol and diol dehydratases are involved in the anaerobic utilisation of small molecules of 1,2-diol (**Scheme 5**), e.g., propane-1,2-diol (**20**) and glycerol (**24**). They catalyse the intermolecular rearrangements to generate aldehyde in the first step of the fermention of small molecules. 3-Hydroxypropionaldehyde (**25**) can be reduced to 1,3-propanediol by consuming 50-66 % of glycerol (**24**) into acetate, ethanol, lactate, H_2 and CO_2, which is the major product in glycerol fermentation pathways.[65] In propane-1,2-diol (**20**) fermentation pathways, the propionaldehyde (**21**) is converted to equal amounts of propanol and propionic acid. The propanol was oxidised to propionic acid eventually. The propionic acid was degraded in to propionate and CO_2.[66] In the bacterial strains which lack of glycerol dehydratase, diol dehydratase instead of glycerol dehydratase is involved in the conversion of 3-hydroxypropionaldehyde (**25**).



Scheme 5: Reaction catalysed by diol dehydratase and glycerol dehydratase

Diol dehydratase (DL-1,2-propanediol hydro-lyase, EC 4.2.1.28) and glycerol dehydratase (glycerol hydro-lyase, EC 4.2.1.30) are delicately different enzymes. Diol dehydratase catalyses the conversion of 1,2-ethanediol (**22**), 1,2-propanediol (**20**), and glycerol (**24**) to acetaldehyde (**23**), propionaldehyde (**21**), and 3-hydroxypropionaldehyde (**25**) respectively.[67] Glycerol dehydratase (glycerol hydro-lyase, EC.4.2.1.30) catalyses the conversion of 1,2-propanediol (**20**) and glycerol (**24**) to propionaldehyde (**21**) and 3-hydroxypropionaldehyde (**25**), respectively.[68] Study also showed that the diol dehydratase "prefers" 1,2-propanediol whereas glycerol dehydratase "prefers" glycerol. 2,3-butanediol can be able to converted into methyl ethyl ketone by diol dehydratase. (**Scheme 5**)

Diol dehydratase was originally discovered in *Klebsiella pneumonia* (ATCC 8724, formerly known as *Aerobacter aerogenes*), which can be formed in, for example, *Klebsiella oxytoca*[69] and *Salmonella typhimurium*[70], while glycerol dehydratase can be detected in the cells of , for example, *Clostridium pasteurianum,*[71] *Cl. Butyricum*[72] and *Citrobacter freundii*[68].

1.7.1 Crystal structure of diol dehydratase

The genes coding for diol dehydratase of *Klebsiella oxytoca* have been expressed in *Escherichia coli*[73] and purified to homogeneity[69]. The X-ray crystallographic structure is available in protein data bank. The enzyme exists as a dimer of a heterotrimer comprising the α, β, and γ subunits, $(\alpha\beta\gamma)_2$. (**Figure 17**) β and γ subunits are bounded to α unit. The interaction between the two α units contributes to the dimerisation of the heterotrimers, αβγ. Glycerol dehydratase from *C. freundii* and *K. pneumonia* also consists of composition $(\alpha\beta\gamma)_2$, which shows 71% (α), 58% (β), and 54% (γ) sequence identity to the diol dehydratase of *Klebsiella oxytoca*.

Each molecule of the coenzyme B_{12}-dependent diol dehydratase contains two molecules of the coenzyme B_{12} with its 5,6-dimethylbenzimidazole coordinating to the cobalt atom, so call 'base-on' mode. This was the first crystal structure that indicated the 'base-on' mode of cobalamin binding.[74] In contrast, the cobalamin binding shows the 'base off/His on' mode in methionine synthase (**Figure 8**) and glutamate mutase, that is, 5,6-dimethylbenzimidazole ligand is replaced by a histidine residue from the enzymes. The cobalamin is bound between α and β subunits, and the substrate is deeply buried in α subunit. The enzyme also requires a certain monovalent metal cation K^+ for its activity,[75, 76] which is also bound in α subunit. The two hydroxyl groups of the substrate coordinate directly to the metal cation. (**Figure 18**) The γ subunit is located far from the cofactor and substrate and in full contact with α subunits. The role of γ subunit seems to support α subunit and maintain the overall structure. In 2009, it was reported that the metal cation that is required for the activity

of diol dehydratase is Ca^{2+} rather than K^{+}.[77] There are not any experimental results supporting this yet. So we still assume the metal cation is K^{+} in this thesis.

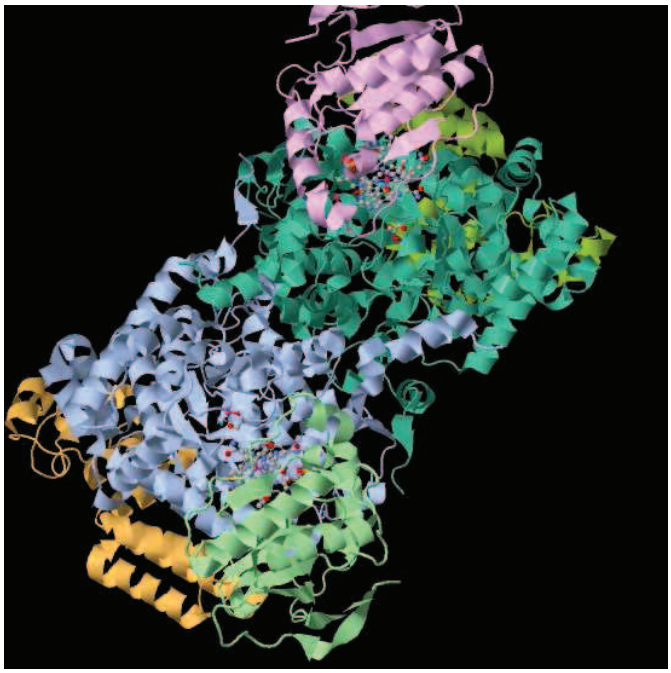


Figure 17: Overall structure of diol dehydratase complexed with (*S*)-1,2-propanediol.(PDB ID: 1UC4)

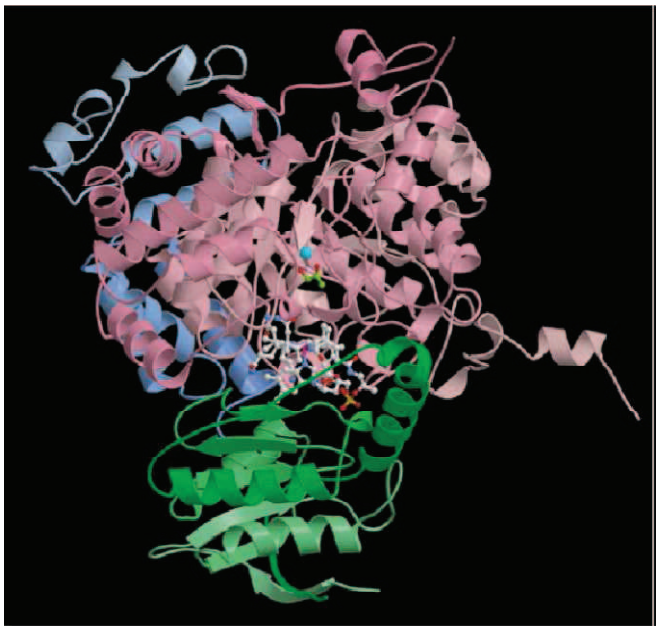


Figure 18: Structure of the heterotrimer αβγ unit-cyanocobalamin complex. Pink, green, and blue colors indicate α, β, and γ subunits repectively. Blue ball indicates the metal ion, K^{+}. Green and red molecular is for propane diol. (Adapted from Ref [78])

1.7.2 Active sites of diol dehydratase

The active site is located in the C-terminal side of a $(\beta/\alpha)_8$ barrel-like structure so called TIM (triose phosphate isomerase) barrel of the α subunit above the corrin ring of the cobalamin.[74] The substrate propane-1,2-diol and the cofactor K^+ are bound inside the barrel. K^+ is heptacoordinated by the two hydroxyls of the substrate and the five oxygen atoms from the five active-site residues. (**Figure 19**) This structure indicates that the K^+ ion and the active-site residues might stabilise the transition state during the hydroxyl group migration from C2 to C1. Due to the negative charge of the active site, the K^+ ion plays a key role in binding substrate but stabilises the transition state rather slightly.

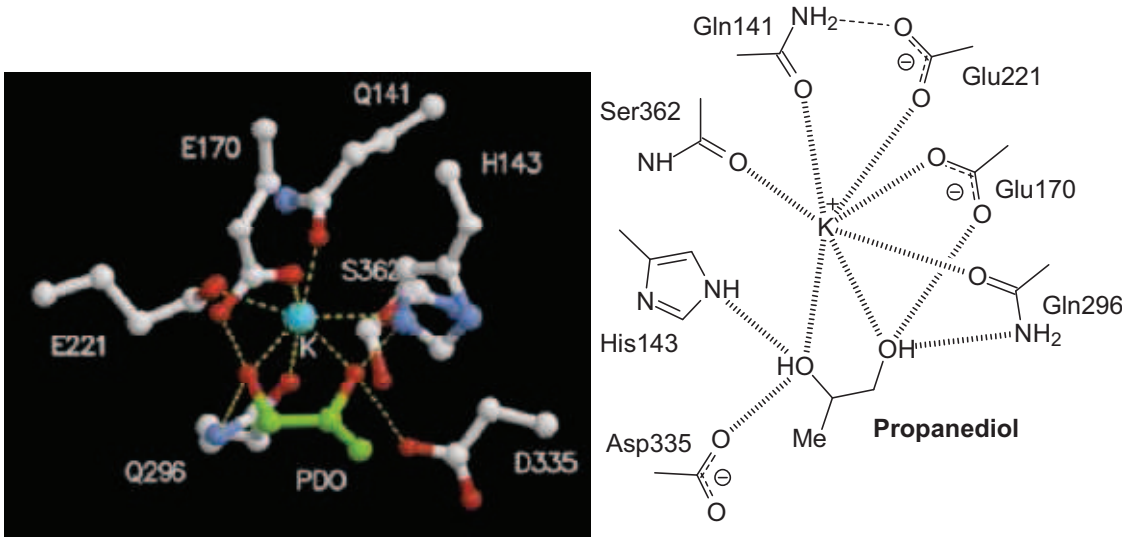


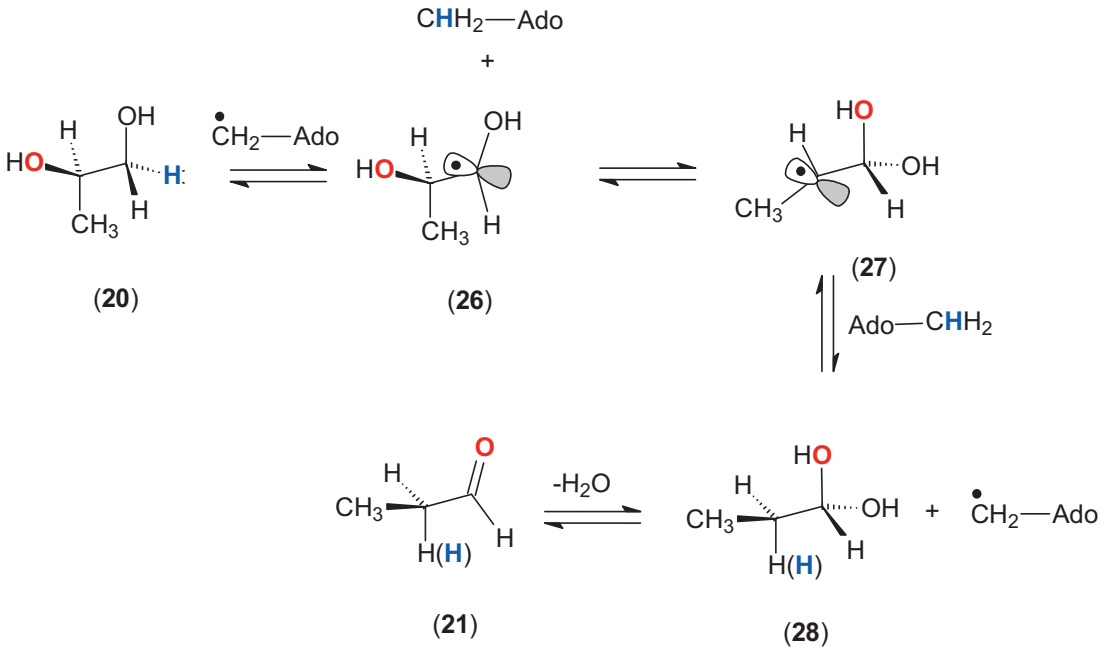
Figure 19: Structure of the diol dehydratase binding site.

This is proved by the theoretical calculations with a simple model of diol dehydratase.[79] The long distance between the cobalt atom and C1 (8.37 Å) and C2 (9.03 Å) of the substrate indicates that the cobalt atom of the cobalamin acts as a 'spectator'[80].

A mutation study of the residues in the active site of the enzyme suggested that E221, E170 and D335 are very important for the substrate binding. The enzyme/substrate complex was not formed without them. H170, H143 and D335 play a key role in the catalysis as they have hydrogen bonds with hydroxyl groups in the substrate. The roles of H170 and D335 are to keep substrate and intermediates appropriately oriented and to proved acid-base catalysis in the migration of the hydroxyl group of the intermediate. H170 also plays a role of stabilizing the transition state for migration of hydroxyl group from C2 to C1 by accepting the proton from the spectator hydroxyl group on C1. It is also suggested that Q296 and Q141 are important for the substrate binding and the roles of Q296 and H143 are to prevent the enzyme from mechanism-based in activation.[81]

1.7.3 Stereochemistry and mechanism of action of diol dehydratase

Like all the AdoCbl dependent enzymatic reactions, the 5'-deoxyadenosyl radical is generated by the homolysis of the coenzyme's Co-C σ-bond. The substrate radical (**26**) is formed *via* a hydrogen abstraction where the 5'-deoxyadenosyl radical abstracts a specific H atom from C-1 of diol substrate (**20**). Then the substrate radical (**26**) rearranges to a product derived radical (**27**) by 1,2-shift of hydroxyl from C-2 to C-1. A product intermediate (1,1-diol, **28**) is produced by 5'-deoxyadenosine giving up its hydrogen atom which is abstracted from C-1 of the substrate. The complete reaction pathway with propane-1,2-diol (**20**) as substrate is illustrated in **Scheme 6.**



Scheme 6: Diol dehydratase reaction minimal mechanism pathway from (*R*)-propane-1,2-diol to propanal showing proposed intermediate radicals and the fate of isotopic labels (CH_2-Ado = adenosyl; Red O = ^{18}O; Blue H = ^{2}H or ^{3}H)

As noted in section 1.5, the use of substrates or coenzyme B_{12} specifically labeled with deuterium or tritium demonstrated that the 1,2-shift of one C-1 hydrogen in the propane-1,2-diol rearrangement was not only intramolecular but also intermolecular, without exchange with solvent hydrogen. AdoCbl functions as the hydrogen carrier through the formation of the methyl group in 5'-deoxyadenosine.

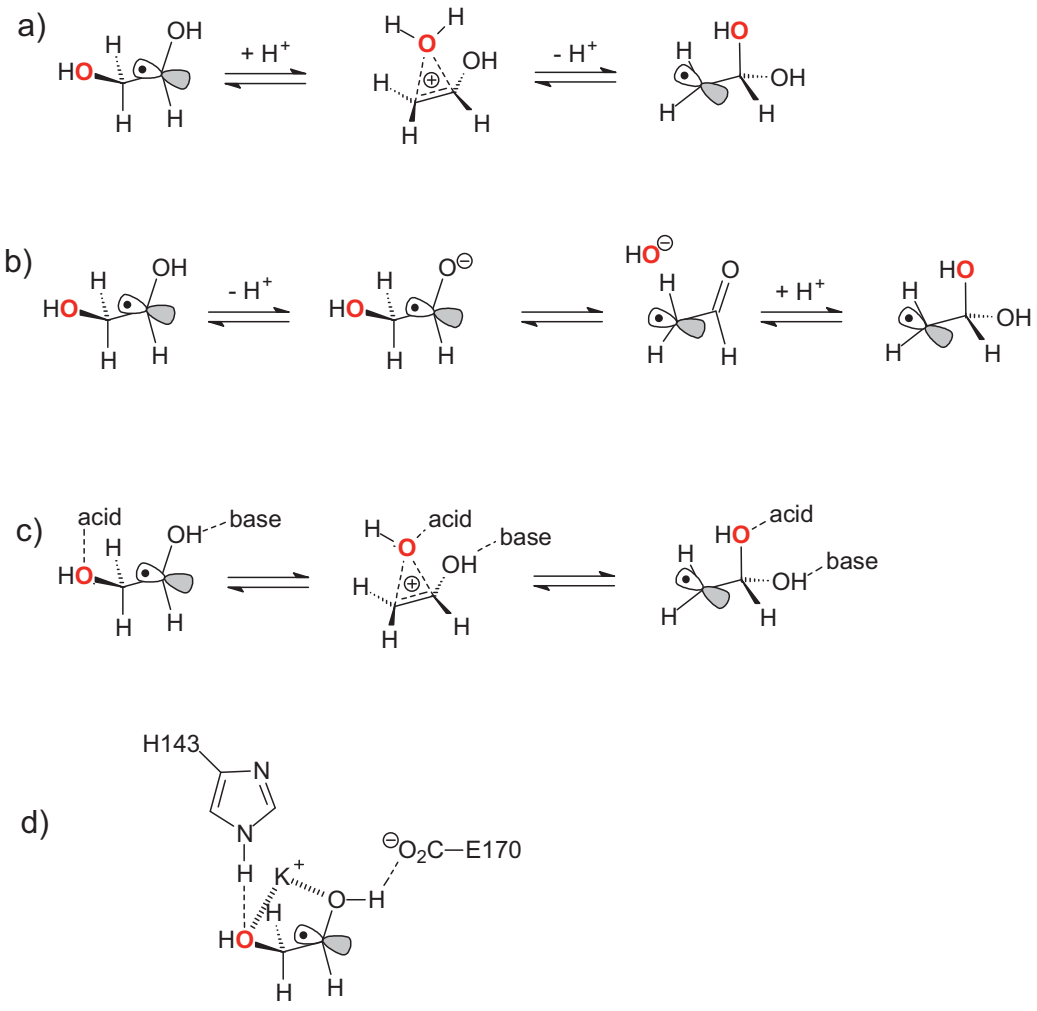
Retey and co-workers[82] demonstrated that the migrating hydroxyl group and the migrating hydrogen atom is stereospecific and that the dehydration of gem-diol is controlled sterically by diol dehydratase, with only one of the two hydroxyl groups on the being eliminated. Using [1-18O] 1,2-propanediol as substrate, [18O] and

unlabeledd propionaldehydes are formed from (*S*)- and (*R*)-1,2-propanediol, respectively. (**Scheme 6**)

The mechanism of the hydroxyl group migration from C2 to C1 still remains unclear. **Scheme 7** has shown several proposed mechanisms. All pathways lead to an intermediate, 2,2-dihydroxyethyl radical, that retrieves a hydrogen atom from 5'-deoxyadenosine and the resulting 2,2- diol dehydrates to give product aldehyde.

Acid catalysed migration of the 1-hydroxyl group in the initially formed substrate-derived radical occurs via a bridged transition state. (**Scheme 7a**)This pathway firstly was suggested by Golding *et.al.*[80] and it is supported by *ab initio* molecular orbital calculations, which showed that the bridged transition state lies only 7.5 kJ mol^{-1} above the substrate radical and 2.7 kJ mol^{-1} above the product radical. Since the dramatic change in pK_a during the proposed conversion of the alcohol (pK_a ~ 16) into a 1-hydroxyalkyl ('ketyl') radical (pK_a ~ 9), the ketyl radical could be formed as intermediate by enzyme mediated protonation.[43] Then the resulting α-formyl radical is converted to the product-related radical (e.g. 2,2-dihydroxyethyl radical) by capturing the hydroxide (or water molecule) (**Scheme 7b**) The product-related radical rather than α-formyl radical will abstract a hydrogen atom from 5'-deoxyladenosine because of the resonance stabilisation of the former one. Furthermore, 'push-pull' mechanism (**Scheme 71c** and **7d**)[83] has been expected to be the favor pathway for this conversion because it is consistent with the X-ray crystal structure in above section. One possibility for this mechanism is the hydroxyl group migration from C2 to C1 through a cyclic transition state where the energy of transition state is reduced by an acidic and basic catalyst and a mediator, K^+. Golding and Radom[84] have demonstrated

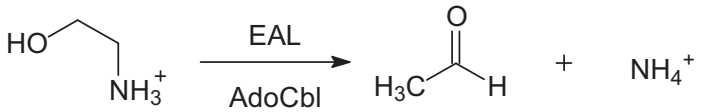
by calculation that the activation energy for the hydroxyl group migration is lowered by partial protonation of the migrating hydroxyl. The other possibility is a stepwise hydroxyl group abstraction/readdition pathway through a radical anion and a ketyl radical. Under this pathway with mediator K^+, enzyme residue E170 acts as a base, which will assist deprotonation of the α-hydroxyl group; H143 acts as an acid which will assist the abstraction and addition of the β-hydroxyl group.



Scheme 7: Proposed mechanisms for diol dehydratase hydroxyl shift, ethane-1,2-diol as substrate: **a)** acid catalysed; **b)** base-catalysed ('ketyl' mechanism); **c)** 'push-pull' mechanism with an acidic and basic catalyst; **d)** 'push-pull' mechanism showing Glu170 acting as a base, His143 acting as an acid and K^+ as a mediator.[85]

1.8 ETHANOLAMINE AMMONIA-LYASE

Ethanolamine ammonia lyase (EC 4.3.1.7, EAL and also known as ethanolamine deaminase) is an adenosyl-cobalamin dependent enzyme that catalyses the conversion of ethanolamine (**29**) to acetaldehyde (**23**) and ammonia (**30**) (**Scheme 8**), and it also catalyses the conversion of other short chain, vicinal amino alcohols to oxo products and ammonia.[86] The aldehyde is proposed to dismutate to acetate and ethanol in the microorganisms.



(**29**) (**23**) (**30**)

Scheme 8: Reaction catalysed by ethanolamine ammonia lyase

The enzyme was first discovered in *Clostridium sp*. by Bradbeer.[87] Since then a few bacterial species, including *Klebsiella aerogenes*,[88] *Escherichia coli*,[last] *Salmonella typhimurium*, [89] *Bacillus megaterium*, [90] and *Enterobacter aerogenes* have been shown to produce EAL when grown on ethanolamine as a nitrogen source.

1.8.1 Structure of ethanolamine ammonia lyase (EAL)

The sequences of EAL from *Salmonella typhimurium,*[91] *E.coli*[92] and *Clostridia sp.*[93] are known. They are composed of two different subunits, large (α) and small (β).[94] The α and β subunits of EAL are encoded by *eut*B and *eut*C genes, respectively.[95] The α subunit roughly is a 453-residue, 50 kDa protein and the β subunit is a 286-residue, 32 kDa protein. Six copies of each subunit are thought to assemble into the $\alpha_6\beta_6$ oligomeric protein, EAL. In active-site titration with adeninylpentylcobalamin, complete inhibition of 1 equivalent of EAL requires 6 equivalents of this relative strong inhibitor.[96] This indicates that six active sites are in one enzyme molecule. Both types of subunits are required for enzyme activity, although *eut*B contains the active site. The full crystal structure of EAL has not been elucidated yet. The structure of *eut*B(α_6) from *Listeria monocytogenes* has been deposited in the Protein Data Bank (**Figure 20**).

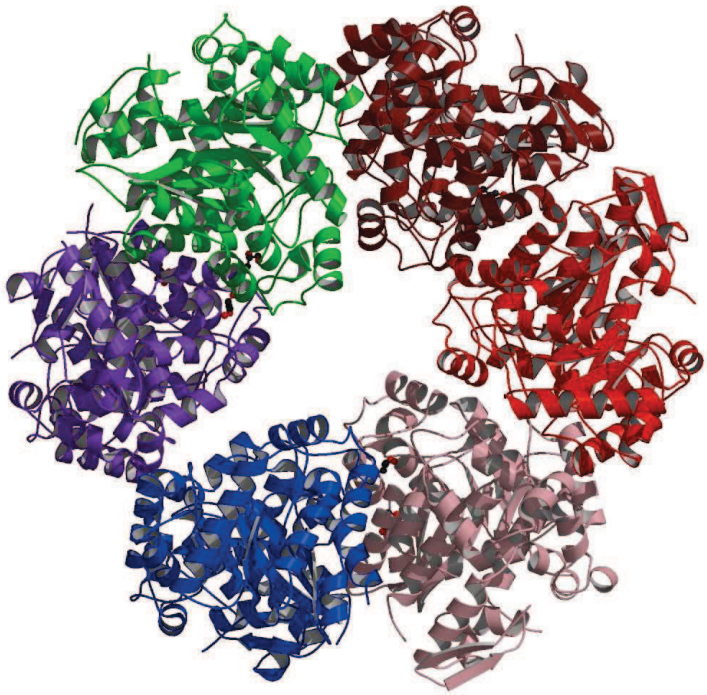
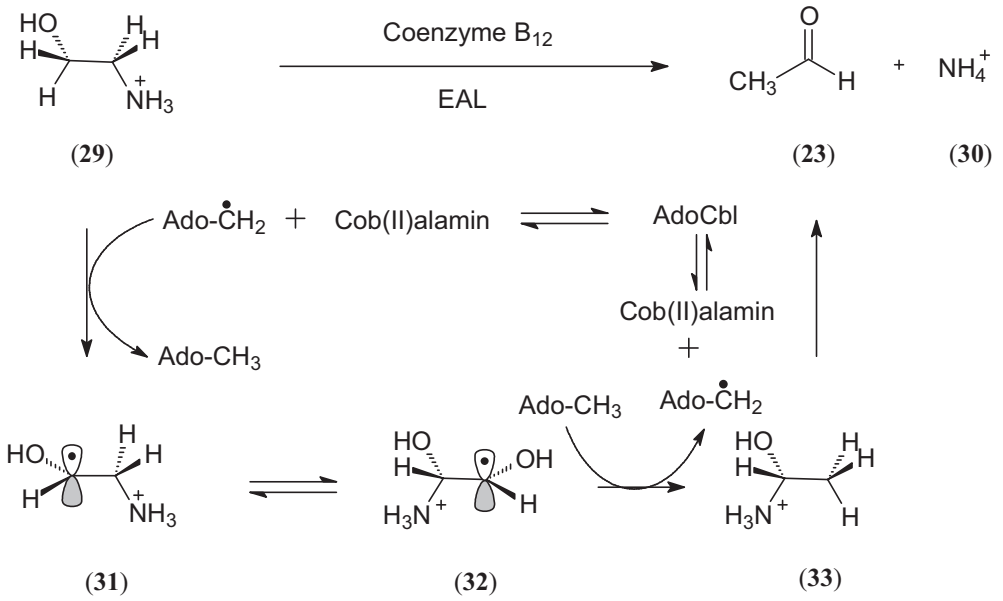


Figure 20: Crystal structure of EAL *eut*B subunit at 2.15 Å resolution (PDB ID: 2qez)

1.8.2 Mechanism of action of EAL

The formation of cob(II)alamin and an organic radical intermediate during the catalysis of EAL was observed by optical and electron paramagnetic resonace (EPR) spectroscopies. This result indicates that the reaction proceeds by a radical mechanism. The formation of an organic radical, 2-aminopropanol-1-yl radical (**32**), was identified in the EPR experiment of isotopically labeled 2-aminopropanol (**29**) in addition to the formation of cob(II)alamin radical. The radical rearrangement step therefore proceeds by the amine migration pathway, in which the amine nitrogen migrates from C2 to C1 to form 2-aminoproanol-1-yl radical (**32**).



Scheme 9: Catalytic cycle of EAL catalysed reaction.

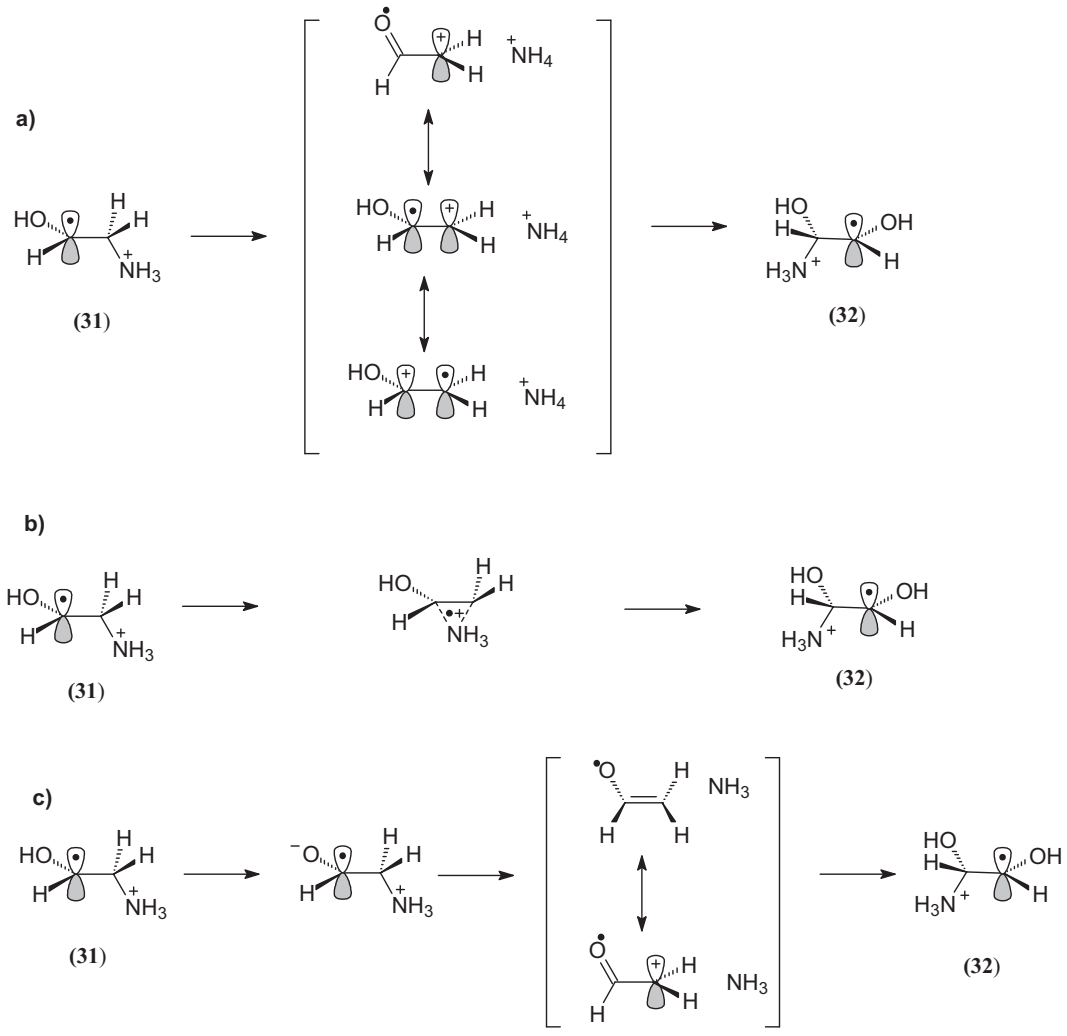
Therefore, the reaction catalysed by ethanolamine ammonia-lyase takes place with the transfer of hydrogen from the carbinol carbon (C1) to the amino carbon (C2) without

exchange with water and this intermediate subsequently undergoes a deamination of the carbinolamine to give the product. (**Scheme 9**)

The proposed catalytic cycle (**Scheme 9**) starts with formation of a highly reactive 5'-deoxyadenosyl radical and cob(II)alamin after homolysis of the cobalt-carbon bond of coenzyme B_{12}. A hydrogen abstraction by the 5'-deoxyadenosyl radical gives the substrate-derived radical (**31**) and 5'-deoxyadenosine. This substrate-derived radical (**31**) rearranges to the intermediate radical (**32**) with the unpaired electron spin density localised on C2. Subsequent hydrogen atom transfer from the C5 methyl group of 5'-deoxyadenosine to C2 of the product radical (**32**) yields a 1-aminoethanol (**33**). This isomerisation is followed by an elimination of ammonia (**30**) that leads to acetaldehyde (**23**). The 5-deoxyadenosyl radical and cob(II)alamin may then recombine.

EAL is therefore an 'eliminase' like diol dehydratase and there are mechanistic parallels. The 3D structure is awaited for understanding the refined mechanism of action of this enzyme on a molecular level. Both hydrogen transfers and EPR observations indicates the carbinolamine radical in the catalysis. Several mechanisms have been proposed for the rearrangement of the substrate-derived radical to the carbinolamine radical. (**Scheme 10**) Each of the rearrangements involves a slightly different transition intermediate. Pathway **a**) and **c**) are amine elimination/readdition, pathway **b**) is amine migration. Formation of a radical cation proceeds via protonation of the substrate derived radical and elimination of ammonium. The cation and ammonium are stabilised by resonance forms. The carbinolamine radical is formed by deprotonation and addition of the ammonium to C1. (**Scheme 10a**) Intramolecular

rearrangement involves a bridged transition state of the amino group. (**Scheme 10b**) Conversion of the substrate-derived radical to a ketyl radical is driven by the pK_a change of deprotonation of the hydroxyl group. The radical is stabilised with resonance forms. The enzyme-mediated protonation and readdition of ammonium produces the product radical. **(Scheme 10c**)



Scheme 10: Proposed mechanism for the rearrangement of EAL catalysed reaction: a) Radical cation mediated rearrangement; b) intramolecular rearrangement; c) ketyl radical rearrangement.[86]

Chapter 2

Towards Models for Coenzyme B_{12}-dependent Glutamate Mutase

2.1 MOLECULAR RECOGNITION AND ENZYME MODEL

Host guest chemistry describes the complexation of a smaller organic molecule (guest) inside a 'pocket' of a larger organic molecule (host).[97] It covers the idea of molecular recognition and interactions of host/guest through noncovalent bonds. In 1894 Fischer[98] put forward the first fundamental theory of molecular recognition, the "lock and key" model. It explained the geometric features required for the enzyme catalysis. Jorgensen[99] has presented many examples where the conformations of enzyme and ligand change upon ligand binding according to rigid lock and key complementarity. The concept of molecular recognition is the foundation for antibody activity,[100, 101] enzyme catalysis,[97, 102] drug discovery[103, 104] and catalyst development. [105]

Molecular recognition is normally achieved through the reversible formation of a complex between the receptor (the host) and the substrate (the guest). Receptors are capable of recognising specific molecules (substrates) with a stereospecific mode. In enzymatic reactions, molecular recognition is the first step of catalysis. In the following stages, the transformation of substrates occurs under the regulation of enzyme, where enzymes maybe stabilise the reaction intermediates and certainly reduce the transition state energies, followed by release of substrate.[106]

Enzymes outperform the chemist in reactions under mild conditions not only in terms of rate enhancement, but also in the selectivity of substrates and reaction pathways. These outperforming properties are the result of the unique binding selectivity at the active site and the stabilisation of the unique transition state. It is essential for the

elucidation of enzymatic reaction mechanisms to understand the enzyme-substrate interactions and how enzymes catalyse the reaction.

Enzyme mimics have been a long-term high-profile target for chemists and biochemists to define the active sites of enzymes, to elucidate the mechanisms of enzymatic reactions and to reproduce the enzyme's function. Kirby[107] has catalogued three different approaches to model enzymatic reactions and the binding of receptor and substrate.

- Enzyme-based mimics.
- Mechanism-based mimics.
- Binding-step based mimics.

The 'enzyme-based mimics' are built by modifying a natural enzyme chemically by protein engineering. This method is to modify enzymes rather than mimicking them. The 'mechanism-based mimics' are achieved by making reactions intramolecular, that is, bringing the functional groups and the reaction intermediates on the same molecule. Finally, the 'binding-step based mimics' are to gather synthetic designed hosts which bring reactants into productive proximity and orientation. Most catalytic antibodies belong to this type of mimic.

Although it is possible to mimic the general binding properties and reactivity of some enzymes, it is very difficult to mimic the selectivity and rate enhancements of their chemical transformations. The fundamental concept of this chapter is building a model scaffold for mimicking the active site of enzyme (i.e. glutamate mutase, proteases and aldolase). This concept is illustrated in **Figure 21**.

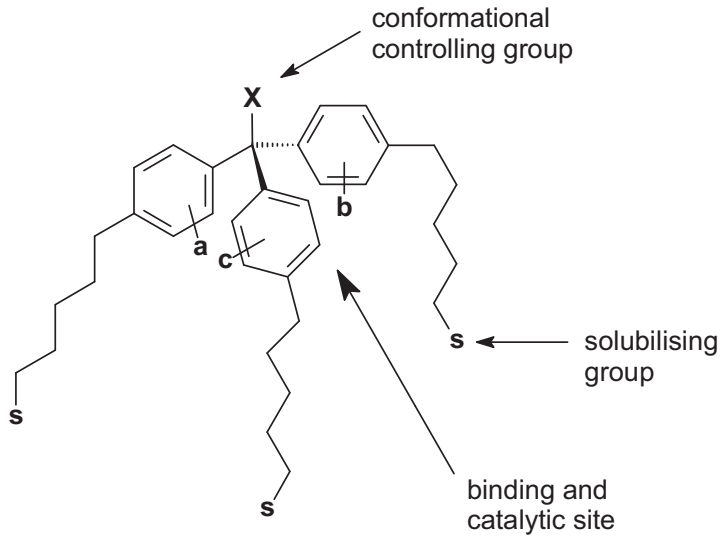


Figure 21: Structural concept of enzyme model mimicking the active site, so called advanced trityl receptor, or tritylases model. [a, b and c are typical amino acid side-chain groups (e.g. guanidinium, amino, carboxy, etc.); X is a conformational controlling group and s are solubilising group][108]

The structural concept is achieved by adopting the recognition principle of many biogenic hosts, i.e. interaction between the guanidinium function of arginyl side chains and carboxylate groups within the glutamate mutase reaction complex. The model is built by attaching appropriate functional groups to a molecular scaffold to provide the optimal constellation of functionalities for enhancement of a specific reaction. The concept of model should fulfill the following criteria:

A- The model compound should be a conformationally flexible bicyclic or tricyclic molecule that behaves in a limited but potentially advangeous way to give a flexible cavity size.

B- The host/guest complexation should lead to a defined structure for the complexation as shown in the natural prototypes.

C- The orientation and spacing of the guest should be defined within narrow limits by the host.

Therefore the study will be made of modelling tritylases with functional groups (e.g. NH_2, imidazole, CO_2H and combinations of these) capable of mimicking selected enzymes (e.g. glutamate mutase, proteases, and aldolases). Beyond their use as enzyme models tritylases have potential as new therapeutic agents and as analytical tools.

In enzymes of immediate interest (e.g. glutamate mutase), the guanidinium groups of arginines in the active site maintain the protein tertiary structure *via* internal 'salt bridges' with carboxylate groups of substrate (e.g. glutamate residues), as well as participating in binding and recognition of anionic substrates (by enzymes, receptor sites and antibodies). The binding patterns of guanidinium groups with oxoanion groups feature two parallel hydrogen bonds in addition to the electrostatic attraction. (**Figure 22**)

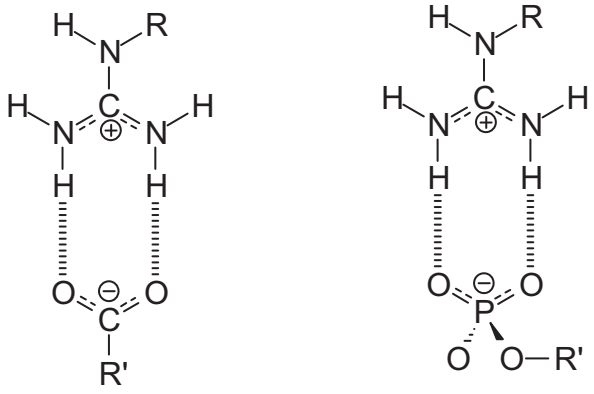


Figure 22: Binding pattern of guanidinium groups with a carboxylate and phosphate found in many X-ray structures of complexes.[109]

Another feature of making the guanidinium an attractive anchor group in receptor systems is its high basicity. Guanidine has a pK_a of 13.5 in water which ensures protonation over a wide range of pH. In arginine residues, the pK_a of guanidinium is attenuated to 12.5. But it varies depending on its neighbour group effects. There is a large amount of literature about guanidinium receptors and their binding properties.[108, 110]

2.2 MODELLING THE ACTIVE SITE OF GLUTAMATE MUTASE

Coenzyme-B_{12} dependent glutamate mutase is a radical enzyme (See section 1.6), which catalyses the isomerisation of (*S*)-glutamate to (*2S*,*3S*)-3-methylaspartate *via* enzyme-bound radical intermediates. With the recent elucidation of the crystal structure of glutamate mutase,[56, 57] it was confirmed that arginine residues play a critical role in the binding to the carboxylate groups of both substrate and product within the active site of enzyme.

In order to simulate the 'arginine claw'[111] of glutamate mutase, molecules coined 'tritylases' have been previously synthesised. These molecules have an enzyme-like cavity into which small molecules can bind and, in principle, undergo reactions catalysed by specifically placed functional groups. The synthesis of the *m,m,p*- and *m,p,p*-tritylase (**Figure 23**) was completed by D. Suarez.[108] In her research, these molecules were shown to bind strongly certain dicarboxylates (e.g. glutamate, glutarate, succinate) and also citrate (e.g. $K_d \sim 10^6$ mol^{-1} dm^3). Computational

modelling indicated a mode of binding for glutamate similar to that observed in glutamate mutase.

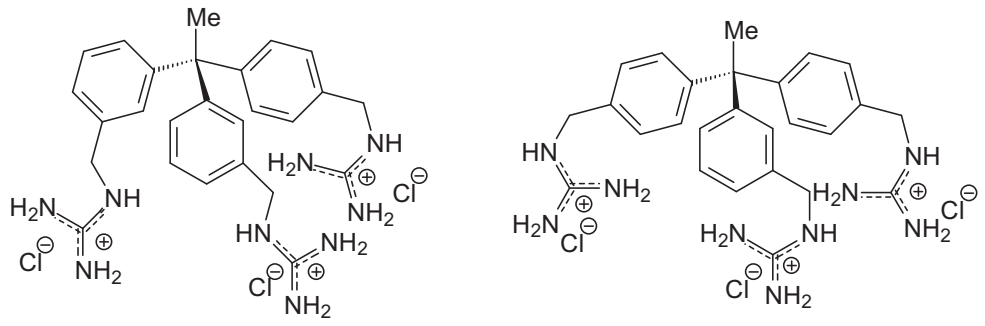


Figure 23: Tritylase model compounds with (meta, meta, para) (**34**) and (meta, para, para) (**35**) arrangement of guanidinium groups

The purpose of introducing the tritalyse and biphenyl model receptor is to probe the role that guanidinium groups of arginyl residues play in glutamate mutase and compare these roles with those in the synthetic systems. In the model system, the binding of a dicarboxylate requires a minimum of two guanidinium groups. In order to learn about the binding function of the guanidinium group, the biphenyl model receptor was introduced because of its relative ease of preparation and flexibility of conformation to bind with designed dicarboxylate compounds.[112]

In a previous study, the biphenyl model receptor (**36**) showed a significant selectivity in binding to the dicarboxylate guests (i.e. glutaric acid and adiptic acid) in CD_3OD with 1 : 1 complexation.[112]

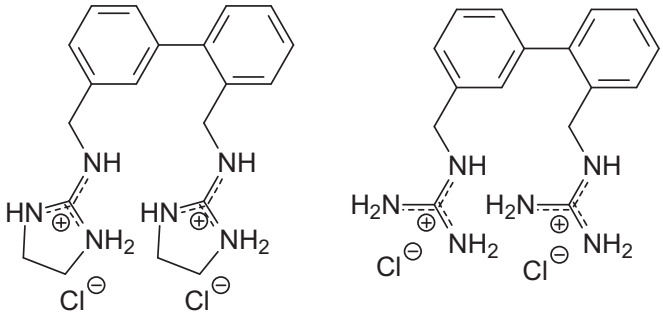


Figure 24: Biphenyl model receptors showing two different types of guanidinium group.

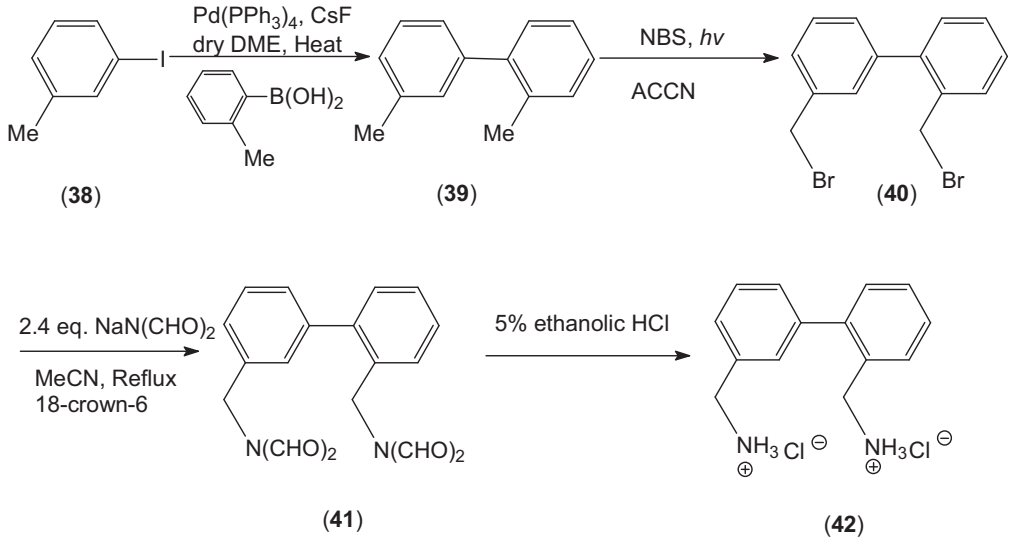
In this work, species (**36**) contains non-natural guanidinium functions was synthesised and recrystallisations of (**36**) from several solvents were attempted while species (**37**) containing natural guanidinium functions was synthesised and its binding properties were investigated by NMR titration.

2.3 SYNTHESIS OF MODIFIED BIPHENYL RECEPTOR, 2,3-BIS-(AMINOMETHYL)-BIPHENYL DIHYDROCHLORIDE

2,3-Bis-[(4, 5-dihydro-*1H*-imidazol-2-yl)-aminomethyl]-biphenyl dihydrochloride **(36)** and 2,3-bis-guanidinomethyl-biphenyl dihydrochloride **(37)** were synthesised successfully. Both compounds were synthesised from the same starting compound 2,3-bis-(aminomethyl)-biphenyl dihydrochloride **(42)**.

The synthesis of 2,3-bis-(aminomethyl)-biphenyl dihydrochloride **(42)** commenced with the formation of 2,3-dimethylbiphenyl **(39)** from a Suzuki palladium-catalysed

cross-coupling reaction. In this reaction, 2-tolyboronic acid was reacted with an organic electrophile, 3-iodotoluene (**38**) in the presence of a Lewis base, caesium fluoride. The palladium catalyst was tetrakis(triphenylphosphine)palladium (0) and the low polarity solvent 1, 2-dimethoxyethane (DME) was used.



Scheme 11: Synthesis of 2,3-bis-(aminomethyl)-biphenyl dihydrochloride (**42**).

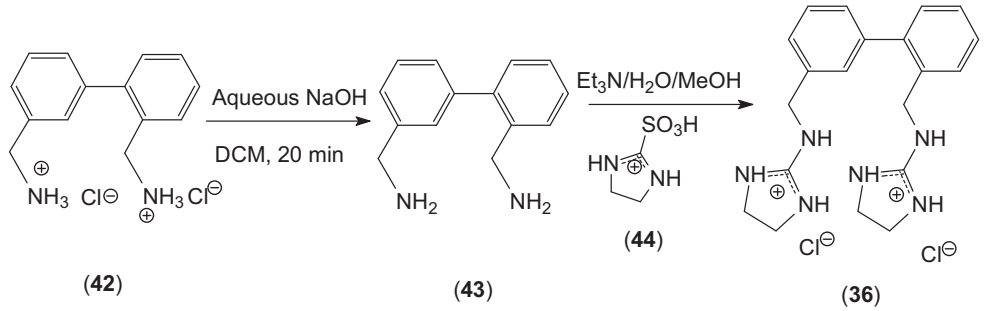
2,3-Dimethylbiphenyl **(39)** was monobrominated at each methyl group in dry trifluorotoluene to obtain 2,3-bis-(bromomethyl)-biphenyl (**40**) using the radical initiator 1,1-azobis(cyclohexanecarbonitrile) (ACCN). A side product was identified as a tribromo-derivative, where two of the original methyl protons were brominated (a singlet at $\delta = 6.62$ ppm corresponding to the methyl proton with two bromine atoms in ^{1}H NMR), in the final product. The ^{1}H and more especially ^{13}C NMR indicated that it cannot be removed completely by chromatography. The product collected from chromatography was only 90 % pure indicated by both ^{1}H and ^{13}C NMR spectra. This was confirmed by EIMS showing a peak with a molecular mass of 414 corresponding

to a tribromoproduct (two tribromo isomeric derivatives are possible). Finally the side products were removed by recrystallisation of the product from petrol after chromatography. This was proved by ^{1}H NMR where a singlet at δ = 6.62 ppm corresponding to the methyl proton with two bromines disappeared.

The substitution of bromines by diformylamide groups proceeded in dry acetonitrile with the use of sodium diformylamide as a convenient substitute for phthalimide in the literature of the Gabriel synthesis of primary amines.[113] 18-Crown-6 was used as catalyst and the yield of the reaction was increased considerably. 2,3-bis-(*N, N*-diformylamidomethyl)-biphenyl (**41**) was obtained as a white solid. ^{1}H NMR spectroscopy indicated that the product was pure. Both of the formyl groups were hydrolysed by 5 % (w/v) hydrochloric acid in ethanol to give 2,3-bis-(aminomethyl)-biphenyl dihydrochloride **(42).**

In the final step, two guanidinium derivative groups were inserted into (**42**) by a one pot-two step reaction using imidazoline 2-sulfonic acid (**44**) as reagent. Imidazoline 2-sulfonic acid (**44**) was prepared by molybdenum-promoted H_2O_2 oxidation of the thiourea.[114] This reaction was found to be very exothermic. The internal temperature of the reaction must be controlled under 5 °C since the temperature is a critically important to this reaction. H_2O_2 was added sufficiently slowly to avoid the internal temperature sharp rising. Reproducible yields were given by careful monitoring of the internal temperature.

As seen in **Scheme 12**, 2,3-bis(aminomethyl)-biphenyl **(43)** was obtained when 2, 3-bis (aminomethyl)-biphenyl dihydrochloride (**42**) was treated with aqueous NaOH in DCM and condernsed with 4,5-dihydro-*1H*-imidazole-2-sulfonic acid **(44)**.

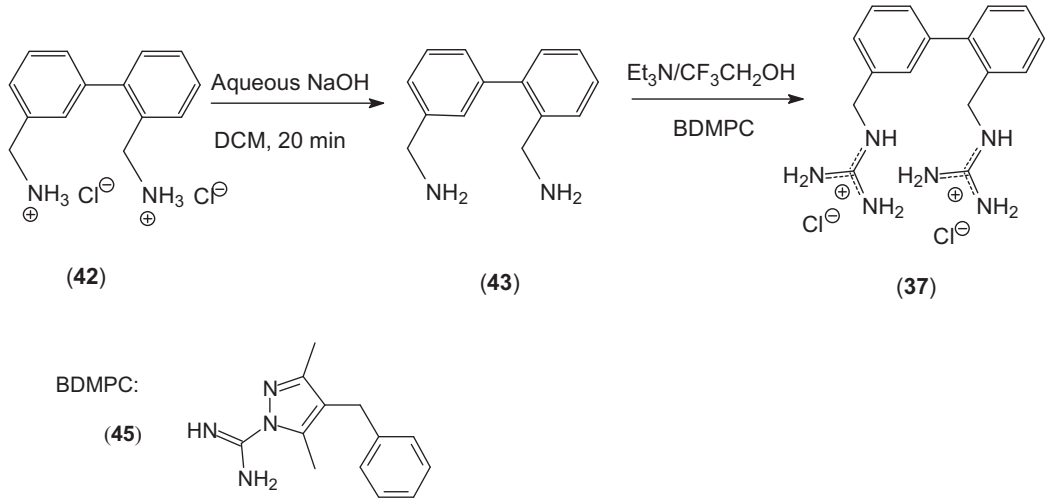


Scheme 12: Synthesis of 2,3-bis-[(4,5-dihydro-1H-imidazol-2-yl)-aminomethyl]-biphenyl dihydrochloride (**36**).

The crude product was purified by chromatography on Amberlite IR-400. The anion exchange resin was set up with hydroxyl groups (a 6 M solution of NaOH was flushed through the column in order to saturate the resin with hydroxyl anions). Water was used as eluent and the product was obtained as the free base. The detection method was TLC using ninhydrin dip (bright yellow colour). Then the free amine was isolated successfully. The last step was to synthesise the hydrochloride salt. The free amine was dissolved in water and conc. HCl was added dropwise and the mixture was stirred for 10 minutes. The solvent was removed under *vacuo* to afford 2,3-bis-[(4,5-dihydro-1H-imidazol-2-yl)-aminomethyl]-biphenyl dihydrochloride (**36**) as a white hygroscopic solid.

Another work was to synthesise 2,3-bis-guanidinomethyl-biphenyl dihydrochloride **(37)**. The synthesis consisted of the guanidine insertion using 4-benzyl-3,5-dimethyl-*1H*-pyrazole-1-carboxamidine (**45**, BDMPC) and is similar to the procedure described above**.** BDMPC (**45**) was prepared by D. Suarez in our research group.[108] The diamine

dihydrochloride (**42**) was neutralised with aqueous NaOH in DCM and the free amine (**43**) was reacted with BDMPC (**45**) in the present of triethylamine in 3,3,3-trifluoroethanol.



(**42**) (**43**) (**37**)

BDMPC:

(**45**)

4-Benzyl-3,5-dimethyl-*1H*-pyrazole-1-carboxamidine

Scheme 13: Synthesis of 2, 3-bis-guanidinomethyl-biphenyl dihydrochloride (**37**).

Recrystallisations from several solvent systems under vapour diffusion were attempted. Unfortunately no X-ray crystal structures have been obtained for either compound (**36**) or (**37**).

2.4 BINDING STUDY OF 2,3-BIS-(AMINOMETHYL)-BIPHENYL DIHYDROCHLORIDE WITH GLUTARIC ACID

The complexation phenomena in host-guest chemistry can be characterised by many physical methods (e.g. spectroscopic methods, electrochemical methods and thermodynamic methods).[115] These results could give further insight into designing a new binding system. Nuclear magnetic resonance (NMR) spectroscopy is normally used for measuring the binding constants in the range 10 - 10^4 M^{-1}; UV-vis spectroscopy is for $10^2 < K < 10^4$ M^{-1}.[116] NMR results can provide information on the conformation change of the host in the complex. This conformation change is difficult to observed from UV-VIS titration and impossible from calorimetric data. The NMR titrations method has been widely in the complexation study of small molecules and cyclodextrins, [117, 118] calixarenes[119] and cryptophanes. [120]

The biphenyl host (**36**) has been proved to be selected for dicarboxylate guest (i.e. glutaric and adipic acids).[112] In this part of the work, the glutaric acid was selected as a substrate in the binding studies to the modified biphenyl model (**37**) by NMR titrations.

2.4.1 Non-linear least-squares method for NMR titration

NMR titration is one of most useful methods for the study of complexation phenomena. Significant shifts of the receptor (or substrate) signals can be observed by ^1H or ^{13}C NMR in the free and complex states. And the concentrations of the receptor and substrate can be evaluated during the experiment. By measuring the chemical

shift of the host (or guest) during the titration, the binding constant can be determined from the following equation. This equation is derived upon the basis of equilibrium of 1:1 host-guest complexation.[121]

$$\delta_{obs} = \delta_H + (\delta_{HG} - \delta_H) \cdot \frac{\left([H]_t + [G]_t + \frac{1}{K}\right) - \sqrt{\left([H]_t + [G]_t + \frac{1}{K}\right)^2 - 4[H]_t \cdot [G]_t}}{2[H]_t} \qquad \text{Equation (1)}$$

Where

δ_{obs} is experimentally measured chemical shift in the host or guest molecule.

δ_H is chemical shift of a nucleus in the host molecule.

δ_{HG} is chemical shift of a nucleus of host in the host-guest complex.

$[H]_t$ is total concentration of host in solution.

$[G]_t$ is total concentration of guest in solution.

K is equilibrium constant for the formation of the host-guest complex.

The quantities δ_H, δ_{HG} and *K* were allowed to vary during the complexation. The δ_{obs} can be measured by NMR spectroscopy during the titration of guest into the host. $[H]_t$ and $[G]_t$ can be determined on the basis of the total amount of host or guest and the total volume of the solutions used during the procedure. This method has been used for a similar model in previous study.[17, 112] Fielding's review[122] of data treatment and Hirose's review[121] of practical aspects for the determination of binding constants were used to assist the present study.

2.4.2 Binding experiments

Firstly, two stocks of solution were prepared:

Host: biphenyl-diguanidinium chloride (**37**), 10 mL of a 1.5×10^{-3} M solution

Guest: bis-tetrabutylammonium glutarate. 2 mL of a 2.5×10^{-2} M solution

The bis-tetrabutylammonium dicarboxylate salt was prepared from the commercially available glutaric acid and tetrabutylammonium hydroxide. Two equivalents of a 2 M solution of Bu_4NOH in MeOH was added to the glutaric acid in methanol. The solvent was removed by rotary evaporator, and the solid was dried under 0.01 Torr vacuo, 60 ℃ for 24 hours.

All the ^1H NMR results were collected from a 300 MHz Bruker Avance BVT3200 instrument at 293 K. A set of 10 titration samples was prepared in this experiment. In all samples the concentration of host was kept constant, whereas the concentration of the guest was varied in the range of 20-80 %. It has been demonstrated that measurements not in this range get very uncertain values.[123]

Titrations were started with 0.4 mL of a known concentration (1.5×10^{-3} M) of the host, biphenyl-diguanidinium chloride, in CD_3OD into a NMR tube. A few μL of a known concentration solution of guest (2.5×10^{-2} M) was added into the NMR tube, the total volume of each sample was kept at 0.6 mL. Then it was shaken and immediately a ^1H NMR spectrum was measured. The difference in the chemical shift of benzylic protons was recorded. (**Table 2**) All the data was plotted by Job's method and the stoichiometry and the association constant of complexation was computed by non-linear curve fitting.

Exp n	**Y1=ppm**	**Δδ**	**Y2=ppm**	**Δδ**	**Guest** μL	**CD_3OD** μL	**X_G**	**No. Eq**
Exp 0	4.500		4.327		0	200	0	0
Exp 1	4.499	0.001	4.325	0.002	3	197	0.11	0.125
Exp 2	4.494	0.005	4.319	0.006	14	186	0.37	0.58
Exp 3	4.489	0.005	4.315	0.004	24	176	0.50	1
Exp 4	4.488	0.001	4.310	0.005	48	152	0.67	2
Exp 5	4.488	0	4.307	0.003	72	128	0.75	3
Exp 6	4.488	0	4.306	0.001	96	104	0.80	4
Exp 7	4.486	0.002	4.304	0.002	120	80	0.83	5
Exp 8	4.485	0.001	4.304	0	144	56	0.86	6
Exp 9	4.486	+0.001	4.304	0	168	32	0.88	7
Exp 10	4.488	+0.001	4.304	0	192	8	0.90	8

Table 2: NMR titration data collection of the complex between 2, 3-bis-guanidinomethyl-biphenyl dihydrochloride (**37**) and bis-tetrabutylammonium glutarate in CD_3OD. (X_G = mole fraction of guest in equilibrium mixture; 400 μL of host was added in each NMR sample tube.)

[Biphenyl Diguanidinium] = 1.5×10^{-3} M [Glutarate] = 2.5×10^{-2} M

The NMR spectrum showed upfield chemical shift changes for the two host benzylic CH_2 protons. However, the changes of induced shifts were too small and cannot used to calcuclate the binding constant. In fact such small values (0.02 compared to 0.15 - 0.20 obtained for the first model receptor[112]) could be a dilution effect only. It could be that the association constant K_a is much smaller, or that therer is no influence in the host methylene group we have investigated (it is not the group involved in binding).

2.4.3 Job's method to determine stoichiometry

In a binding study, the plot of [C] against X (molar fraction) is normally used as the signals of free and complexed molecules are exchanging rapidly.[124] The equation (2) is obtained in a host-guest complexation with fast exchange rate.[121]

$$[H]_t \cdot (\delta_{obs} - \delta_H) = a \cdot [C] \cdot (\delta_{HG} - \delta_H) \qquad \text{Equation (2)}$$

Where the stoichiometry (a) is a constant, [C] is the total concentration of the complex. Therefore, the equation (2) means that $\delta_{obs} - \delta_H$ is proportional to [C], as $a \cdot (\delta_{HG} - \delta_H)$ and $[H]_t$ both are constants.

The data were plotted in the form $\Delta\delta$ (measured shift change of benzylic proton) versus X_G (guest molar fraction). Despite the shift changes being lower than 0.1 ppm, in the plot of series 1 (one of two benzylic proton groups) the position of maximum indicates the stoichiometry of the complex is 1 : 1. (**Figure 25**)

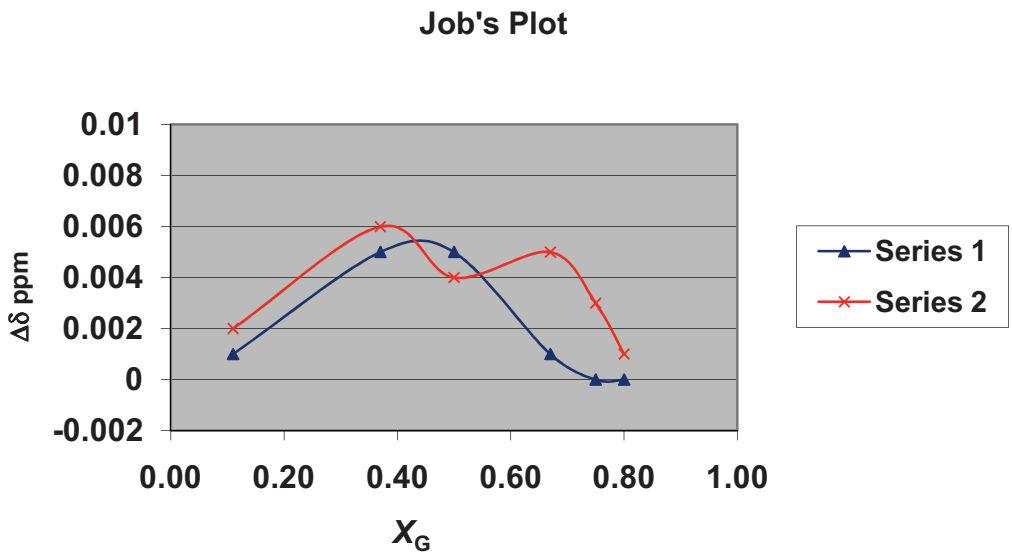
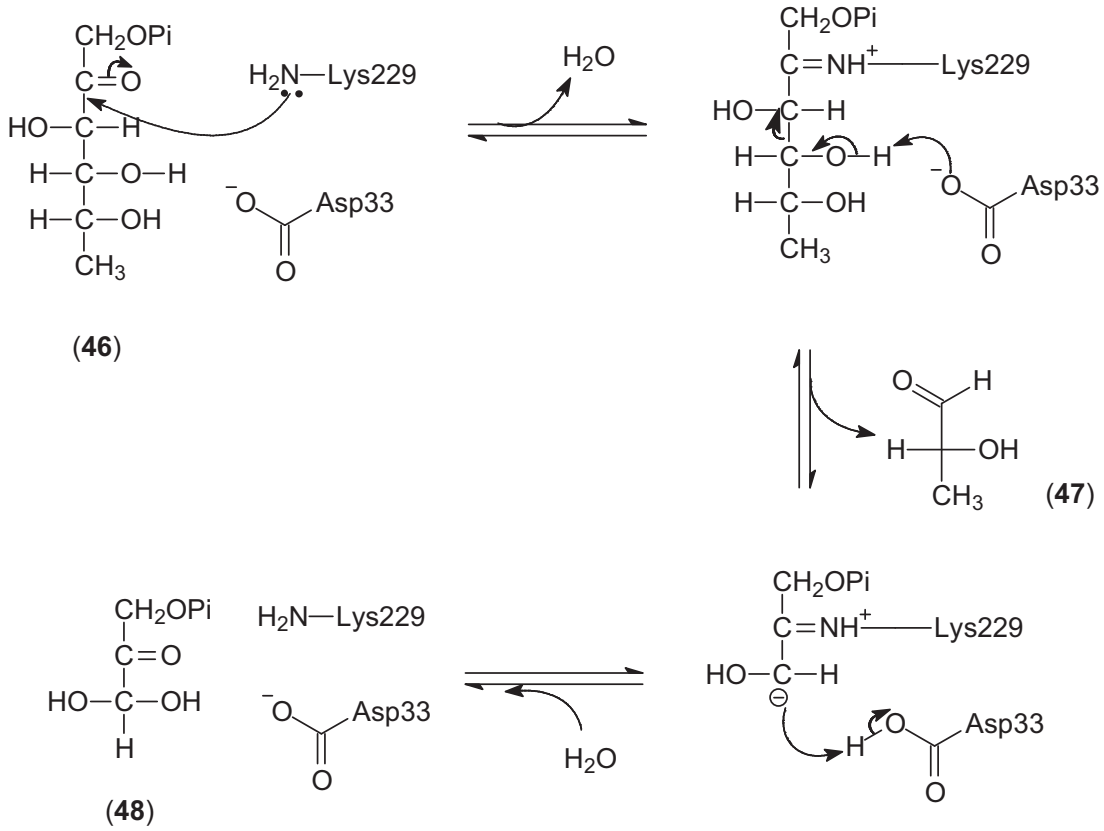


Figure 25: Job's plot for complexation of host and guest: Δδ, measured shift change of benzylic proton and X_G, guest molar fraction

2.5 MODIFIED TRITYLASE MODEL

Aldolase is an enzyme that cleaves an aldol, and includes three isozymes (aldolase A, B and C). The enzyme catalyses the reverse cleavage of fructose 1,6-biphosphate and fructose 1-phosphate (**46**) to form dihydroxyacetone phosphate (**48**) and either glyceraldehyde 3-phosphate or glyceraldehyde (**47**), respectively. Aldolase A is the only form present in muscle and red blood cells, whilst aldolase B characteristically occurs in the liver, kidney, and small intestine; aldolase C is present in the brain and neuronal tissue. Despite the isozyme-specific activity, the structures of aldolase A, B, and C are the same in their overall fold and active site structure.

The active site structure consists of Asp33, Arg42, Lys107, Lys146, Glu187, Ser271, Arg303, and Lys229. Lys 229 acts critically as it forms the Schiff-base intermediate.[125] Two distinct mechanisms of the reactions catalysed by aldolase occur in Nature.[126] Class I aldolase produced in animals uses covalent catalysis through the formation of a Schiff base intermediate between an active site lysine and the carbonyl group of the substrates. Class II aldolase produced in bacteria and fungi requires a divalent metal ion as a cofactor. The mechanism for the aldol cleavage of fructose-1-phosphate in the Class I aldolase is shown in **Scheme 14**. The Schiff-base intermediate formed between the lysine of active site and carbonyl group of the substrate increases the acidity of the β-hydroxyl group and facilitates the cleavage as shown in **Scheme 14**.



Scheme 14: Mechanism for the aldol cleavage of fructose 1-phosphate by aldolase B.[127]

Aldolase was thought of as a potential enzyme that the scaffold may fit as it contains lysine and arginine residues playing a key role in catalysis. Acidic groups (e.g. $-NH_2$) on the benzylic positions would complement the basic nature of the active site giving good interactions. (**Figure 26**) These could be studied further using UV techniques or isothermal titration calorimetry. NOESY spectra may be useful to show the distance of the residues to the substrate and quality of the binding.

In this research, we aimed to create a modified model (**49, 50**) for the active site of glutamate mutase, in particular with the features that constitute the arginine claw. This

modified model can be used not only for glutamate mutase but also some other enzymes (e.g. aldolase). The extra 'arm' of the molecule could bend and fit into the pocket. This will avoid the aggregation in the solution forming micelles and perhaps to help obtain a crystal structure of the molecule and the complex.

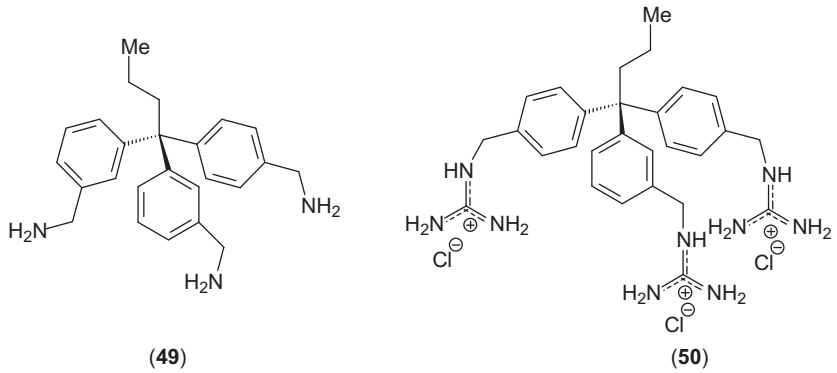
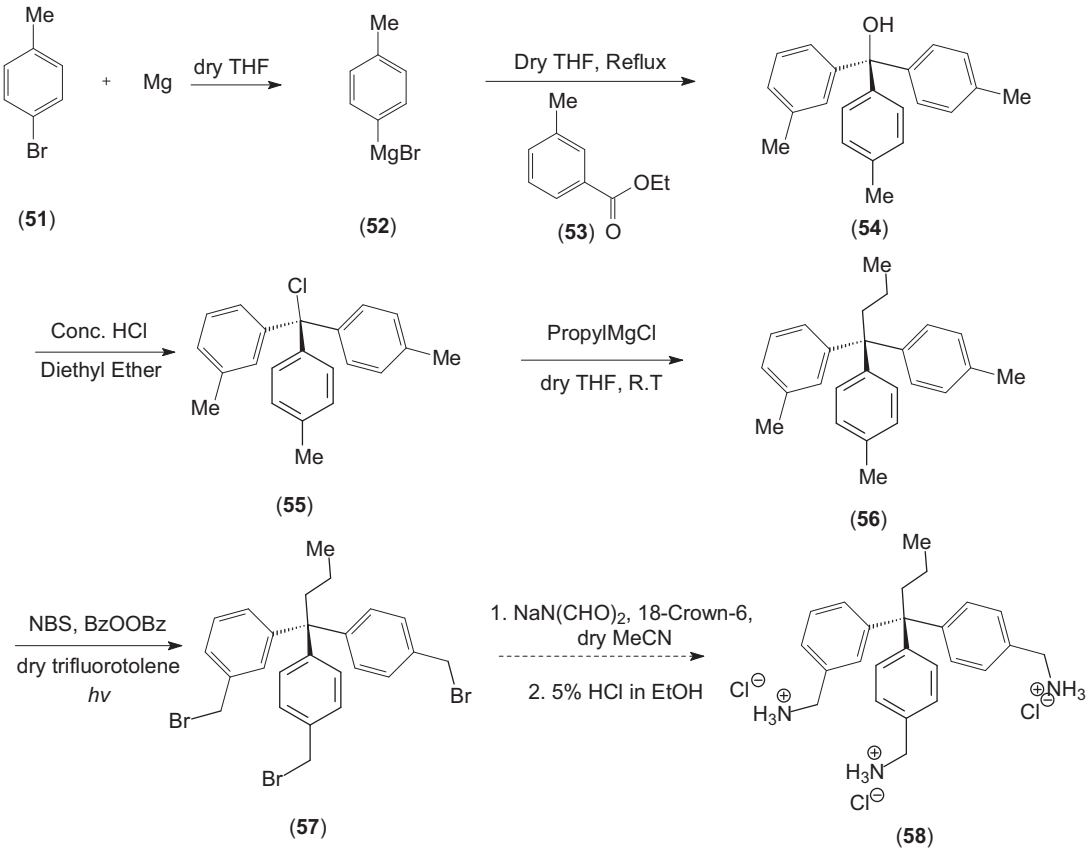


Figure 26: Modified tritylase model compounds with (meta, meta, para) arrangement of amine groups (**49**) and (meta, para, para) arrangement of guanidinium groups (**50**).

2.6 SYNTHESIS TOWARDS 3-(AMINOBENZYL)-BIS-(4-AMINOBENZYL)-BUTANE TRIHYDROCHLORIDE

From previous studies it is known that the stabilising groups can be substituted to add desirable groups, e.g. guanidinium residues, which will mimic the binding of anionic substrates.[108] In this study the *meta, para, para* isomer was synthesised using the procedure explored. Different reagents and conditions were explored in an effort to obtain a better yield with a purer product. This study was carried out for a 10-week summer project by the author and Hayley J. Lumb, an undergraduate student.

The conformational controlling group (**Figure 21**) stops any bromination to the tertiary position, but can this be used to add desirable groups on. If a longer chain (e.g. propyl) can be added to the tertiary carbon the flexibility of the molecule will be increased. This extra 'arm' may potentially control conformation of the tricyclic system.



Scheme 15: Synthetic route to 3-(aminobenzyl)-bis-(4-aminobenzyl)-butane trihydrochloride (**58**).

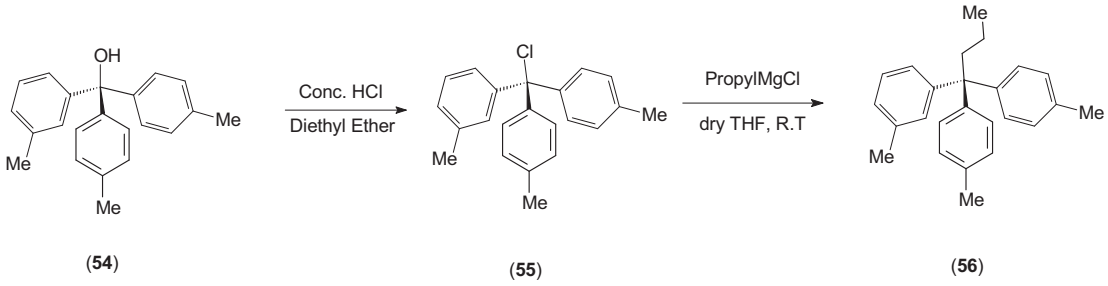
The synthetic route (**Scheme 15**) to the modified receptor, 3-(aminobenzyl)-bis-(4-aminobenzyl)-butane trihydrochloride (**58**), started with the nucleophilic addition of an excess Grignard reagent prepared from 4-bromotoluene (**51**) and magnesium

turnings in dry THF with ethyl *m*-toluate (**53**). The reaction proceeded at the same rate without iodine added to the reaction mixture and produced the same yield. Although the TLC showed four spots of different R_f values the corresponding, ^{1}H NMR showed little impurities, so this product was not purified by column chromatography. The desired product *para*-tolyl-di-*meta*-tolyl methanol (**54**) was obtained after purification by recrystallisaton from ether/petrol in over 80 % yield. The ^{1}H NMR analysis showed a singlet at δ = 2.25 ppm corresponding to the OH proton in the compound. This was also confirmed by IR spectrum which showed a small but sharp peak at 3600 corresponding to the alcohol group.

A synthesis to convert the alcohol to the chlorine to block the tertiary position was reported by D. Suarez.[108] The procedure used gaseous HCl, calcium chloride, and dry trifluorotolene in a nitrogen atmosphere. The gaseous HCl is costly and hazardous so an alternative method[128] was sort in this work. (**Scheme 16**)

The purified alcohol (**54**) was dissolved in minimum amount of diethyl ether, an excess of conc. HCl was added and left to react at room temperature for 24 hours in the dark. IR was again used to confirm the product and the characteristic OH peak (sharp peak at 3600) was not present. ^{1}H NMR was also used to confirm that the chlorine atom had taken the tertiary position and there was no singlet at δ = 2.25 ppm, which corresponded to the OH proton in the compound. This gave a slightly lower yield at 60 %, although the reaction takes longer this is a plausible synthesis as the reagents are easily available and cheap. This product must be stored in the dark under nitrogen, as this compound can easily convert to the compound (**54**) in air.

The next step is a nucleophilic substitution by a Grignard reagent at the tertiary position of chlorine atom. As the chlorine compound (**54**) is unstable, it had to be used quickly after preparation. In the previous work, the tertiary group was converted to a methyl group by using methyl magnesium bromide. To investigate if a higher structure could be made that would help the molecule to bend and close the pocket, a different Grignard reagent, propyl magnesium chloride, was used.

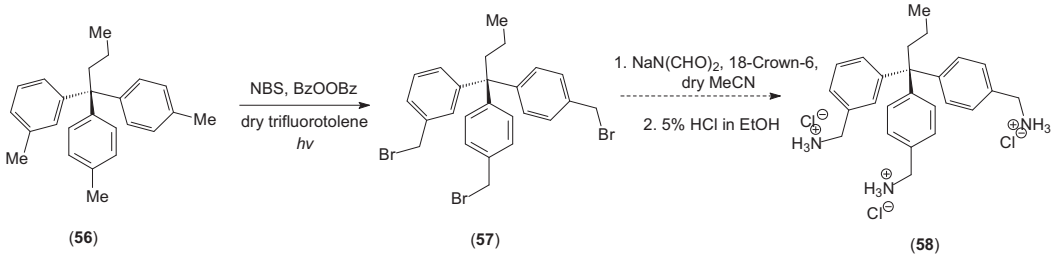


(**54**) (**55**) (**56**)

Scheme 16: Synthetic route for blocking the tertiary position.

The first attempt used the same ratio and procedure as explored by D. Suarez[108] at room temperature; this gave a 40 % yield. A ratio of 3:1 propyl magnesium chloride to the *m*-tolyl-di-*p*-tolyl-chloromethane (**55**) showed to obtain a better yield of 45-50 %. This is still lower than the stated value due to the additional steric hindrance of the propyl group to the tricyclic. The reaction was heated to 70 ˚C over 48 hours to investigate if temperature increased the yield. This was unsuccessful as the yield stayed at 40 %. ^{1}H NMR was used to analyse the sample, this was to confirm that the reaction had taken place and the reaction did not go back to the alcohol. The spectrum showed the propyl group with a shouldered triplet at δ = 0.90 ppm, multiplet at δ = 1.00 ppm and another triplet at δ = 2.42 ppm. Also two singlets at the same shift δ = 2.13 ppm showing the two *para* and a *meta* methyl group. A GCMS analysis was also

undertaken to confirm the product, this showed a large amount of fragmentation at the tertiary carbon position. The largest peak shown is the tritylase cation ($M^+ - 285$).



(**56**) (**57**) (**58**)

Scheme 17: Synthetic route towards the triamine trihydrocloride (**58**).

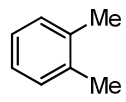
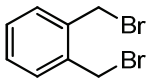
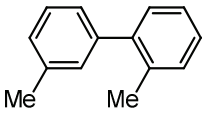
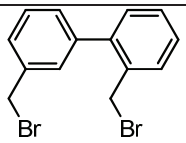
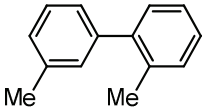
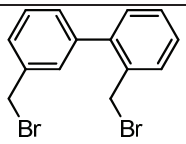
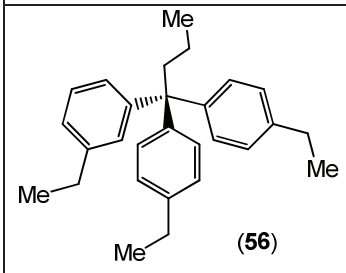
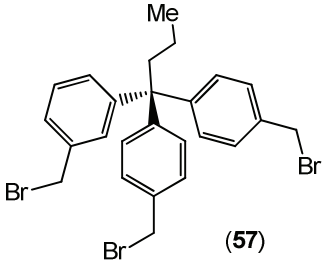
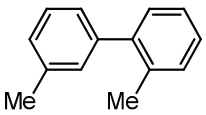
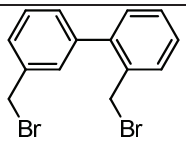
To obtain 1-(3-bromobenzyl)-1,1-di(4-bromobenzyl)-butane (**58**), a new bromination method was developed which will be discussed in later section. In this reaction, benzoyl peroxide was chosen as a radical initiator under the photochemical conditions with trifluorotoluene as solvent and *N*-bromosuccinimide (NBS) as brominating agent. The product was obtained at 50 % yield after column chromatography ^{1}H NMR analysis of the brominated compound (**57**) showed two new singlets at $\delta = 4.4$ ppm in a 2:1 ratio intensity. These show the three CH_2 groups at the benzylic positions next to the bromine atom. The same peaks and splitting were observed for the propyl group as before.

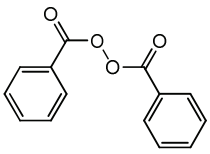
The following step consisted of a one pot two step synthesis commencing from a Gabriel-type synthesis[113] to convert the brominated compound (**57**) to 1-(4-diformylamidomethylphenyl)-1,1-di(3-diformylamidomethylphenyl)-butane (**58**) using sodium diformylamide. 18-Crown-6 was used as catalyst and increased considerably the yield of the reaction. This reaction was not completed due to lack of time.

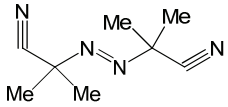
2.7 BENZYLIC BROMINATION

In the bromination of 2, 3-dimethylbiphenyl (**39**), a novel method developed in our research group was employed. In this method instead of using traditional solvents, benzene or tetrachloromethane, a less toxic trifluoromethylbenene (α, α, α-trifluorotoluene, benzotrifluoride) was used as the solvent.[129] *N*-Bromosuccinimide (NBS) was used as a bromination agent and 2,2'-azo-bis-isobutyronitrile (AIBN) or benzoyl peroxide is as radical initiator. In this work, AIBN was not commercially available any more. An alternative radical initiator to AIBN, 1,1'-azobis(cyclohexanecarbonitrile) (ACCN) was chosen in the experiments. This compound was regarded as a more efficient radical initiator than AIBN and has been employed to initiate primary radical reactions.[130]

Additionally, in order to establish the complete method, a series of experiments of different radical initiator with different benzylic brominations was required. Of course, the AIBN result is from previous study.[112] (**Table 3**) The results did not show ACCN was more efficient radical initiator than AIBN in our experiments.

 (**59**)	ACCN	33 %	 (**60**)
 (**39**)	AIBN	84 %	 (**40**)
 (**39**)	BPO	57 %	 (**40**)
 (**56**)	BPO	50 %	 (**57**)
 (**39**)	ACCN	71 %	 (**40**)


Benzoyl peroxide, BPO


1,1-azobisisobutyronitrile, AIBN

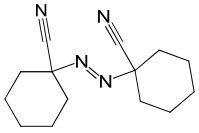

1,1-azobis(cyanocyclohexane), ACCN

Table 3: Benzylic dibromination with 2.2 eq. of NBS and different radical initiators in trifluorotoluene.

2.8 CONCLUSIONS

Two biphenyl model receptors, 2,3-bis-[(4,5-dihydro-1*H*-imidazol-2-yl)-aminomethyl]-biphenyl dihydrochloride (**36**) and 2,3-bis-guanidinomethyl-biphenyl dihydrochloride (**37**) were successfully synthesised. The dibromination at the benzylic positions was achieved using a different radical initiator, ACCN, rather than AIBN. The purity of the dibromo-product (**40**) was improved by successfully removing the tribrominated side products.

The binding property of the modified receptor model (**37**) to a dicarboxylate (glutaric acid) in CD_3OD was investigated by ^1H NMR titration at fast host-guest exchange rate. Titrations were run at constant host concentration (1.0×10^{-3} M). The increasing amount of guest in the samples induced upfield chemical shift changes of the protons in both benzylic CH_2 groups of the host. It indicated formation of a complex. Both chemical shifts happened at same time, so we cannot calculate the association constant, *K*, base on the equation (**1**). The maximum change of chemical shift with no more than 0.1 ppm indicates that the binding constants are too small to be evaluated or the solvent system affects the complaxation of the host and guest. So new investigation method (such as isothermal titration calorimetry, or UV absoption) should be used to determine the binding constants in the future work.

Synthesis of 3-(aminobenzyl)-bis-(4-aminobenzyl)-butane trihydrochloride (**58**) gave another route to the 'tritylse' model receptor. A new methodology to insert a different conformation controlling group has been explored. Further experiments to insert guanidinium groups to the compound are required. The new model fits the original

scaffold but may have the ability to mimic different enzymes, not just B_{12}-dependent enzymes (e.g. aldolase). UV techniques or isothermal calorimetry (ITC) for binding studies with different dicarboxylates could be employed to investigate its binding properties. NOESY spectra could be used to show the distance of the residues to the active site and quality of the binding.

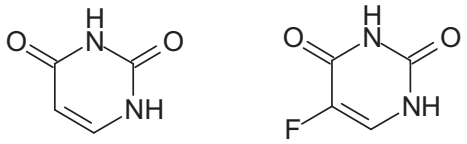
Chapter 3

Synthesis and Model Study of Fluorinated Substrate Analogues for Diol Dehydratase and Ethanolamine Ammonia-lyase

3.1 INTRODUCTION TO FLUORINE CHEMISTRY

3.1.1 Overview

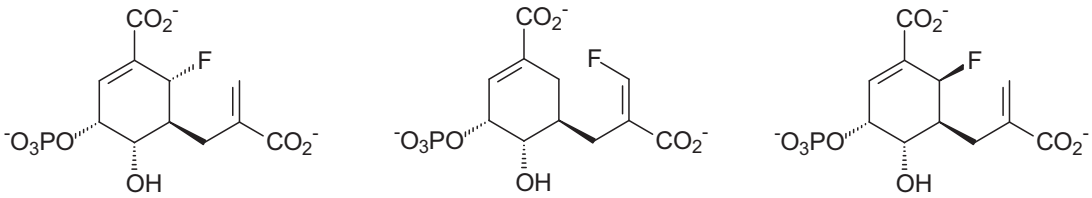
Enormous numbers of fluorinated compounds have been widely used in medicinal chemistry and chemical biology, because the fluorine atom(s) or fluorinated group(s) could give the molecules some unique property that cannot be given by any other element. The most notable example in medicinal chemistry is 5-fluorouracil (**62**), which is an anticancer drug developed in 1957.[131] Uracil (**61**) does not show any anticancer activity, but the introduction of a single fluorine atom to the molecule brought a potent anticancer drug. They are of similar shape. However, 5-fluorouracil (**62**) can inhibit the activity of RNA replication enzymes while uracil (**61**) is a normal component of RNA.[132]



(**61**) (**62**)

Figure 27: Uracil (**60**) and 5-fluorouracil (**61**).

In chemical biology, fluorinated substrates have been used as mechanistic probes or inhibitors. They could give multifarious information about the mechanism of catalytic cycles and enzymatic transformations.[133] Fluorinated analogues (**63**, **64** and **65**) have been used for study of the mechanism of the reaction catalysed by chorismate synthase.[134]



(**63**) (**64**) (**65**)

Figure 28: Fluorinated analogues for 5-enolpyruvylshikimate 3-phosphate

The C-F bond is one of the strongest known and it is relatively short (typically 1.35 Å, e.g. 1.39 Å in fluoromethane). The adjacent C-C single bonds are strengthened by the fluorine substitution, whereas adjacent allylic C=C double bonds are weakened.[135] Some characteristic properties of fluorinated molecules are described in this context.

The electronegativity and van der Waals radius of fluorine (3.98, 1.47 Å) compare better with values for the oxygen of a hydroxyl group (3.44, 1.52 Å) rather than with hydrogen (2.20, 1.20 Å). [136, 137] So fluorine can serve as an effective hydroxyl group mimic in molecules. In the 1980s, Blackburn and coworkers [138] reported that fluoromethylene or difluoromethylene groups could be used in the design of stable analogues of phosphate esters. Fluorine often replaces H in organic molecules since the bond dissociation energy of a C-F bond (451.9 kJ mol^{-1}) is actually more similar to a C-H bond (431.0 kJ mol^{-1}) than to a C-O bond (377.0 kJ mol^{-1}).[136]

The insertion of a fluorine atom in a molecule can appreciably alter its physicochemical properties (bond strength, lipophilicity, conformation, electrostatic potential, dipole, and pK_a). [139] The next sections list several key features which are

important for the fluorinated molecules to be effective in medicinal chemistry and chemical biology.

3.1.2 The effect of fluorine on pK_a

Since fluorine is a strong electronegative element, it can induce electronic effects on its neighbouring functional groups such as hydroxyl and amino groups by affecting their electron density. Substantial changes in pK_a values of alcohols, amines, and carboxylic acids are observed with fluorine substituted into these molecules. The inductive effect reduces the pK_a value of these functional groups. (**Table 1**)

Compound	**pK_a**	**Compound**	**pK_a**
CH_3CH_2OH	16.0	$CH_3CH_2NH_3^+$	10.7
CF_3CH_2OH	12.37	$FCH_2CH_2NH_3^+$	9.0
$CH_3CH_2CH_2OH$	16.0*	$F_2CHCH_2NH_3^+$	7.3
$CF_3CH_2CH_2OH$	14.6*	$CF_3CH_2NH_3^+$	5.7
$CH_3CH_2CO_2H$	4.87	$CH_3CH_2CH_2NH_3^+$	10.7
$CF_3CH_2CO_2H$	3.06	$CF_3CH_2CH_2NH_3^+$	8.7

Table 4: Selected pK_a values of various functional groups affected by fluorine substitution. (*: estimated value.) [140]

As seen in the table, the linear amines become much less basic with the fluorine substitution at the β position. The inductive effect of the trifluoromethyl group (CF_3) could much influence pK_a even with γ-substitution. β-Substitution of ethanol with CF_3

group leads to a dramatic increase in the acidity of the resulting alcohol. Even carboxylic acids become more acidic with the introduction of a CF_3 group.

3.1.3 The effect of fluorine on protein-ligand interactions

Pauling assigned fluorine the highest electronegativity value of all of the elements.[141] This electronegativity and the presence of three lone pairs might suggest that the fluorine will act as a good acceptor in hydrogen bonding. However, extensive study suggested that the C-F unit has a very low proton affinity and is highly non-polarisable in respect of hydrogen bonding as the F is such a strong electronegative element that holds all the lone pairs.

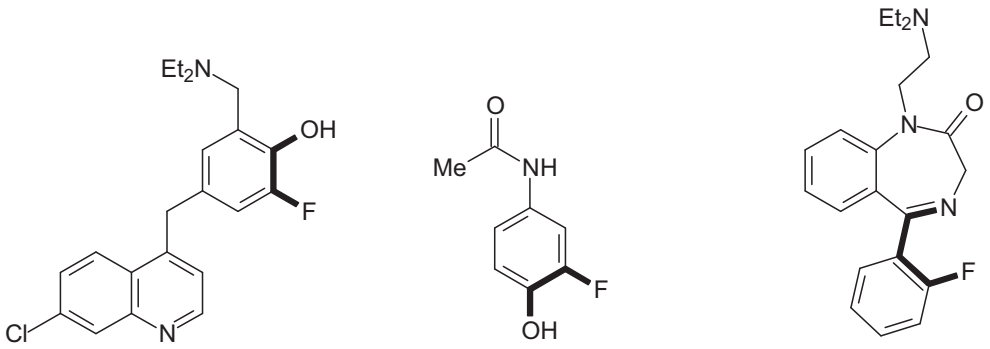
Generally, it is found that fluorine substitution could increase the binding affinities of a compound by electrostatic interactions. In chemical biology, the fluorine substitution of a hydroxyl group has been used as a standard method to probe protein-ligand interactions.[142] The fluorine could affect the binding affinity in a protein-ligand complex either by modulation of the polarity of other groups of the ligand that interact with the protein or directly by interaction of the fluorine with the receptor.

As stated in the last section, fluorine substitution could modulate the pK_a of the binding group to enhance protein-ligand interactions. In addition, hydrogen bonds can be formed by hydrogen in H-C with fluorine although they are much weaker than those observed with oxygen or nitrogen.[143, 144] However, arguments continue on the existence of hydrogen bonds between the C-F group and hydroxyl (-OH) or amine (-NH) donors.[145] In contrast to hydrogen bonding, the electrostatic interaction of

fluorine is widely accepted to contribute to the enhanced binding affinity of some fluorinated compounds to an enzyme's active site. Olsen *et al*[146] have described that the interactions of C-F···C=O can play an important role in the binding mode of a fluorine inhibitor with the active site of thrombin. This fluorine inhibitor binds five times more strongly to the active site than the corresponding non-fluorinated compound. A very similar binding mode has been discovered in some fluorinated inhibitors of p38 kinase.[147] (PDB ID: 1AU9)

3.1.4 The effect of fluorine on metal coordination

Evidence has shown that C-F bond can coordinate with metal ions. It is assumed that fluorine atoms are Pearson-type hard donors since fluorine has similar van der Waal radius and electronegativity to oxygen.[148] The fluorine can be regarded as an efficient donor atom in coordination with hard metal ions (e.g. Na^+, K^+, Mg^{2+}, Ca^{2+}). Recent studies have shown that in many situations the C-F bond can coordinate with metal ions.[149] However, it remains to be shown whether this interaction is strong enough to influence the biochemistry of fluorinated compounds in biological systems. As there are several metals (e.g. Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+} and Fe^{3+}) that are abundant in organisms and participate in many biological functions,[150] it is very important to demonstrate whether fluorinated molecules could coordinated with metal ions in biological systems. Plenio[18] has selected a few fluorinated compounds that are potential candidates as chelating ligands with fluorine coordination. (**Figure 29**)



Fluoramodiaquin
Anti-Malarial
(**66**)

Fluoroparacetamol
Analgesic
(**67**)

Flurazepam
Anxiolytic
(**78**)

Figure 29: Fluorinated drugs as potential metal chelating ligands.

3.1.5 The effect of fluorine substituent on conformation

Substitution of a hydrogen atom or a hydroxyl group by fluorine in biologically active molecules has been carried out in various areas of bioorganic and medicinal chemistry, as the fluorine van der Waals radius, (1.47 Å) lies between that of oxygen (1.52 Å) and hydrogen (1.20 Å). O'Hagan *et al.*[149] has demonstrated that the substitution of a single fluorine for a hydrogen atom introduces very small steric and geometric effect to the molecule. However, the substitution of CF for CH group in enzyme substrate analogues could have a weaker binding affinity for their target receptor.[151] Substitution of a methylene by a difluoromethylene group (CH_2 is replaced by CF_2) can be more dramatic than the single substitution. It has been noted that the melting points of the fluorinated compounds are lower than the corresponding non-fluorinated compounds.[152] This phenomenon could be caused by an increased conformational flexibility of the hydrocarbon caused by CF_2 substitution.[153]

The substitution of a methyl group by trifluoromethyl group within a molecule may lead to a much more dramatic steric change than difluoromethylene as its van der Waals volume is estimated to be close to that of an ethyl or isopropyl group although of a significantly different shape.[154, 155]

As a result of the steric variations and the strong electronegativity of fluorine, the effect of fluorine substitution on molecular conformation is quite elusive and sometimes difficult to predict. For example, trifluoromethoxybenzene does not adopt a similar planar conformation like methoxybenzene. It has its trifluoromethoxyl group out of the plane (dihedral angle for C-C-O-C up to 90°).[155]

3.2 FLUORINATED SUBSTRATE ANALOGUES FOR B_{12} DEPENDENT DIOL DEHYDRATASE AND ETHANOLAMINE AMMONIA-LYASE

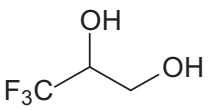
Diol dehydratase from e.g. *Klebsiella oxytoca* is a radical enzyme that converts simple 1,2-diols into corresponding aldehydes and water. The enzyme requires adenosylcobalamin (coenzyme B_{12}) as cofactor and a metal cation (e.g. K^+). The 5'-deoxyadenosyl radical from homolysis of the coenzyme's Co-C bond abstracts a specific H atom from C-1 of diol substrate giving a substrate radical that rearranges to a product radical by 1,2-shift of hydroxyl from C-2 to C-1. Ethanolamine ammonia-lyase (EAL) from e.g. *Clostridia* sp. converts ethanolamine into acetaldehyde and ammonia by a similar pathway. (See Chapter 1 for details)

It is important to understand the reaction mechanism of these enzyme-catalysed reactions since the insights may facilitate to design the methods to control or mimic these reactions. Using fluorinated substrate analogues as mechanistic probes have become a common method to study enzyme reactions. Numerous experiments have indicated that many fluorinated substrate analogues are good enzyme inhibitors.[156]

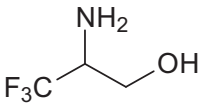
Although intensive research efforts have elucidated the fate of the hydrogen atoms during the enzymatic dehydration catalysed by diol dehydratase and EAL, the complete mechanism of the reaction is still subject to considerable speculation.

In order to provide evidence for the mechanistic pathways which we discussed in chapter 1, we have proposed two fluorinated substrate analogues, (**Figure 4**). In a previous study, 3,3,3-trifluoropropane-1,2-diol (**68**) was shown to be a substrate for glycerol dehydratase (ca. 4 % of the activity of propane-1,2-diol).[157] This result is surprisingly in the context of the proposed mechanism for diol dehydratase, although *ab initio* molecular orbital calculations[158] have indicated that CF_3 has a minimal effect on the stabilities of the proposed intermediates. Due to the similarity of glycerol dehydratase and diol dehydratase, it is possibly a substrate for diol dehydratase as well.

D. Smith's calculation[158] indicated the CF_3 in 2-amino-3,3,3-trifluoropropanol (**69**) has a minimum effect on the stabilities of the proposed intermediates in the reaction catalysed by EAL. Hence, 2-amino-3,3,3-trifluoropropanol (**69**) might be a substrate for EAL.



(**68**)



(**69**)

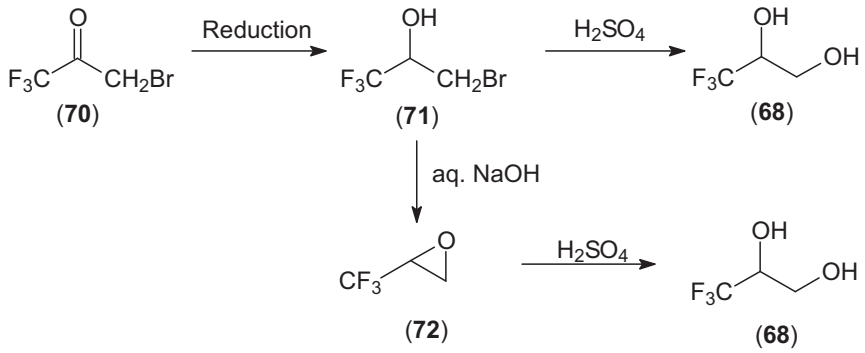
Figure 30: Fluorinated substrate analogues of diol dehydratase and EAL.

In this study, the initial objective was to synthesise 3,3,3-trifluoropropane-1,2-diol (**68**) and 2-amino-3,3,3-trifluoropropanol (**69**). Model studies were carried out to examine the effect of a trifluoromethyl substituent on the corresponding substrates.

3.3 SYNTHESIS OF 3,3,3-TRIFLUOROPROPANE-1,2-DIOL AND 2-AMINO-3,3,3-TRIFLUOROPROPAN-1-OL

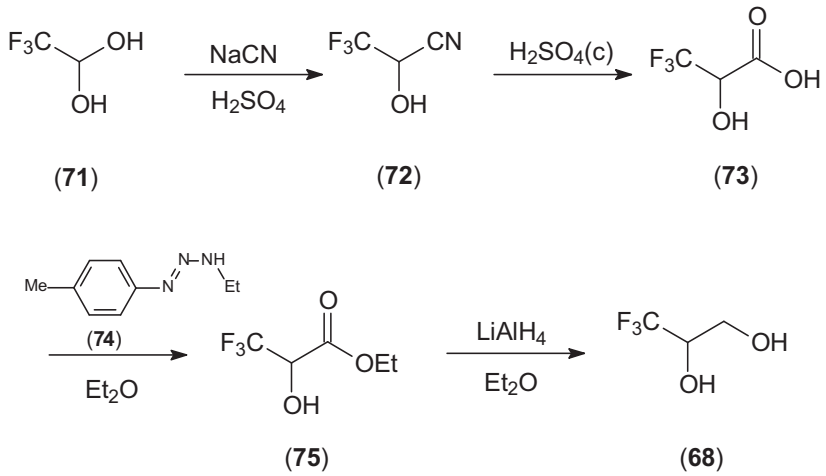
3.3.1 Synthesis of 3,3,3-trifluoropropane-1,2-diol (68)

In McBee's work (**Scheme 18**) [159] , 3,3,3-trifluoropropane-1,2-diol (**68**) was synthesised from 3-bromo-1,1,1-trifluoroacetone (**70**). This method involved reduction of ketone to the corresponding alcohol (**71**) which was converted to 3,3,3-trifluoro-1,2-epoxypropane (**72**) in the presence of aqueous sodium hydroxide. 3,3,3-trifluoropropane-1,2-diol (**68**) was obtained from acidic hydrolysis of the bromo alcohol (**71**) or acid hydrolysis of the epoxy compound (**72**). This method is not quite satisfactory, due to the low boiling point of 3,3,3-trifluoro-1,2-epoxypropane (**72**).



Scheme 18: Synthesis of 3,3,3-trifluoropropane-1,2-diol (**68**) starting from 3-bromo-1,1,1-trifluoroacetone (**69**).

Watson demonstrated [160] an alternative synthesis of 3,3,3-trifluoropropane-1,2-diol (**68**), which involved $LiAlH_4$ reduction of ethyl 2-hydroxyl-3,3,3-trifluoropropionate (**75**). (**Scheme 19**)

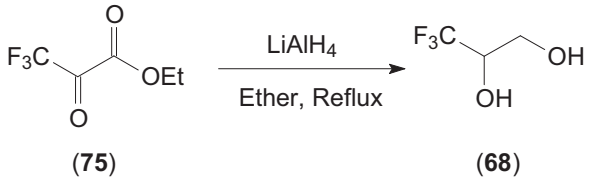


Scheme 19: Synthesis of 3,3,3-trifluoro-1,2-propanediol (**68**) via 2-hydroxyl-3,3,3-trifluoroacetic acid (**75**).

2-Hydroxyl-3,3,3-trifluoropropionate acid (**73**) was prepared starting from the reaction of sodium cyanide-sulfuric acid with 2,2,2-trifluoroacetaldehyde hydrate (**71**). The intermediate cyanohydrin (**72**) was hydrolysed to the racemic acid (**73**). The esterification of the acid was accomplished by reaction with 1-ethyl-3-*p*-toluenetriazene (**74**) in diethyl ether. The final product, 3,3,3-trifluoropropane-1,2-diol (**68**) was obtained by reduction of the ester using lithium aluminium hydride. This method involved the hazardous reagent sodium cyanide and the carcinogen triazene (**74**), and is also multistep.

In this study (**Scheme 20**), the 3,3,3-trifluoropropane-1,2-diol (**68**) was simply synthesised from lithium aluminium hydride reduction of commercially available ethyl 3,3,3-trifluoropyruvate (**75**) in a good yield (84 %). It was characterised by ^{1}H NMR spectroscopy which showed two double doublets at δ = 3.60-3.67 and 3.74-3.80 ppm corresponding to the protons of CH_2 group and a multiplet at δ = 4.03-4.14 ppm

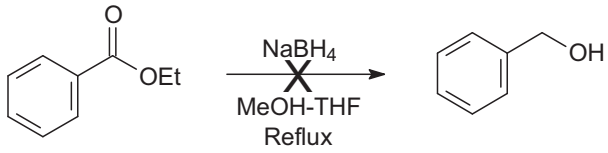
corresponding to the proton of the CH group. The ^{19}F NMR showed a doublet at δ = -79.2 ppm corresponding to the fluorine of the trifluoromethyl group.



(**75**) (**68**)

Scheme 20: Synthesis of 3,3,3-trifluoropropane-1,2-diol (**68**) *via* reduction.

The first synthetic attempt towards 3,3,3-trifluoropropane-1,2-diol (**75**) was based on the work of de Souza *et. al*[161] (**Scheme 21**). Sodium borohydride was used as reductant in methanol/THF. A model reaction was carried out to reduce ethyl benzoate (**76**). However, ^{1}H NMR indicated that benzyl alcohol (**77**) was not the product. Comparing the NMR spectra, the compound obtained from the reaction was recovered starting material, ethyl benzoate (**76**).



(**76**) (**77**)

Scheme 21: Attempted synthesis of benzyl alcohol (**77**) via sodium borohydride reduction

So lithum aluminium hydride reduction was used. Several workup procedures have been examined to optimise the best yield of the reaction. For all entries of **Table 5**,

ethyl 3,3,3-trifluoropyruvate (**75**) was reacted with excess of lithium aluminium hydride under reflux for 4 hours in dry diethyl ether. Then different methods were chosen to destroy the excess of lithium aluminium hydride. The data obtained are summarised in **Table 5**.

We proposed that the reason why many of the reactions gave such a low yield is that Al^{3+} formed a complex with the diol groups. This was proved by the addition of H_2SO_4 which is a strong acid. The acid could destroy this complex to give free diols, but water and sodium hydroxide could not. The experiments showed a significant increase of yield with addition of H_2SO_4 before separating the mixture.

Entry	**Addition after reaction**	**Removing traces of water**	**Purification**	**Yield**
a	H_2O	$MgSO_4$	Continuous extraction	10.3%
b	$Na_2SO_4 \cdot 10H_2O$	Na_2SO_4	Filtration	17%
c	H_2O	Na_2SO_4	Filtration	5.3%
d	H_2O and $NaOH_{(aq)}$	Na_2SO_4	Filtration	< 30%
e	H_2O and H_2SO_4	Na_2SO_4	Distillation after column	84%

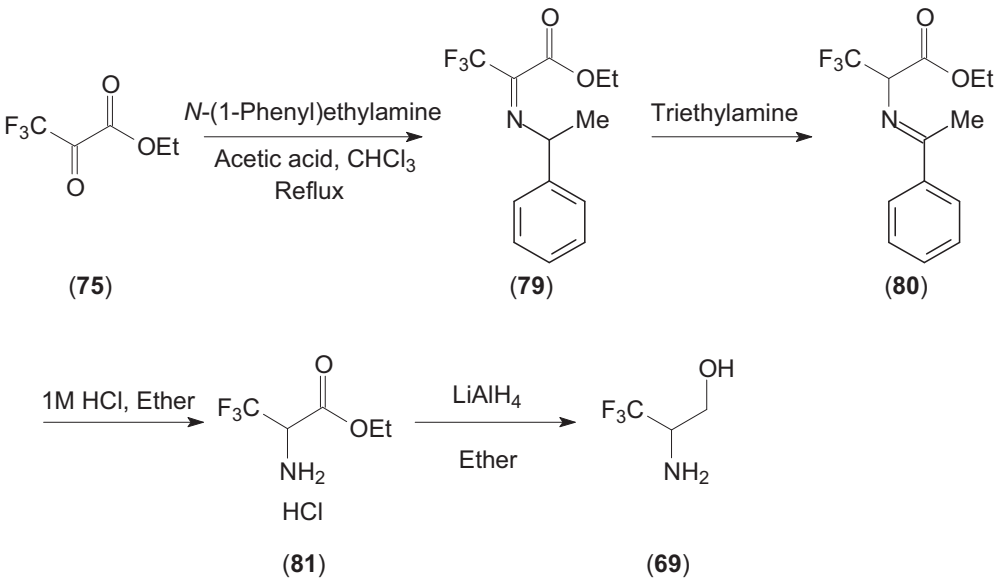
Table 5: Summary for the preparation of 3,3,3-trifluoro-1,2-propanediol (**68**) with different workup procedures.

In addition, after water was added to destroy the excess of aluminium hydride, a white semi-solid was found from the filtration. The organic phase was concentrated to give the product diol in a low yield. In order to improve the yield, methanol was used here to try to wash out more diol from the filtration. But it is interesting that, the gummy white solid was dissolved in the methanol. This observation indicated that the gummy white solid could be the complex of diol and aluminium. This complex is different from that obtained from most of the reductions.

3.3.2 Synthesis of 2-amino-3,3,3-trifluoropropanol (69)

2-Amino-3,3,3-trifluoropropanol (**69**) was prepared from lithium aluminium hydride reduction of 3,3,3-trifluoroalanine ethyl ester hydrochloride (**81**), which was synthesised by following Soloshonok's method. [162, 163] (**Scheme 22**)

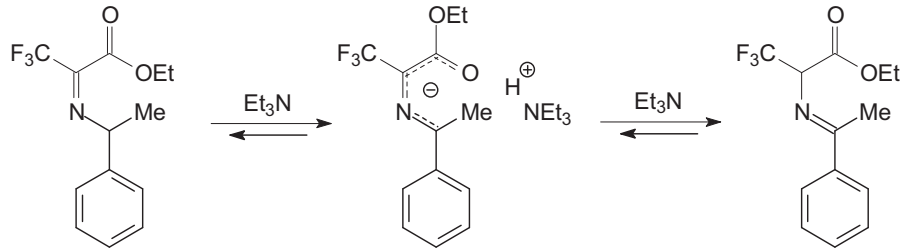
Ethyl 2-*N*-(1-phenylethyl)imino-3,3,3-trifluoropropionate (**79**) was obtained from the reaction of ethyl 3,3,3-trifluoropyruvate (**75**) treated with methylbenzylamine and acetic acid in chloroform under reflux. The compound was characterised by ^{1}H NMR (appearance of doublet at δ = 1.48 ppm corresponding the methyl protons of phenylethyl and a quartet at δ = 4.88 ppm corresponding the protons CH of the imine) Isomerization of the (1-phenyl)ethylimine (**79**) to (1-phenyl)ethylidene (**80**) proceeded in triethylamine under mild condition.^{1}H NMR showed a singlet at δ = 2.35 ppm corresponding the methyl protons of phenylethyl group and a quartet at δ = 4.88 ppm corresponding the protons CH of CF_3CH.



Scheme 22: Preparation of 2-amino-3,3,3-trifluoropronanol (**69**).

The 1,3-proton shift reaction is a key step of this method. It uses the intramolecular reduction-oxidation process in the aza-allylic system of the imines (**79**) and (**80**). The mechanism of this azomethine-azomethine isomerization involves an aza-allylic anion as intermediate. (**Scheme 23**) The equilibrium of this isomerisation is nearly entirely shifted to the imine (**80**), since this imine is much more thermodynamically stable than the imine (**79**).

3,3,3-Trifluoroalanine ethyl ester hydrochloride (**81**) was easily released from the Schiff base (**80**) by a mild acidic hydrolysis. This compound was purified by recrystallisation from acetonitrile and proved to be pure by NMR analysis. It was dried in high vacuum before being used in next step with lithium aluminium hydride.



(**79**) (**80**)

Scheme 23: Mechanism of the azomethine isomerisation.[162]

Numerous reactions have been carried out to obtain the final compound, 2-amino-3,3,3-triflluoropropanol (**69**). Initially, we had a low yield of the product, even obtaining nothing after removing the solvent under reduced pressure. A few white crystals were observed at top of the flask after leaving the flask containing crude product for few days at room temperature. So we concluded that 2-amino-3,3,3-triflluoropropanol (**69**) is a volatile compound and could sublime at room temperature. Water was used to destroy the excess of lithium aluminium hydride. Pure compound was collected as white crystals from sublimation of the crude compound at 30 °C, 20-30 mmHg. The mixture could not be left too long under reduced pressure when removing the solvent.

2-Amino-3,3,3-triflluoropropanol (**69**) was characterised by ^{1}H NMR, which showed a broad singlet at δ = 1.65 ppm corresponding to the amine, a multiplet at δ = 3.23-3.29 ppm corresponding to the proton of the CH group and two double doublets at δ = 3.50-3.46 and 3.75-3.80 ppm corresponding to the protons of the CH_2 group. The ^{19}F NMR showed a doublet at δ = -76.7 ppm corresponding to the fluorine of the trifluoromethyl group.

3.4 EPR STUDY OF 2-AMINO-3,3,3-TRIFLUOROPROPANOL WITH EAL

The EPR studies were carried out by Dr Kurt Warncke's group in Emory University, Atlanta, USA. EPR spectra (**Figure 31**) were obtained by mixing (in the following order) 165 μL potassium phosphate, 62 μL EAL and 60 μL substrate. After 13 μL AdoCbl was added, the total volume of the solution was taken to 300 μL. The solution was transfered to an EPR tube. Freezing times were as noted in the Experiment Section and below.

As seen from the data, the CF_3-substrate did not lead to the significant formation of a paramagnetic intermediate, which had spectral features expected from the 2-amino-3,3,3-trifluoropropanol. There is a narrow-line free radical-type signal at g = 2, but this is present in relatively low abundance [as assessed by comparison with the control native radical pair EPR spectrum, which was obtained with (*S*)-2-aminopropanol] (see **Figure 31**), and there does not appear to be a corresponding Co(II) signal, either of the uncoupled (to the g = 2 radical) or the coupled (as in native systems) type. The g = 2 signal does not develop with time, either.

In the second round, for which data are shown, prolonged incubation with the CF_3-substrate was carried out, and the sample has been sonicated. Again, no EPR signals corresponding to the desired radical species were observed.

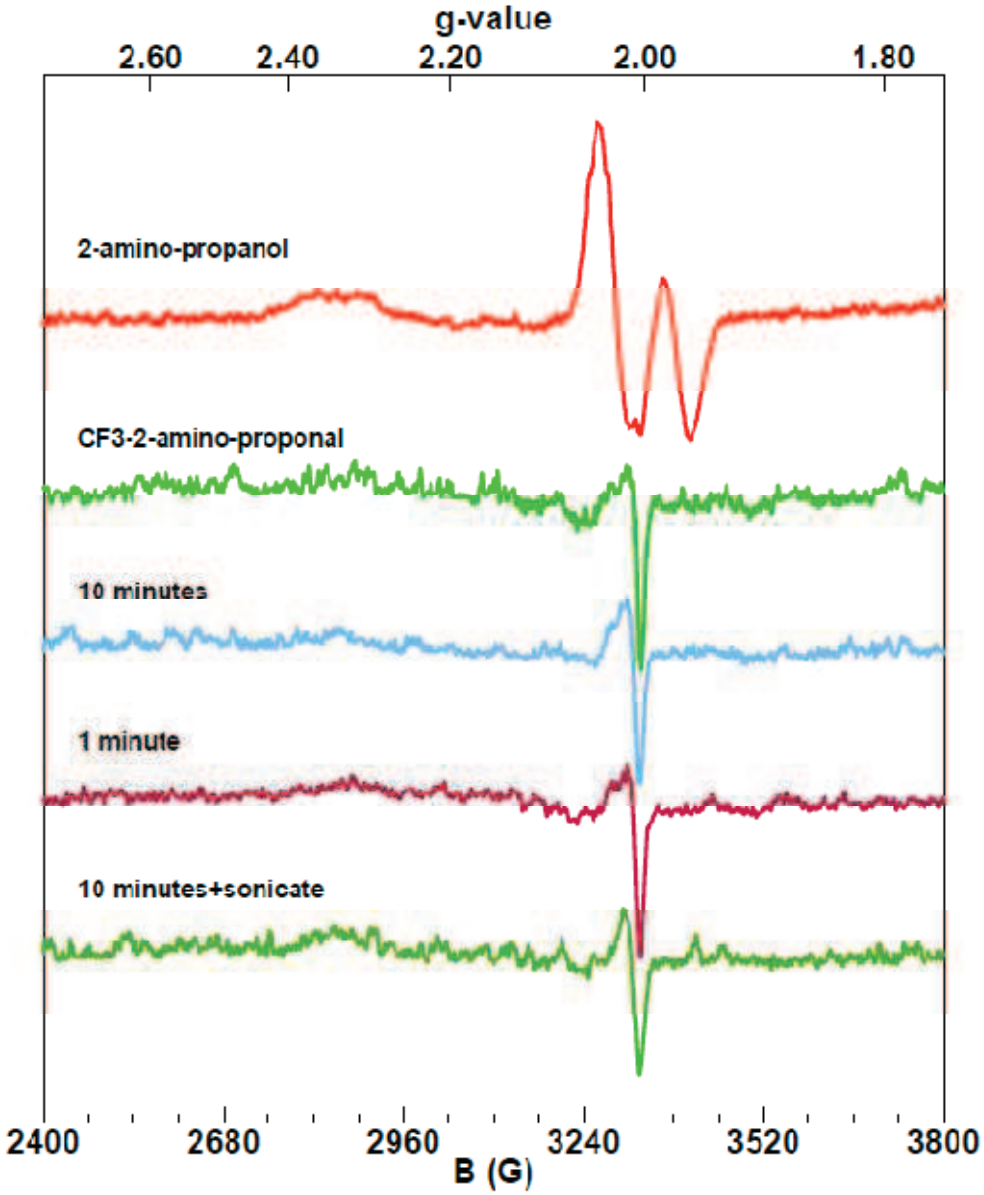


Figure 31: EPR spectra of 2-amino-propanol and 2-amino-3,3,3-trifluoropropanol. (Conditions: 10 mg/mL EAL (120 μM sites), 240 μM AdoCbl, 20 μM substrate, 10 mM potassium phosphate, pH 7.4.) One sample was frozen in isopentane immediately after the AdoCbl addition. Later this sample was thawed and allowed to incubate for 10 min at room temperature before freezing again. The second sample was frozen 1 min after the addition of AdoCbl. Later this sample was thawed and incubated for 10 min and sonicated before refreezing.

The negative results obtained might be explained by the inductive effect of the trifluoromethyl group and the size of the group. As stated in section 4.1 the pK_a of the amine group has been significantly reduced from 10.7 to *ca.* 5.7 by the CF_3 substituent, and the pK_a of the alcohol group has been reduced from 16 to *ca.* 14.6. These changes could lead to no binding or weak binding between the 2-amino-3,3,3-trifluropropanol and EAL in contrast to the binding with 2-amino-propanol.

The van der Waals volume of the trifluoromethyl group was estimated to be close to that of an ethyl or isopropyl group although of significantly different shape.[154, 155] Thus, 2-amino-3,3,3-trifluoropropanol may not fit into the active site of the EAL due to the much bigger size of trifluoromethyl group compared to than methyl group. This also could explain the absence of the CF_3-substrate radical. Since glycerol dehydratase can catalyse the conversion of glycerol to 3-hydroxypropionaldehyde, it can tolerate the bigger size of trifluoromethyl group. Hence, 3,3,3-trifluoropropane-1,2-diol was shown to be a substrate, albeit weak for glycerol dehydratase.[160]

So we propose to synthesise 2-amino-3-fluoropropanol in future work. The EPR study will bring us the further insight of the effect that can be brought by fluorine.

3.5 INTRODUCTION TO RADIATION CHEMISTRY

The hydroxyl radical, a most damaging reactive oxygen species, plays an important role in creating radical centres in biological systems through either hydrogen atom abstraction or hydroxyl radical addition.[164] It can be generated by radiolysis of water.

In practice, ^{60}Co γ–rays or fast electrons from an accelerator with energies in the range 2 - 20 MeV were used for the radiation of water. Major reactions in the radiolysis of water are summarised below (the estimated time by which the reaction is complete is given):

$$H_2O \xrightarrow[\text{radiation}]{\text{ionizing}} H_2O^{\bullet+} + e^-; \text{ and } H_2O^* \qquad 10^{-16}\ s \qquad (1)$$

$$H_2O^{\bullet+} + H_2O \longrightarrow HO^\bullet + H_3O^+ \qquad 10^{-14}\ s \qquad (2)$$

$$H_2O^* \longrightarrow HO^\bullet + H^\bullet \qquad 10^{-13}\ s \qquad (3)$$

$$e^- + nH_2O \longrightarrow e_{aq}^- \qquad 10^{-12}\ s \qquad (4)$$

The reaction given in **Eq. 1** represents the two major processes under the ionizing radiation in liquid water, which are ionization and electronic excitation of water molecules. The water radical cation, H_2O^+, is known as a strong acid. It loses a proton rapidly to another water molecule (**Eq. 2**). The excited water molecules can break up into an H atom derived radical and hydroxyl radical. (**Eq. 3**) The reaction given in **Eq. 4** shows that the electron becomes solvated in the liquid water within 10^{-12} s.

Next, these products either react with one another to form molecular or secondary radical products, or randomly diffuse into the bulk of the solution. Finally, by about 10^{-7} s, the radiolysis of neutral water can be described by the reaction:[165]

$$H_2O \xrightarrow[\text{radiation}]{\text{ionizing}} 0.28\ e_{aq}^- + 0.062\ H^\bullet + 0.28\ HO^\bullet + 0.047\ H_2 + 0.073\ H_2O_2 + 0.28\ H_3O^+$$

Where the values are the radiation chemical yields (*G* values) in units of μmol J^{-1}.

With the help of nitrous oxide (N_2O) as discovered by Dainton and Peterson,[166] the solvated electrons are converted into hydroxyl radicals according to the following reaction:

$$e_{aq}^- + N_2O \longrightarrow N_2 + O^- \xrightarrow{H_2O} N_2 + {}^\bullet OH + OH^-$$

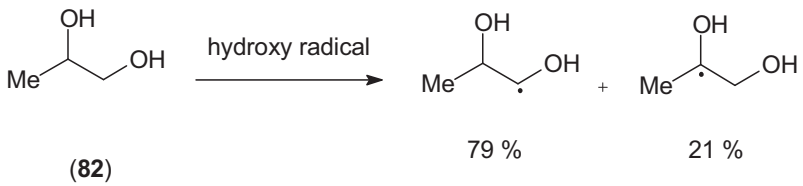
Therefore, a method achieved by saturating water with nitrous oxide (a saturated solution contains about 25 mol m^{-3} N_2O at 1 atm, 25 °C) could yield 90 % of the radicals available, which are hydroxyl radicals ($HO^\cdot$) and 10 % hydrogen radicals ($H^\cdot$).

3.6 PRODUCT STUDY OF THE HYDROXYL RADICAL ATTACK ON 3,3,3-TRIFLUOROPROPANE-1,2-DIOL AND PROPANE-1,2-DIOL

3.6.1 Introduction

As a result of their high reactivity, hydroxyl radicals often abstract carbon-bound hydrogen atoms more where the carbon-hydrogen binding energy are relatively low in preference to stronger bonds. For example, with propan-2-ol 95 % of the reaction occurs at the secondary (α) carbon atom.[167] Hydrogen abstraction from oxygen of alcohol groups always occurs in quite low yield.

The radiation study was carried out by the author at CNR, Bologna supervised by Dr. Chryssostomos Chatgilialoglu. Radicals were generated by continuous γ-irradiation of 3,3,3-trifluoropropane-1,2-diol (**68**) in water saturated with nitrous oxide. The properties of the radicals formed were compared with the corresponding radicals from propane-1,2-diol (**82**). The reaction of hydroxyl radical with propane-1,2-diol (**82**) gave a mixture of radicals (**Figure 32**).[167]



(**82**)

Figure 32: Relative yields of radicals from hydrogen radical abstraction for propane-1,2-diol (**82**)[167]

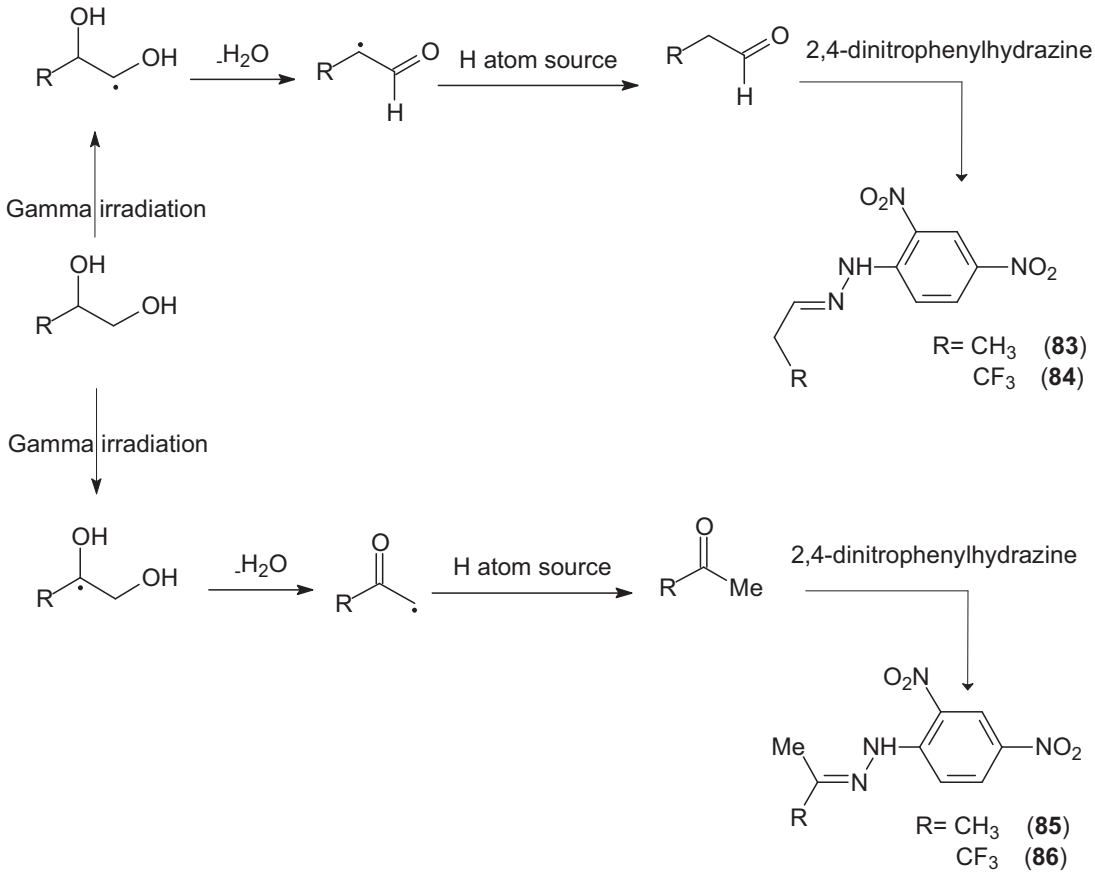
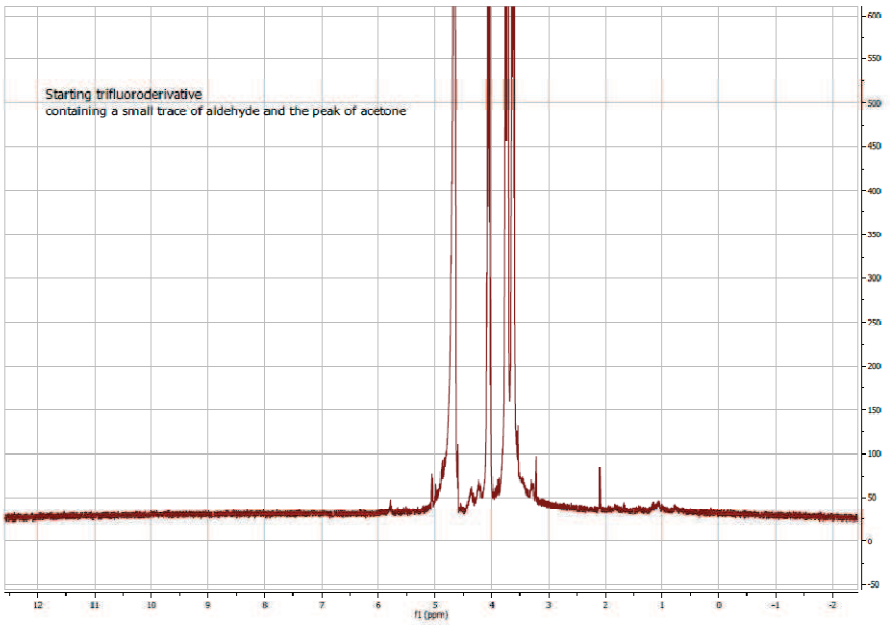


Figure 33: Potential products from the radicals derived from 3,3,3-trifluoropropane-1,2-diol (**68**) and propane-1,2-diol (**82**).

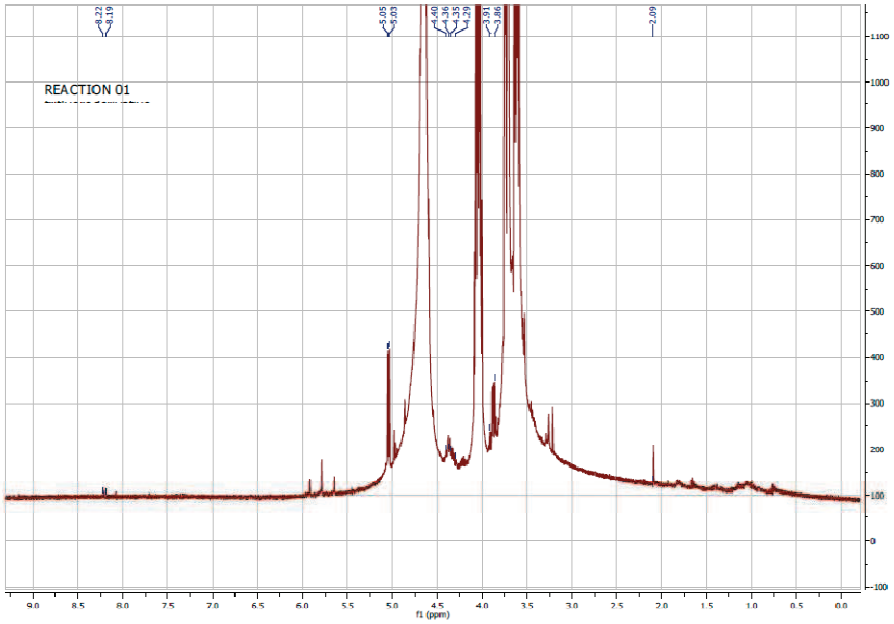
3.6.2 Product study of the crude irradiation product (3,3,3-trifluoropropane-1,2-diol)

A solution of 3,3,3-trifluoropropane-1,2-diol (0.6-1 mmol) in D_2O (0.6-1 ml) was saturated with N_2O. The solution was γ-irradiated with a dose of 1000 Gy to 3000 Gy. ^{1}H-NMR analysis of the crude irradiated sample indicated that there is a novel carbonyl compound formed in the reaction. The ^{1}H-NMR analysis of the starting material showed an impurity of acetone (at δ = 2.06 ppm). (**Spectrum 1**) After the irradiation (**Spectrum 2**), there was evidence of the formation of 1,1-difluoro-3-

hydroxypropanone (a triplet at δ = 5.50 ppm, J_{HF} = 50 Hz corresponding to the CHF_2 group and a singlet at δ = 5.05 ppm corresponding to the CH_2 group). Freeze drying was carried out to remove D_2O. GC analysis of the residue determined that, starting material and products had been removed by this operation.

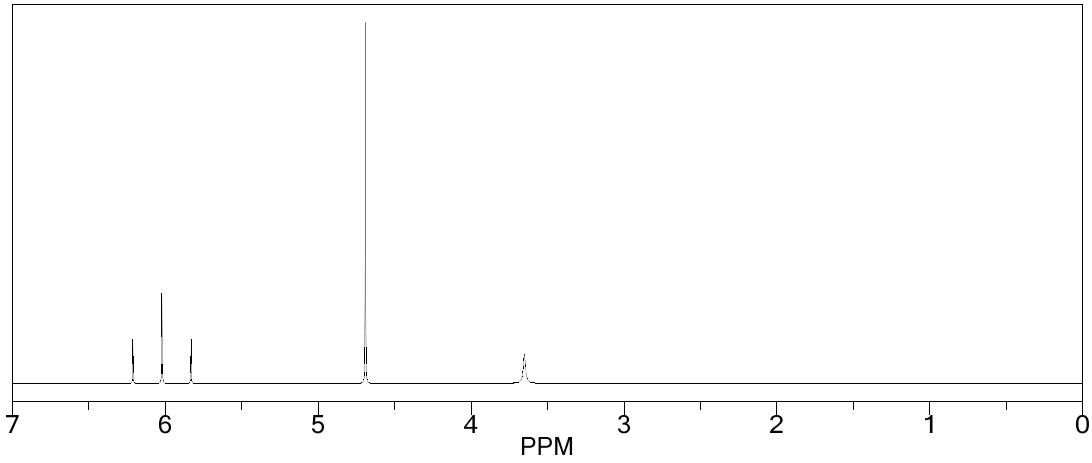


Spectrum 1: Starting material of irradiation, 3,3,3-trifluoropropane-1,2-diol



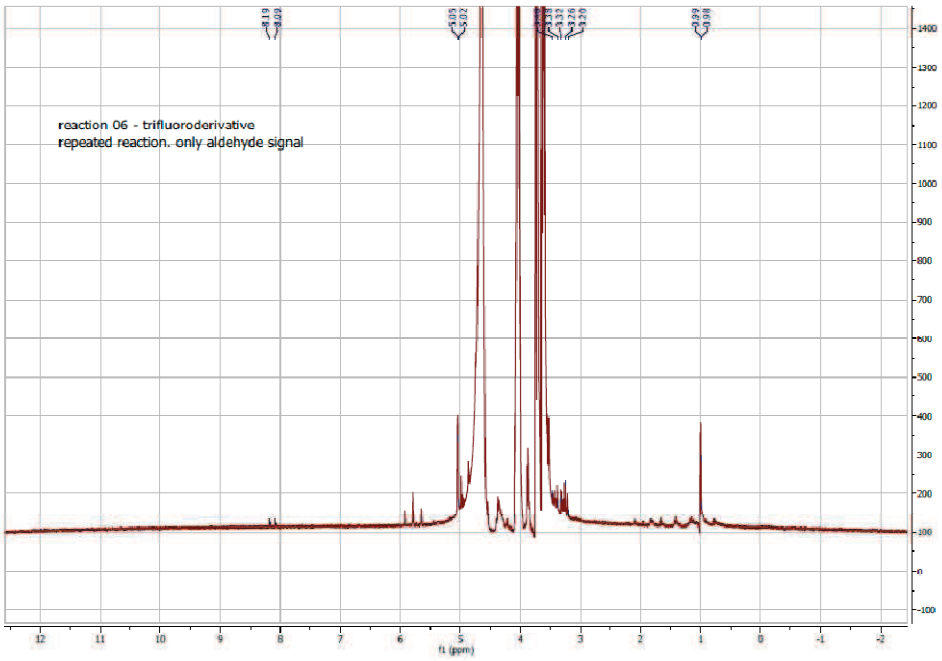
Spectrum 2: Irradiation mixture of 3,3,3-trifluoropropane-1,2-diol with a dose of 1000 Gy

No evidence of the formation of expected 3,3,3-trifluoropropanal and 1,1,1-trifluoroacetone was found in the ^{1}H NMR analysis. Authentic 3,3,3-trifluoropropanal in D_2O showed a triplet at δ = 5.22 ppm corresponding to the CH group and a multiplet at δ = 2.41 ppm corresponding to the CH_2 group. These resonances were not observed in the reaction mixture from irradiation. The estimated ^{1}H NMR of 1,1-difluoro-3-hydroxypropanone was shown in **Spectrum 3**.



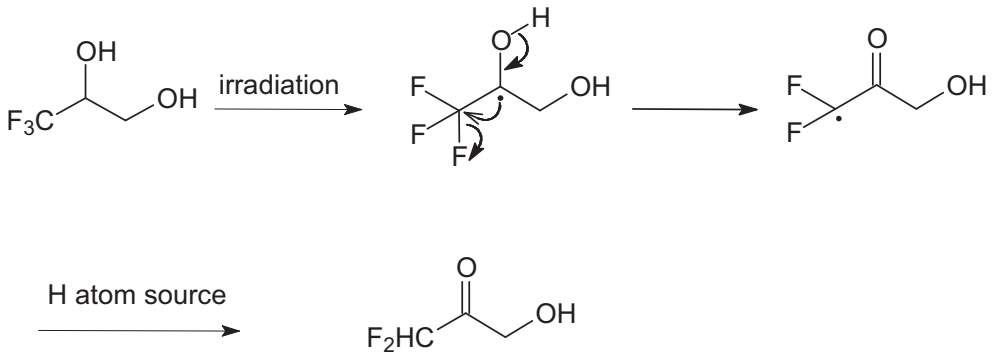
Spectrum 3: Estimated ^{1}H NMR of 1,1-difluoro-3-hydroxypropanone by ChemBioOffice 2008.

The reaction was repeated with dose of 3000 Gy in D_2O. ^{1}H-NMR analysis of the crude irradiated sample determined that again there was formation of 1,1-difluoro-3-hydroxypropanone (a triplet at δ = 5.50 ppm, J_{HF} = 50 Hz and a singlet at δ = 5.05 ppm). No evidence of the formation of 3,3,3-trifluoropropanal and 1,1,1-trifluoroacetone was found in this spectrum. (**Spectrum 4**)



Spectrum 4: Irradiation mixture of with a dose of 3000 Gy

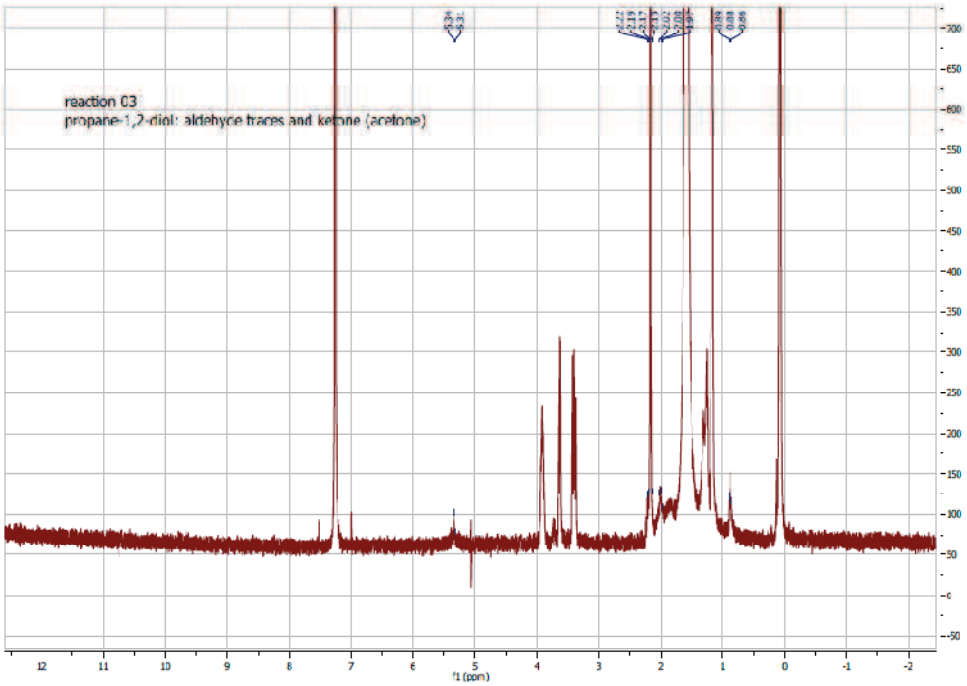
A possible mechanism for the formation of 1,1-difluoro-3-hydroxypropanone in the reaction is shown in **Scheme 24**. The coupling constant J_{HF} for the CHF_2 of 1,1-difluoro-3-hydroxypropanone was ca. 50 Hz, which is the same as that of 1,1-difluoroacetic acid. 1,1-Difluoro-3-hydroxypropanone has not been described in the literature and so its definitive identification requires an independent synthesis.



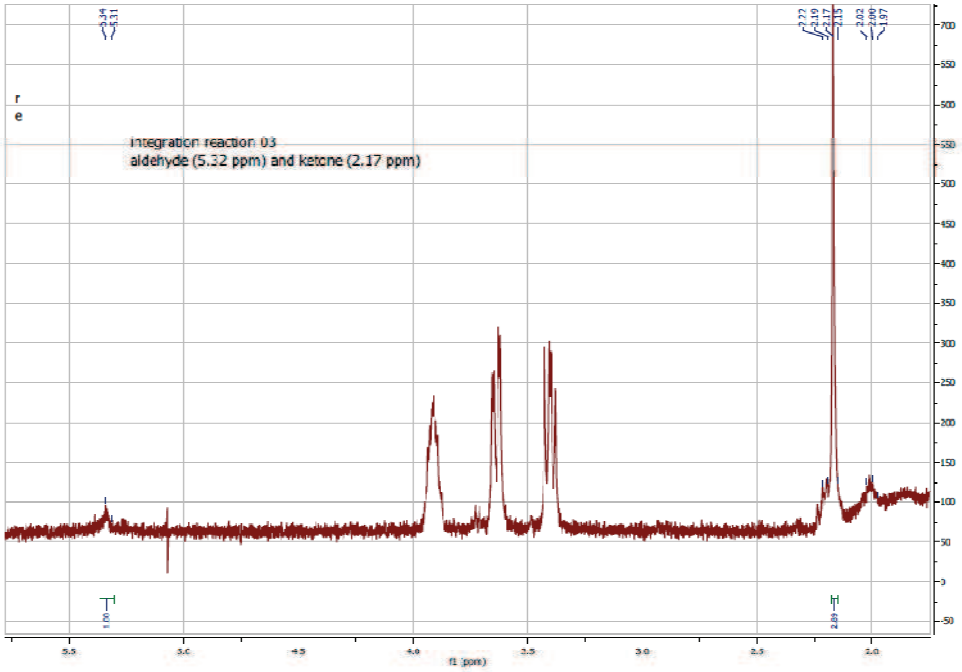
Scheme 24: Proposed mechanism of formation of 1,1-difluoro-3-hydroxypropanone

3.6.3 Product study of the crude irradiation product (propane-1,2-diol)

A solution of propane-1,2-diol (0.20-1 mmol) in D_2O (1 mL) was saturated with N_2O. The solution was γ-irradiated with a dose of 1000 to 3000 Gy. A few drops of 0.5 *N* hydrochloric acid was added to the crude solution. 1 mL of $CDCl_3$ was added to extract the mixture. ^{1}H-NMR analysis of the extract sample determined that there are aldehyde and ketone products formed in the reaction, with aldehyde hydrate at δ = 5.34 ppm and acetone δ = 2.17 ppm respectively. Propane-1,2-diol showed a doublet at δ = 1.15 ppm corresponding to the CH_3 group, a doublet at δ = 3.45 ppm and a triplet at δ = 3.60 ppm corresponding to the CH_2 group and a multiplet at δ = 3.92 ppm corresponding to the CH group. (**Spectrum 5 and 6**)

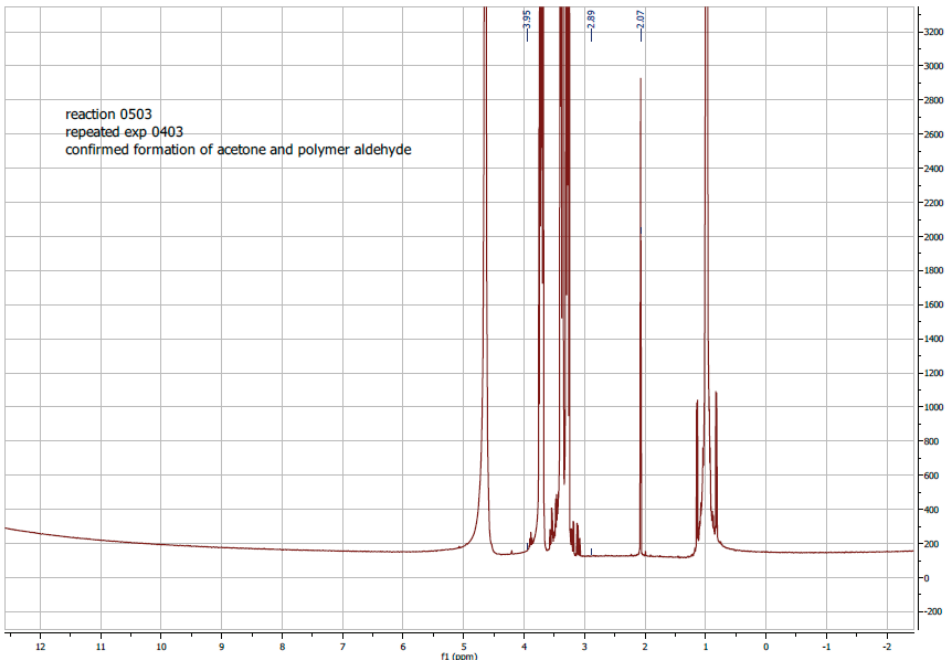


Spectrum 5: Irradiation mixture of propane-1,2-diol with a dose of 1000 Gy



Spectrum 6: Expansion of Spectrum 4

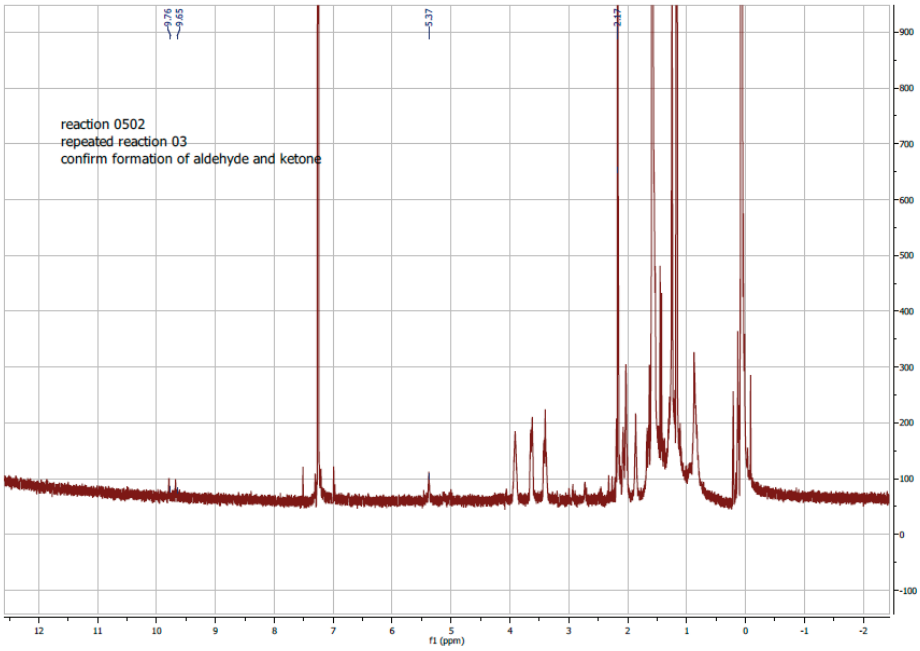
So the reaction was repeated with 3000 Gy in D_2O. ^{1}H-NMR analysis of the crude irradiated sample determined that acetone (δ = 2.07 ppm) and possibly a polymeric product derived from aldehyde (multiplets in the region of δ = 3-3.9 ppm) was formed in the reaction. (**Spectrum 7**)



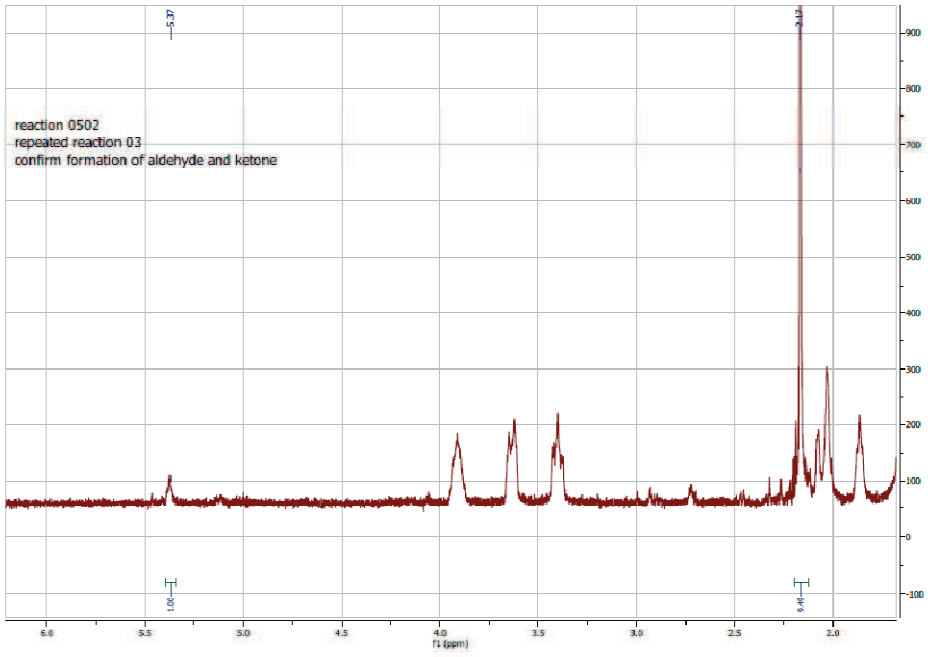
Spectrum 7: Irradiation mixture of propane-1,2-diol with a dose of 3000 Gy

The crude product was acidified, and extracted with $CDCl_3$. ^{1}H-NMR analysis of the extract sample determined that there were an aldehyde and an acetone formed in the reaction. (**Spectrum 8 and 9**)

GC analysis of the organic extract solution from the acidic crude sample indicated that there is a new compound formed in the reaction (retention time, 3 min; the solvent is at 4 min). Propanal was found in the GCMS analysis of the sample.



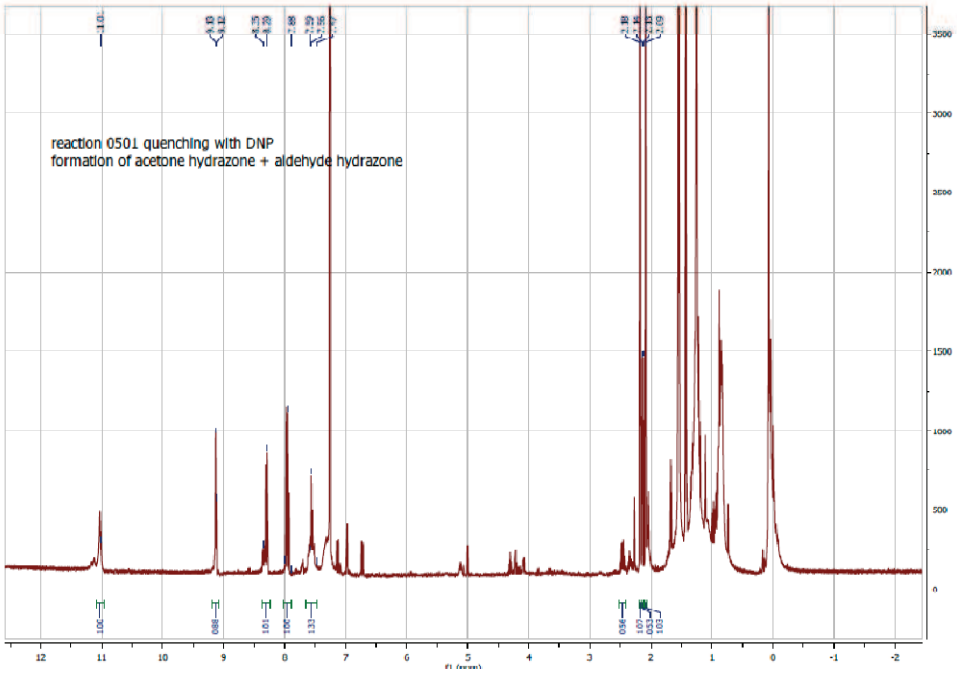
Spectrum 8: Extracts from acidic irradiation mixture of propane-1,2-diol with a dose of 3000 Gy



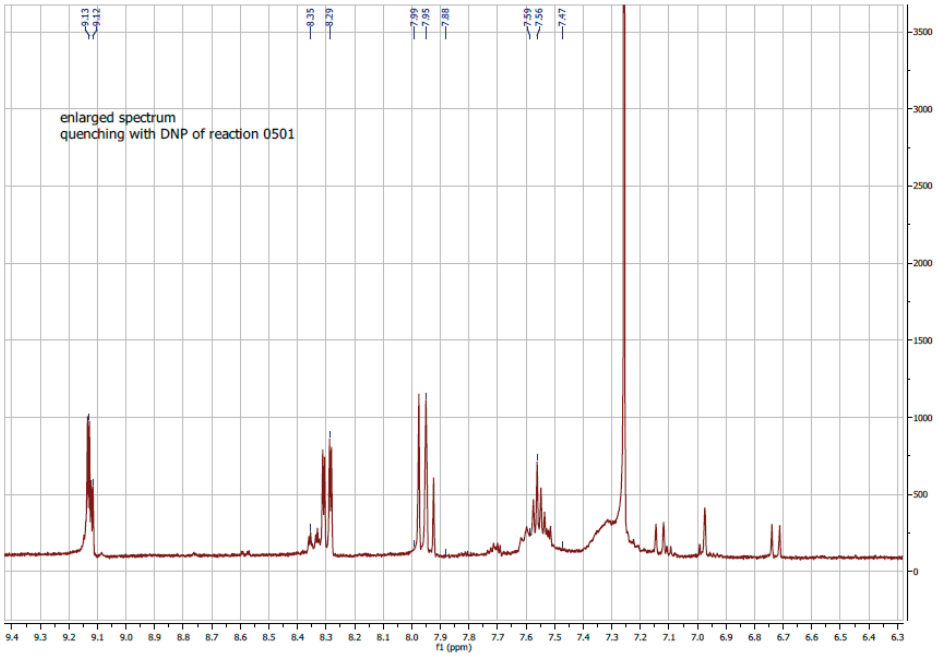
Spectrum 9: Expansion of Spectrum 8

3.6.4 Product study of the DNP derivatives

In another experiment, 2,4-dinitrophenylhydrazine was used to trace the formation of carbonyl groups. A solution of propane-1,2-diol in H_2O was γ-irradiated with a dose of 3000 Gy in the presence of N_2O. 2,4-Dinitrophenylhydrazine was added to convert the product to 2,4-DNP derivatives. TLC study (eluting with diethyl ether and n-hexane, 3:7) indicated that new compounds were formed. ^{1}H-NMR analysis of the solid determined that there were propanal-2,4-dinitrophenylhydrazone and acetone-2,4-dinitrophenylhydrazone formed. (**Spectrum 10 and 11**, characteristic peaks: at δ = 11.0 ppm, propanal-2,4-dinitrophenylhydrazone and δ = 2.18 and 2.09 ppm acetone-2,4-dinitrophenylhydrazone)



Spectrum 10: 2,4-DNP derivatives of irradiation mixture of propane-1,2-diol with a dose of 3000 Gy



Spectrum 11: Expansion of Spectrum 10

The reaction was repeated in D_2O with 3,3,3-trifluoropropane-1,2-diol as starting material. ^{1}H-NMR analysis of the crude irradiated sample determined again there are 1,1-difluoro-3-hydroxypropanone was formed in the reaction. As before, no evidence of the formation of 3,3,3-trifluoropropanal and 1,1,1-trifluoroacetone was found in the spectrum. 2,4-Dinitrophenylhydrazine was added to generate 2,4-DNP derivatives. ^{1}H-NMR analysis of the resulting solid was not clear. Purification of the 2,4-DNP derivatives was unsuccessful due to the small amount present in the irradiation mixture.

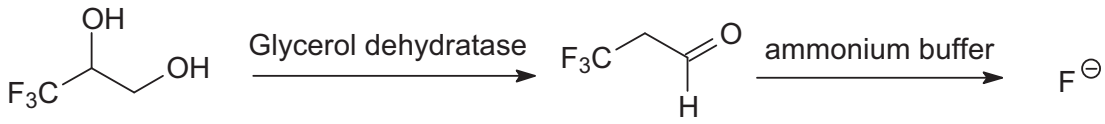
3.6.5 Results and future work

The NMR results indicated that propionaldehyde and acetone were formed by the continuous γ-irradiation from propane-1,2-diol (**82**); 1,1-difluoro-3-hydroxypropanone was formed by the continuous γ-irradiation of 3,3,3-trifluoropropane-1,2-diol (**68**). The expected 3,3,3-trifluoropropanal and 1,1,1-trifluoroacetone could not be determined from the ^{1}H NMR analysis of the irradiation mixuture of 3,3,3-trifluoropropane-1,2-diol (**68**). GC and GCMS results proved that propionaldehyde was formed from propane-1,2-diol.

Due to lack of time, the work was incomplete. More 3,3,3-trifluoropropane-1,2-diol (**68**) (more than 2 g) needed to be synthesised. Pulse radiolysis will be used to study the radical products and their relative yields. Further product study under continuous γ-irradiation will be carried out for 3,3,3-trifluoropropane-1,2-diol (**68**) and propane-1,2-diol (**82**) using DNP derivatives as a probe. (See Appendix for the latest results which was carried out from 08/09/10 to 06/11/10)

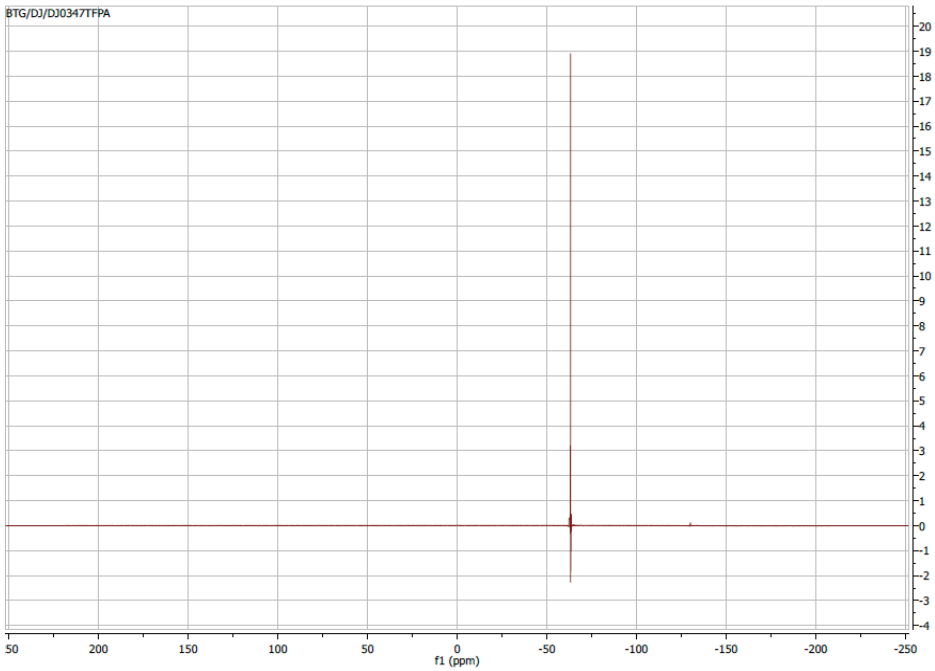
3.7 REACTION OF 3,3,3-TRIFLUOROPROPIONALDEHYDE WITH AMMONIUM ACETATE.

Previous study reported that using ammonium phosphate as a buffer, fluoride ion is a major product of the enzymatic reaction of 3,3,3-trifluoropropane-1,2-diol (**68**) with glycerol dehydratase.[160] This was proved to be related to the pH and use of ammonium buffer. There was no fluoride ion formed with tricine or potassium phosphate as buffer. The experiment also demonstrated that ammonium buffer at pH 6.5 had no effect on the products of the enzymatic reaction of 3,3,3-trifluoropropane-1,2-diol (**68**). However, ammonium buffer affected the products at pH 8.0. It was proposed that fluoride ion is derived by an ammonia-catalysed elimination on 3,3,3-trifluoropropionaldehyde.

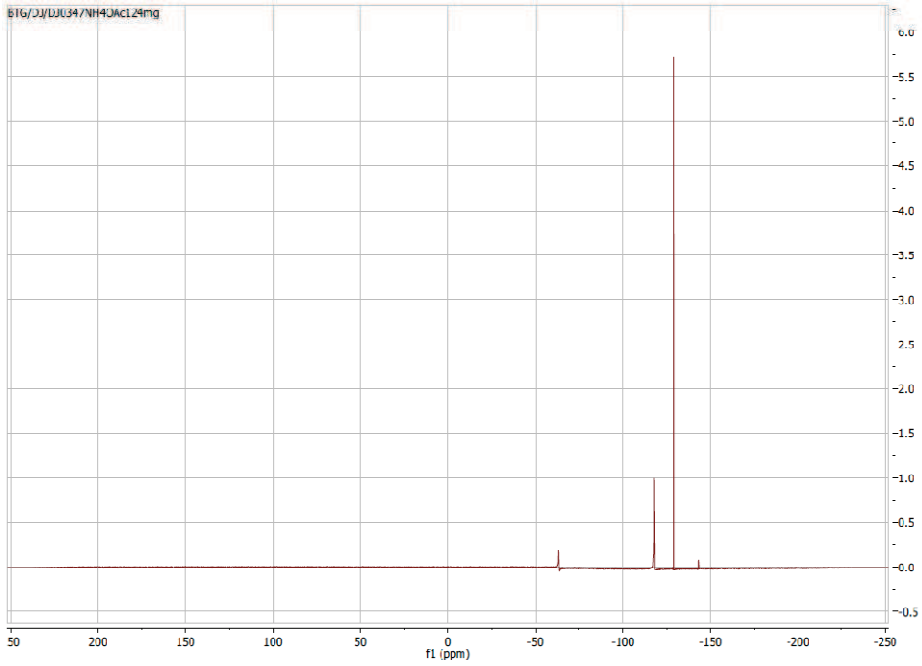


Scheme 25: Formation of fluoride ion in the enzymatic reaction of 3,3,3-trifluoropropane-1,2-diol (**68**) with glycerol dehydratase.

In order to try to understand better the mechanism of fluoride formation, we have studied the non-enzymatic reaction of 3,3,3-trifluoropropionaldehyde with ammonium acetate in D_2O (pH ≈ 7[168]).



Spectrum 13: ^{19}F NMR of authentic 3,3,3-trifluoropropionaldehyde



Spectrum 14: ^{19}F NMR of 3,3,3-trifluoropropionaldehyde treated with ammonium acetate after 72 hours.

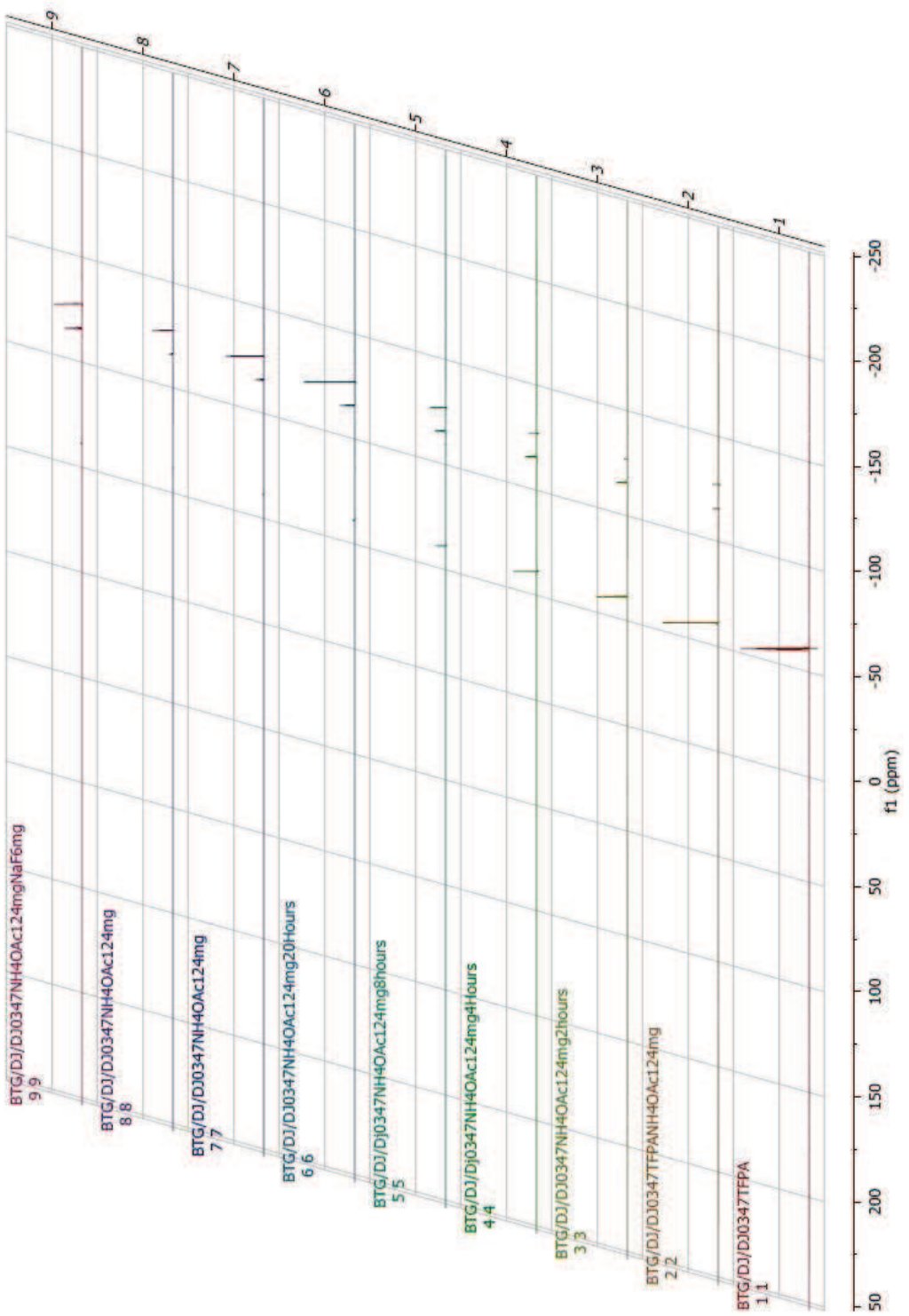
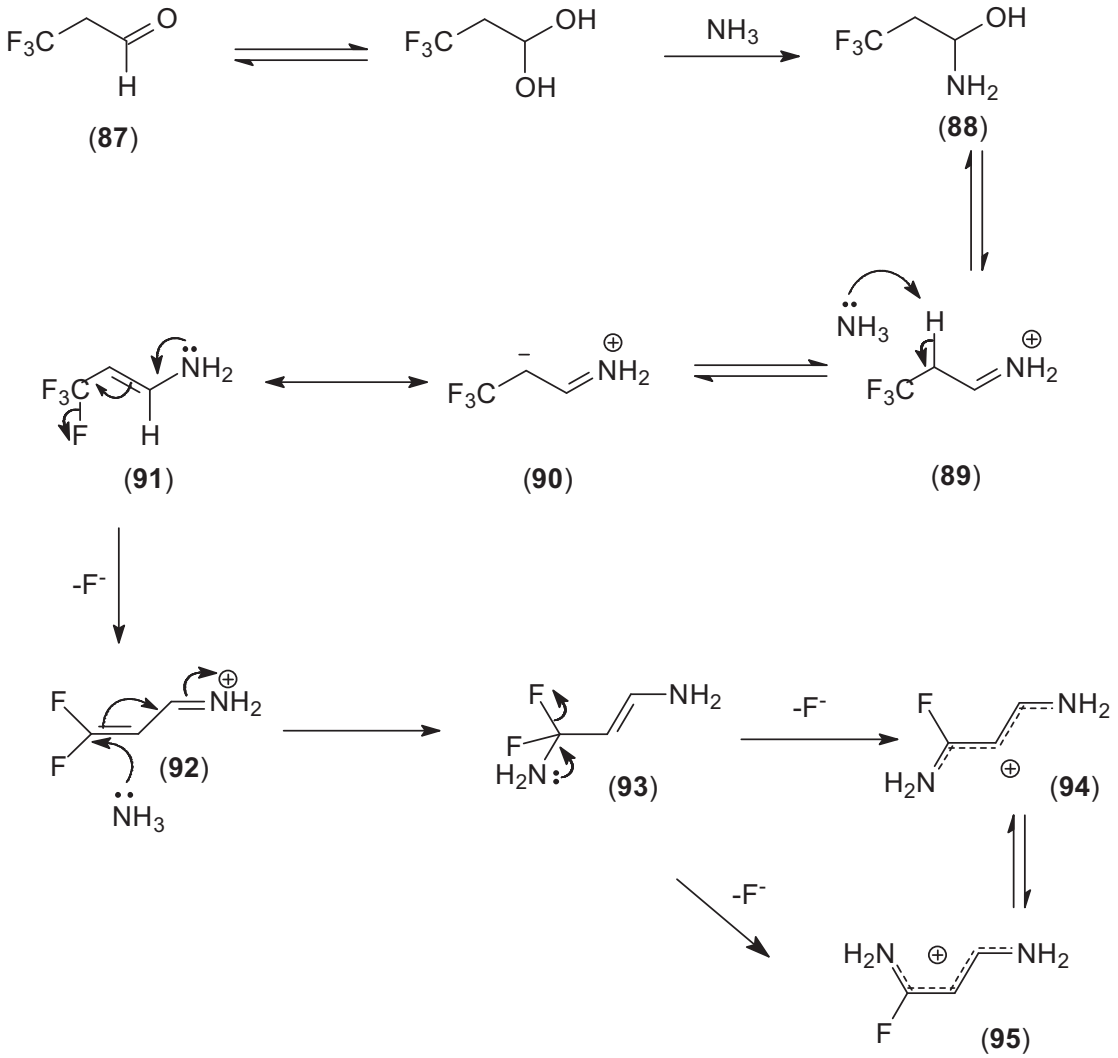


Figure 34: Spectrum 1 is trifluoropropionaldehyde; spectra 2-8 were taken at reaction times 20 min, 2 h, 4 h, 8 h, 20 h, 48 h, and 72 h; spectrum 9 was obtained from addition of NaF to the 72 h reaction.

The ^{19}F NMR indicated that fluoride ion was a product being observed as a singlet at δ = -118 ppm (relative to $CFCl_3$). It was identified by the addition of sodium fluoride, which enhanced the signal at δ = -118 ppm. The singlet at δ = -63 ppm assigned to the CF_3 group of 3,3,3-trifluoropropionaldehyde was found to be diminished quite fast. Two new peaks were detected in the ^{19}F NMR spectra, a major peak at δ = -129 ppm and a minor resonance at δ = -145 ppm (**Spectrum 13, 14**). **Figure 34** shows all the spectra taken at the times as noted. The time course study showed that the resonance assigned to fluoride initially grew but later declined in intensity.

In conclusion, we support Watson's proposal[160] that the reaction of ammonia and 3,3,3-trifluoropropionaldehyde with the elimination of fluoride ion is not controlled by the enzyme. ^1H NMR spectra of authentic 3,3,3-trifluoropropionaldehyde (**87**) indicated that the aldehyde is fully hydrated in aqueous solution. A possible mechanism for the elimination of fluoride ion in ammonia buffer from 3,3,3-trifluoropropionaldehyde is shown in **Scheme 26**. The end-products shown (**94** and **95**) are expected to be relatively stable and may be responsible for the observed resonances at δ = -129 and -145 ppm. It was assumed that the configurations of (**94**) and (**95**) were maintained because of a strong resonance interaction in the π–system. However, the definitive assignment of structures requires further work.



Scheme 26: Proposed mechanism of the reaction of 3,3,3-trifluoropropionaldehyde and ammonium acetate under basic conditions.

3.8 CONCLUSIONS

Fluorine-substituted probes for coenzyme B_{12}-dependent enzymatic reactions (3,3,3-trifluoropropane-1,2-diol (**68**) for diol dehydratase and 2-amino-3,3,3-trifluoropropanol (**69**) for EAL) have been synthesised.

2-Amino-3,3,3-trifluoropropanol (**69**) was found to be devoid of any activity against EAL according to a coupled assay with alcohol dehydrogenase.(collaboration with Dr G. H. Reed, University of Wisconsin-Madison). An EPR study (collaboration with Dr K. Warncke, Emory University) has concluded that the CF_3-substrate did not lead to the significant formation of a paramagnetic intermediate. This may be due to the CF_3 group reducing the basicity of the amino group so that there is a diminished interaction with the active site in EAL. Or this may be caused by the significant steric effect introduced by the trifluoromethyl group (the CF_3 group is approximately equivalent in size to an ethyl or isopropyl group[169]).

A model study in which the hydroxyl radical was used to attack the substrate analogue (**68**) of diol dehydratase showed that 1,1-difluoro-3-hydroxypropanone was formed by attacking on 3,3,3-trifluoropropane-1,2-diol (**68**), the expected 3,3,3-trifluoropropionaldehyde and 1,1,1-trifluoroacetone could not be determined from the ^{1}H NMR analysis of the irradiation mixuture. Propanal and acetone were formed from the attack on propane-1,2-diol (analysis by ^{1}H NMR, GC and GCMS, and by formation of the corresponding 2,4-dinitrophenylhydrazones).

Chapter 4

Synthesis of a Bromo-precursor of an Intermediate Radical for Glutamate Mutase

4.1 INTRODUCTION

Coenzyme B_{12}-dependent enzymes have been associated with radical reactions for many years. In non-enzymatic environments, model studies of these possible radical intermediates may provide evidence for the mechanistic pathways. By generating radicals as proposed in the enzymatic mechanism using suitable radical precursors, their interconversion may be observed and defined in a close physiological but non-enzymatic environment.

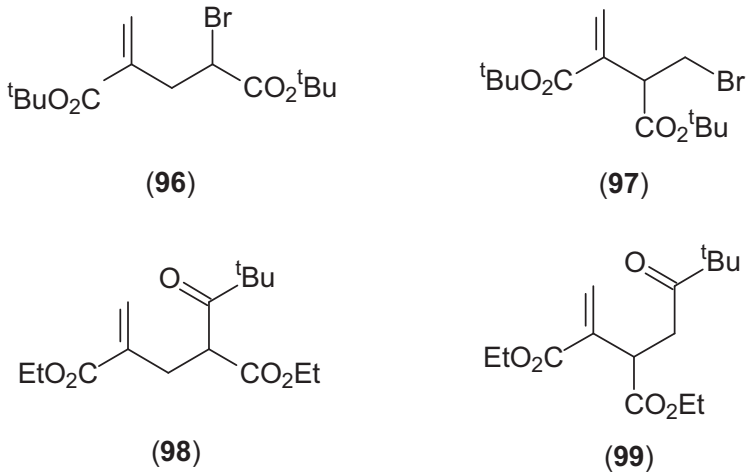


Figure 35: Halide-based and ketone-based radical precursors related to the reaction catalysed by 2-methyleneglutarate mutase.

In previous studies, the intermediate radicals were generated using bromides as precursors by treatment with silyl or tin hydrides.[170, 171] The bromo-precursors (**96**) and (**97**) for radicals in the reaction catalysed by coenzyme B_{12} dependent 2-methyleneglutarate mutase have been obtained successfully.[172] (**Figure 35**)

Another well-known process for the generation of radicals is the Norrish Type I fragmentation of ketones, which can lead to the cleavage of a carbon-carbon bond adjacent to a carbonyl group (**Figure 36**). A typical model involves the *t*-butyl ketone (^{t}BuCOR) that leads to the radical R and *t*-butyl radical by carbon-carbon bond cleavage between the *t*-butyl carbonyl and the desired R groups upon photolysis with light of wavelength ca. 320 nm.[173, 174](**Figure 36**)

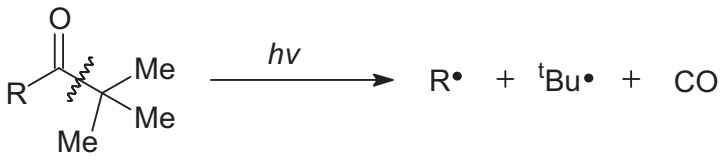


Figure 36: Photochemical cleavage by Norrish Type I fragmentation reaction

The type of precursors for radicals in the reaction catalysed by 2-methyleneglutarate have been shown in **Figure 35**. These two compounds (**98**) and (**99**) have been synthesised by Lopez and the model study showed that the expected radicals were generated from these precursors by using a mercury lamp.[112]

Coenzyme-B_{12} dependent glutamate mutase is a radical enzyme, which catalyses the reversible isomerisation of (*S*)-glutamate to (*2S*,*3S*)-3-methylaspartate (**Scheme 2**) *via* enzyme-bound radical intermediates (see Chapter 1).[175] It is generally accepted that the mechanism of glutamate mutase involves removal of H_{si} from glutamate to give a 4-glutamyl radical that undergoes fragmentation to acrylate and a glycine radical.[60, 61] Recombination of these species leads to the 3-methylene-aspartate radical and hence (2*S*,3*S*)-3-methylaspartate. The generally accepted mechanism has been shown in **Scheme 4** and discussed in Section 1.6.2.

In this study, the stereochemical difference between the three hydrogen atoms of the methyl group at C-3 position of 3-methylaspartate was not considered. The 3-methylene-aspartate radical (**102**) could be generated from the prepared precursors (**100** and **101**), where a keto group or a bromine is attached to the C-3 position. Therefore the synthesis of precursors for the radical (**102**) was required. (**Figure 37**)

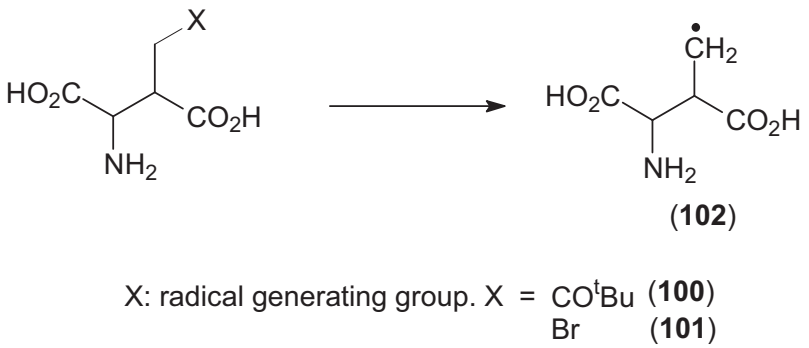


Figure 37: Formation of 3-methylene-aspartate radical (**102**) by irradiation of precursors.

It is proposed to synthesise precursors for the intermediate radicals in the glutamate mutase reaction and determine whether these exhibit a tritylase-catalysed rearrangement. After binding to the tritylase, radicals will be released by photolysis (e.g. Br-R $\rightarrow$ R•) and their tritylase-controlled chemistry will be evaluated. This study is important for assessing the new concept of cob(II)alamin as a conductor in coenzyme B_{12}-dependent mutases.

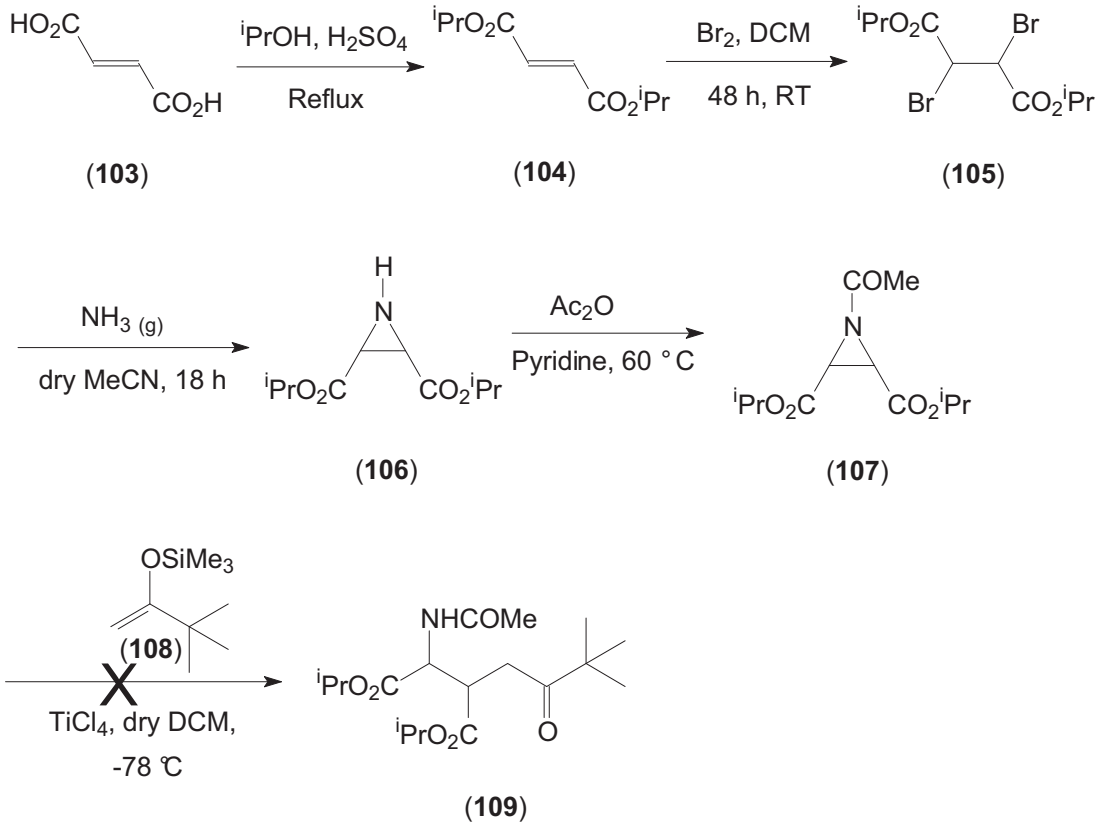
This chapter describes research and development of a synthetic methodology towards a radical precursor for 3-methylaspartate. A variety of different synthetic routes have been attempted to the radical precursors (**100**) and (**101**). Various problems have been

encountered in the synthetic approaches. However, the groundwork has been laid for the successful completion of the synthesis of the desired precursors.

4.2 SYNTHETIC APPROACH TO THE RADICAL PRECURSOR 2-AMINO-3-(2,2-DIMETHYL-PROPIONYL)-SUCCINIC ACID

The synthesis of 2-amino-3-(2,2-dimethyl-propionyl)-succinic acid (**100**) followed a previous study which was carried out in our research group before.[108] The synthesis strategy was based on Mukaiyama aldol addition of enol-silyl ethers[176] aiming for ring-opening process on the acetyl-protected aziridine.(**Scheme 27**)

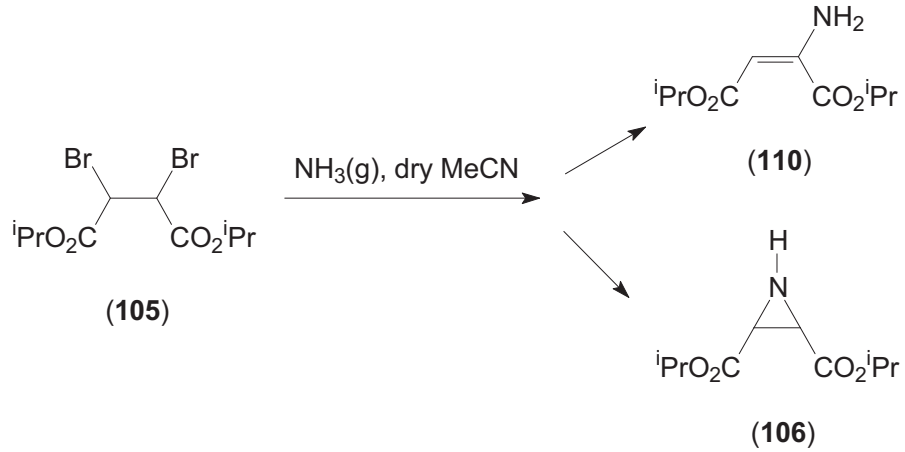
Protection of carboxylic acid groups with isopropanol under acid catalyst led to (*E*)-but-2-enedioic acid diisopropyl ester (**104**). ^{1}H NMR showed two doublets at δ = 1.21 ppm, which correspond to the methyl protons of the isopropyl groups, a septet at δ = 5.02 ppm corresponding to the CH protons of the isopropyl groups, and a singlet at δ = 6.73 ppm corresponding to the vinyl protons. The integration suggested that the product did not need any further purification. The bromination of alkene group (**104**) with bromine under mild conditions proceeded easily, and gave 2,3-dibromo-succinic acid diisopropyl ester **(105)** in very good yield (*ca* 80 %). A singlet at δ = 4.62 ppm in the ^{1}H NMR spectrum corresponded to the protons at C-2 and C-3 (CHBr).



Scheme 27: Synthetic route to 2-acetylamino-3-(3, 3-dimethyl-2-oxo-butyl)-succinic acid diisopropyl ester (**109**) *via* Mukaiyama chemistry

Treatment of 2,3-dibromosuccinic acid diisopropyl ester **(105)** with saturated ammonia gas in acetonitrile at a lower temperature gave aziridine-2, 3-dicarboxylic acid diisopropyl ester **(106)**. ^{1}H NMR analysis showed the characteristic signals at δ = 1.75 and 2.75 ppm corresponding to the N-H proton and the C-H protons of the aziridine ring, respectively. The method was based on the literature for synthesis of the corresponding aziridine methyl ester.[177, 178, 179] A side product (*E*)-2-amino-but-2-enedioic acid diisopropyl ester **(110)** was isolated from the reaction. (**Scheme 28**) It was shown in ^{1}H NMR as a characteristic singlet at δ = 5.40 ppm corresponding to the vinyl proton. The integration of ^{1}H NMR suggested that the ratio of compound **110** to

106 was 5 : 2. A possible mechanism of the conversion is that the elimination of HBr from compound (**105**) gave diethyl 2-bromofumarate, which reacted with NH_3 by an addition-elimination mechanism.



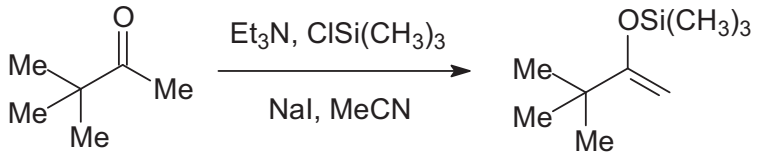
(**105**)

(**110**)

(**106**)

Scheme 28: Synthesis of aziridine-2, 3-dicarboxylic acid diisopropyl ester (**106**) and its side product (*E*)-2-amino-but-2-enedioic acid diisopropyl ester (**110**)

The protection of aziridine 2,3-dicarboxylate (**106**) with an acetyl group was performed by using acetic anhydride following a standard procedure for protection of amines.[180] The ^{1}H NMR showed an additional singlet at δ = 2.09 ppm and no peak was observed corresponding to an N-H proton. 1-Acetyl-aziridine-2,3-dicaboxylic acid diisopropyl ester (**107**) was reacted with the silyl enol ether of pinacolone (**108**) in the presence of titanium tetrachloride following the standard procedure of Mukaiyama aldol addition. However the reaction did not occur and the starting material (**107**) was isolated from the reaction mixture. It suggested that Mukaiyama aldol addition could not apply on the ring open of aziridine ring. The silyl enol ether of pinacolone (**108**) was obtained by reaction of trimethylsilyl chloride on pinacolone (**111**) following the procedure reported by Cazeau *et al*. (**Scheme 4**)[181] (**Scheme 29**)



(**111**) (**108**)

Scheme 29: Synthesis of (2,2-dimethyl-1-methylene-propoxy)-trimethyl-silane (**108**)

A likely explanation for the failure of this reaction is that the three carbonyl groups strengthen the aziridine ring so that it could not be opened by a nucleophilic attack in the presence of Lewis acid. There are a few examples in the literature where the aziridine ring could be opened[184, 185] (**Figure 38**). These examples indicated that the difference of N-substituent and the substituents on both carbons would stabilise the transition state. However, all three groups are electron withdrawing groups in this work.

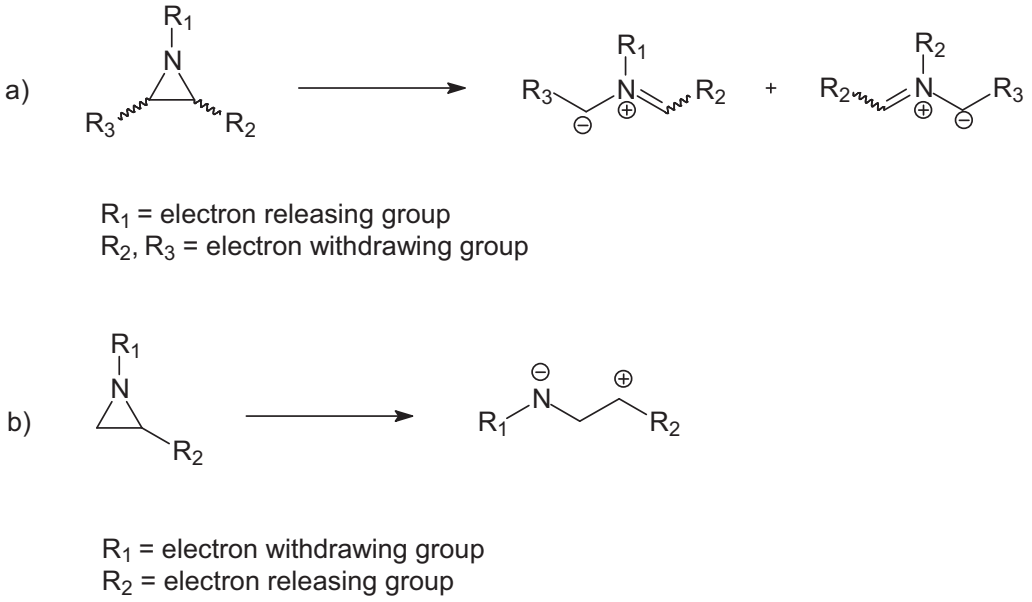


Figure 38: Transition states for the aziridine ring-open reaction. In mechanism a N-substituent is an electron releasing group;[182] in mechanism b it is an electron withdrawing group.[183]

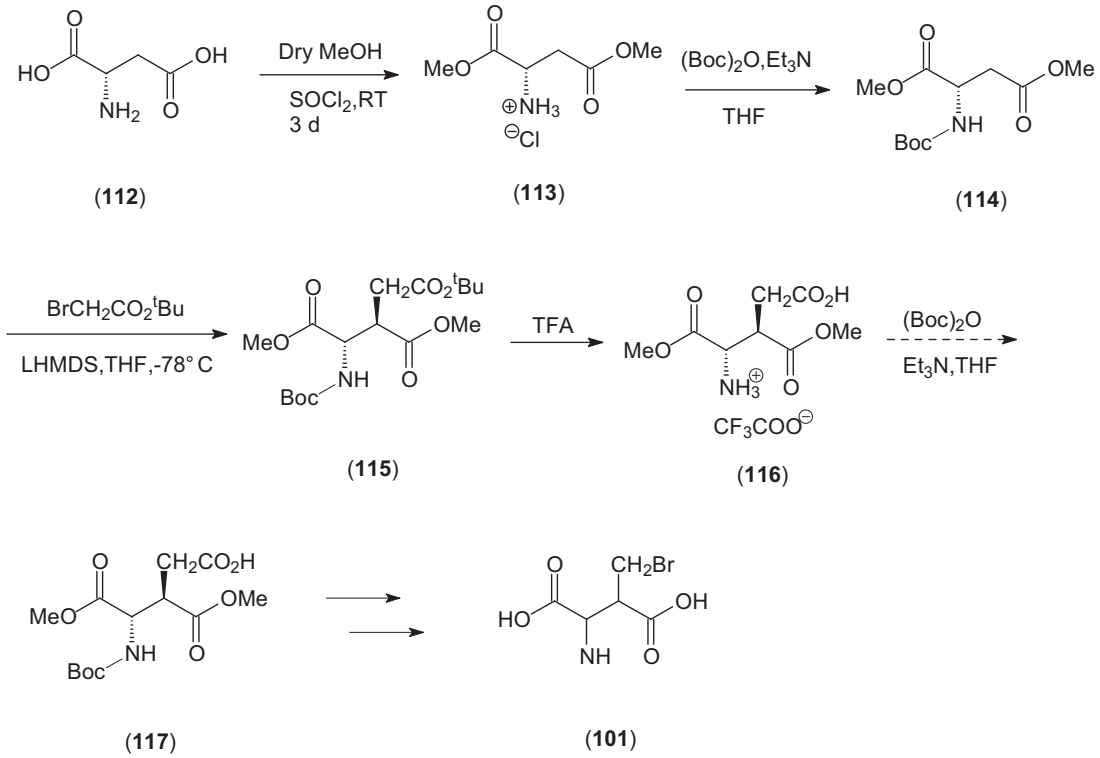
4.3 SYNTHETIC APPROACH TO THE RADICAL PRECURSOR 2-AMINO-3-BROMOMETHYL-SUCCINIC ACID

Due to the failure of synthesis of the radical precursor (**100**) with *t*-butyl ketone as radical initiator, bromine group was chosen as the radical initiator. Several synthetic pathways toward the radical precursor, 2-amino-3-bromomethyl-succinic acid (**101**), have been attempted. The first synthetic approach towards the radical precursor involved a β-alkylation of aspartic acid with a consequent trapping of a methyl radical generating group (bromomethyl group) at β position of aspartic acid (**Scheme 30**).

4.3.1 Synthetic approach *via* β-alkylation of aspartic acid

The synthetic route towards the radical precursor of 3-methylaspartate is outlined in **Scheme 30**. The first step of this synthesis leads to dimethyl (*S*)-aspartate hydrochloride (**113**) by reacting commercially available (*S*)-aspartic acid (**112**) with methanol in the presence of thionyl chloride. This reaction was performed easily on large scale (*ca* 20 g of product). The product without further purification was proved pure by ^{1}H NMR analysis as two singlets at δ = 3.56 and 3.65 ppm corresponding to the two methoxyl protons; and two triplets at δ = 3.18 and 4.51 ppm, which correspond to the protons at the carbon adjacent the carbonyl group. The protection of amino group with a *t*-butyloxycarbonyl (Boc) group was carried out by treatment with Boc anhydride in the presence of triethylamine to gave (*S*)-dimethyl 2-(*tert*-butoxycarbonylamino)succinate (**114**). The compound was characterised by ^{1}H NMR which showed a singlet at δ = 1.43 ppm corresponding to the methyl protons of the *t*-

butyl group and a doublet at δ = 5.49 ppm corresponding to the NH of the carbamate group.



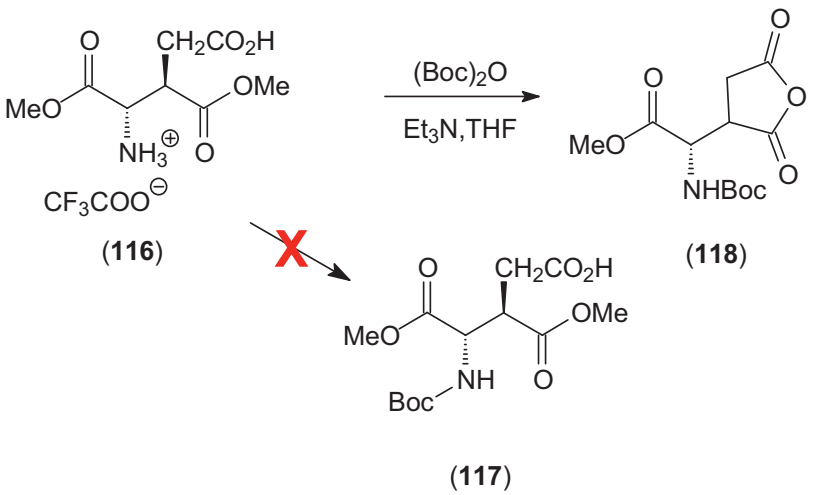
Scheme 30: Synthetic route towards (*S*)-2-amino-3-carboxy-pentanedioic acid 5-*tert*-butyl ester (**117**) *via* β-alkylation of aspartic acid.

The successful preparation of (1*S*,2*R*)-3-*tert*-butyl-1,2-dimethyl-1-(*tert*-butoxy carbonylamino)propane-1,2,3-tricarboxylate (**115**) was started with the enolate dianion, which was obtained by treating compound (**114**) with 2.2 molar equiv of lithium hexamethyldisilazane (LHMDS). The enolate dianion was reacted with *tert*-butyl bromoacetate to give the desired compound (**115**). ^{1}H NMR showed characteristic peaks at δ = 1.40 ppm a singlet corresponding to the *t*-butyl protons, at δ = 2.42 – 2.68 ppm two double boublets corresponding to the protons of CH_2 group,

at δ = 3.55 ppm a multiplet corresponding to the C-H proton where the alkylation occurs, at δ = 4.58 ppm a double doublet corresponding to the C-H proton at the amino group, and at δ = 5.26 ppm a broad band corresponding to the N-H proton.

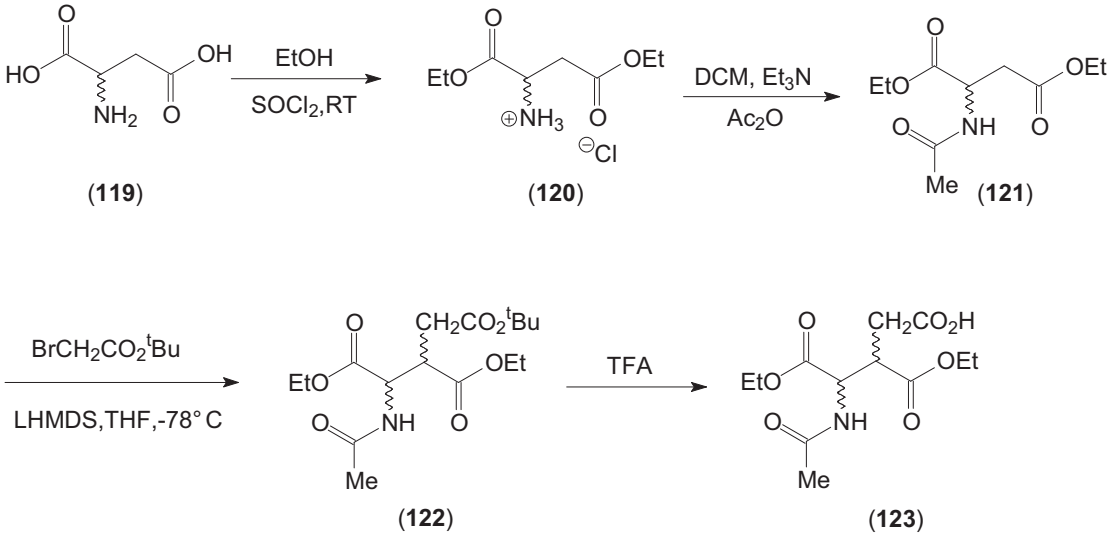
The study carried out by Baldwin *et al*[184] indicated that the bulkier group was in favour of the trans-alkylation with respect the *t*-butyl carbamate group. No stereoselectivity resulted in the β-methylation reaction of (**114**) under the same conditions, which gave a 1:1 mixture of diastereoisomers, while *t*-butyl bromoacetate provided 7:1 ratio of diastereoselectivity. This is consistent with the result observed by Rapoport and co-workers[185]. Several studies[186] also revealed that similar alkylation reactions of β-amino esters proceeded with high ratios of stereoselectivity, particularly when bulkier amine protecting groups were employed.

Treatment of compound (**115**) with trifluoroacetic acid provided the cleavage of *t*-butyl ester and deprotection of amine. As shown in ^{1}H NMR spectra the peaks corresponding to the methyl groups in the *t*-butyl disappeared. In order to protect the amino group, Boc anhydride and triethylamine were employed to obtain (**117**). This reaction resulted in unwanted intramolecular anhydride formation. (2*S*)-Methyl-2-(*tert*-butoxycarbonylamino)-2-(2,5-dioxotetrahydrofuran-3-yl)acetate (**118**) was obtained after purification instead of (**117**)(**Scheme 31**). ^{1}H NMR analysis showed evidence of this formation as one singlet for the methoxyl group had disappeared and one singlet at δ = 1.44 ppm corresponding to the methyl protons of the *t*-butyl group.



Scheme 31: Attempt to protect the amino function of (**116**)

This synthetic route therefore was modified by using a different protecting group for the amino group. The modified synthesis (**Scheme 32**) commenced with the esterification of a racemic mixture of aspartic acid (**119**) with ethanol in the presence of thionyl chloride. The ethyl esters (**120**) were obtained pure and no further purification was required. Diethyl *N*-acetylaspartate (**121**) was obtained after column chromatography from treatment of (**120**) with triethylamine and acetic anhydride in dichloromethane. ^{1}H NMR showed that the characteristic peaks at δ = 2.04 ppm as a singlet corresponding to the methyl protons in acetyl group and a doublet at δ = 6.25 ppm corresponding to the amino group.



(**119**) (**120**) (**121**)

(**122**) (**123**)

Scheme 32: Modified synthetic route to the radical precursor *via* 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**).

Racemic diethyl *N*-acetyl aspartate (**121**) was deprotonated with lithium hexamethyldisilazide (LHMDS) and the resulting enolate dianion was alkylated with *t*-butyl bromoacetate at -78 °C. The ^{1}H NMR gave a similar spectrum to compound (**115**). It also showed only one diastereoisomer in the product. The triester (**122**) was cleaved with trifluoroacetic acid to 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**). ^{1}H NMR showed that the characteristic peak as a singlet at δ = 1.44 ppm a singlet corresponding to the methyl protons of the *t*-butyl group had disappeared. The mono-acid (**123**) then was crystallised from the mixture of diastereoisomers. The compound was finally assigned as the (2*S*,3*R*)/(2*R*,3*S*) isomer by X-ray crystal structure analysis. (**Figure 39**) **Figure 40** shows the (2*S*,3*R*)/(2*R*,3*S*) isomers of 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**).

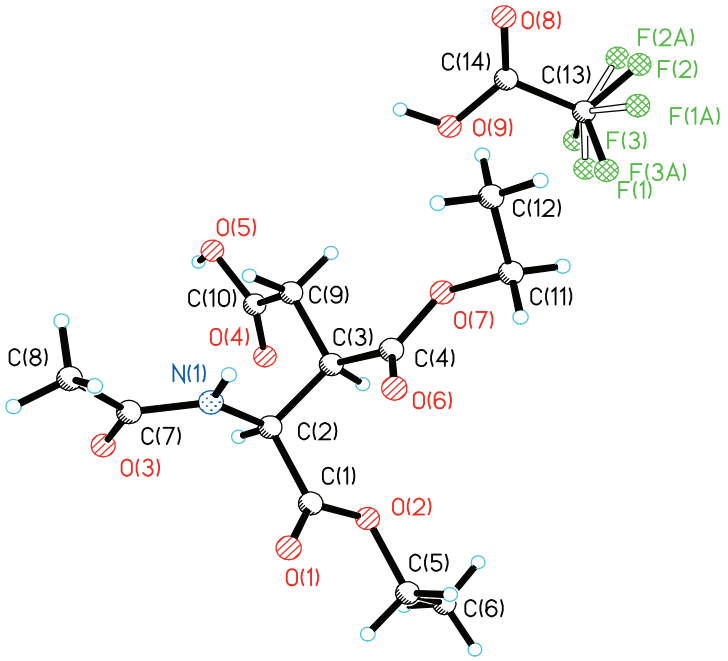


Figure 39: Crystal structure of 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**) (see Appendix)

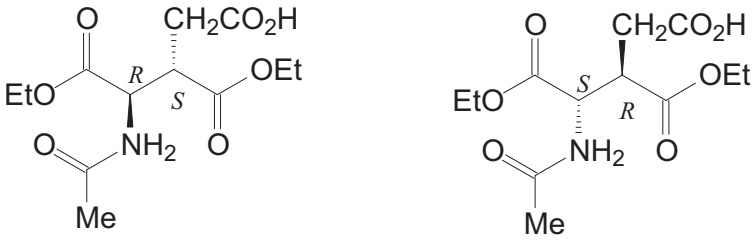
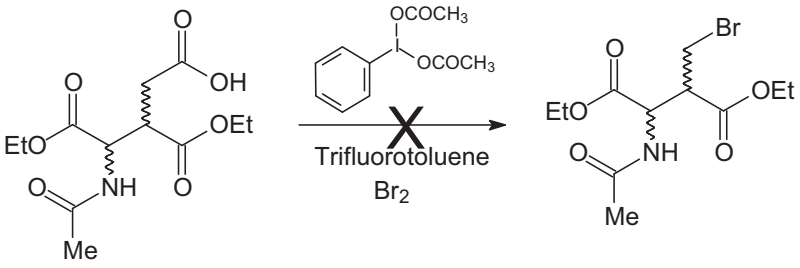


Figure 40: (2*S*,3*R*)/(2*R*,3*S*) isomers of 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**)

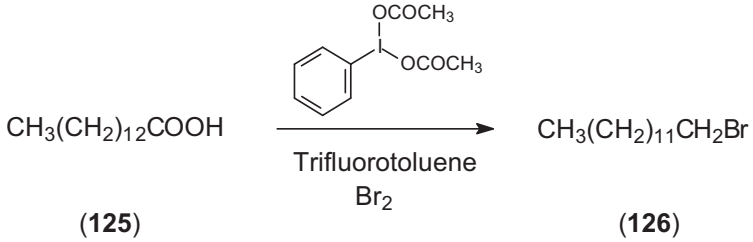
The following step aimed for bromodecarboxylation of the carboxylic acid by iodosobenzene diacetate and bromine following the procedure published by *Camps et al*[187]. (**Scheme 33**) A mixture of starting material (**123**), iodobenzene diacetate and bromine was irradiated under reflux under nitrogen.



(**123**) (**124**)

Scheme 33: Attempt synthesis of radical precursor (**124**) *via* bromodecarboxylation of carboxylic acid (**123**).

Unfortunately, the bromodecarboxylation of the carboxylic acid was unsuccessful. ^{1}H NMR indicated that no starting material or expected product was found from the reaction mixture. A model study was carried out to prove the procedure of the bromodecarboxylation. Myristic acid (**125**) was used as a starting material in the reaction following the same procedure. (**Scheme 34**)

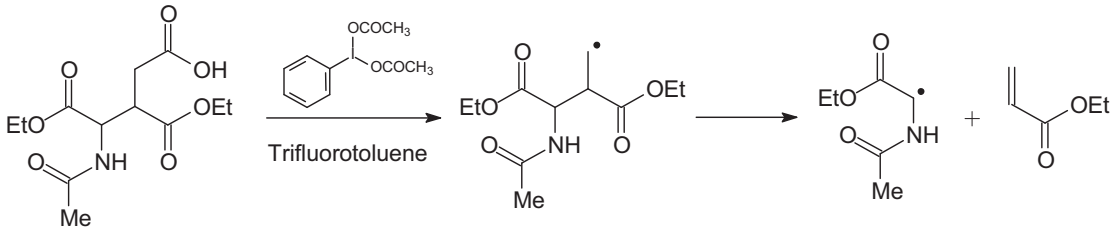


(**125**) (**126**)

Scheme 34: Model study of bromodecarboxylation.

1-Bromotridecane (**126**) was obtained from the reaction without purification. The compound was characterised by ^{1}H NMR which showed a triplet at δ = 0.93 ppm corresponding to the methyl protons, a broad band at δ = 1.30 ppm corresponding to the protons in the CH_2 of the long chain, and a triplet at δ = 3.43 ppm corresponding to the protons in bromomethyl group (CH_2Br).

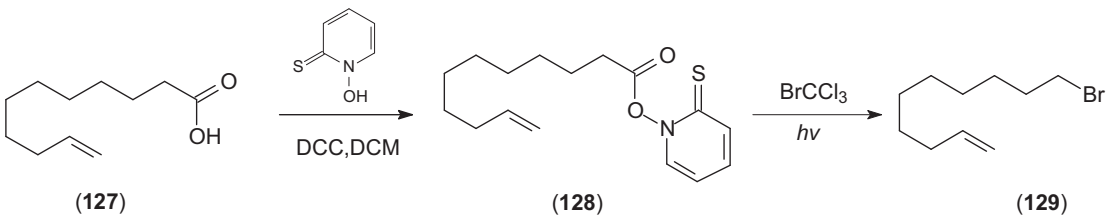
It is quite possible that this reaction involved a radical intermediate which was highly unstable and decomposed during the reaction. It was proposed that the radical intermediate fragmented into two species (**Scheme 35**). Further experiments are required for identification of the radical intermediates.



(**123**)

Scheme 35: Proposed decomposition of the radical intermediate.

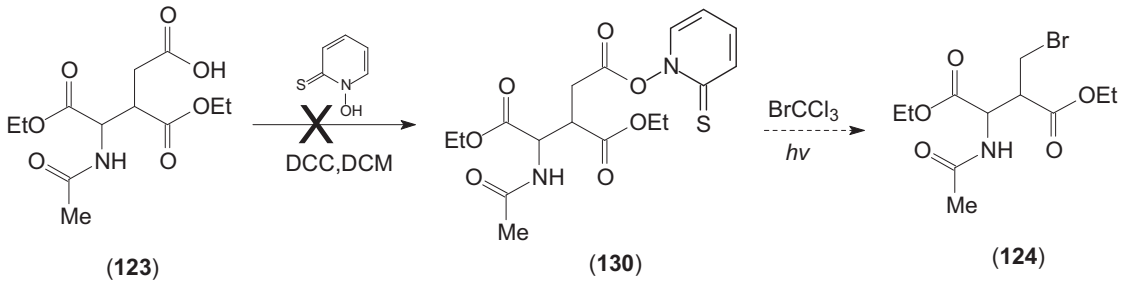
In order to obtain the final product (**124**), an alternative method involved an intermediate base in the method developed by Barton *et al*[188]. This method used bromotrichloromethane as the bromine source and it works very well with various carboxylic acids. (**Scheme 36**)



(**127**) (**128**) (**129**)

Scheme 36: Synthesis of dec-9-enyl bomide *via* Barton's ester.[189]

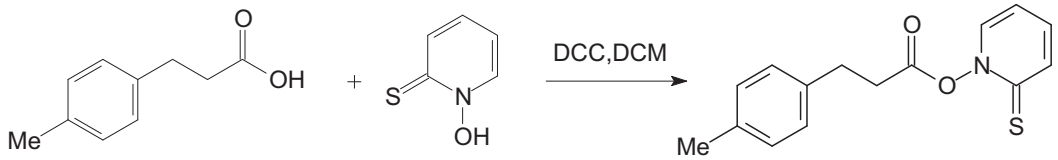
N-Hydroxylthiopyridone was reacted with the carboxylic acid (**123**) in the presence of DCC. The reaction failed: no required compound was observed from the reaction mixture although no starting material was recovered from the reaction. (**Scheme 37**)



(**123**) (**130**) (**124**)

Scheme 37: Synthetic approach to radical precursor *via* Barton's ester.

A model study was carried out to test the formation of Barton's ester. (**Scheme 38**) A similar procedure to last experiment was carried out to synthesise 2-thioxopyridin-1(2H)-yl 3-*p*-tolylpropanoate (**133**) from 3-*p*-tolylpropanoic acid (**131**). The expected product (**133**) was isolated from the reaction mixture. It was characterised by ^{1}H NMR which showed that a singlet at δ = 2.26 ppm corresponding to the methyl protons on the phenyl group, a multiplet at δ = 2.92-3.06 ppm corresponding to the protons in the CH_2 groups, and some peaks at δ = 6.50-7.70 ppm corresponding to the protons in phenyl and thioxopyridinyl group.



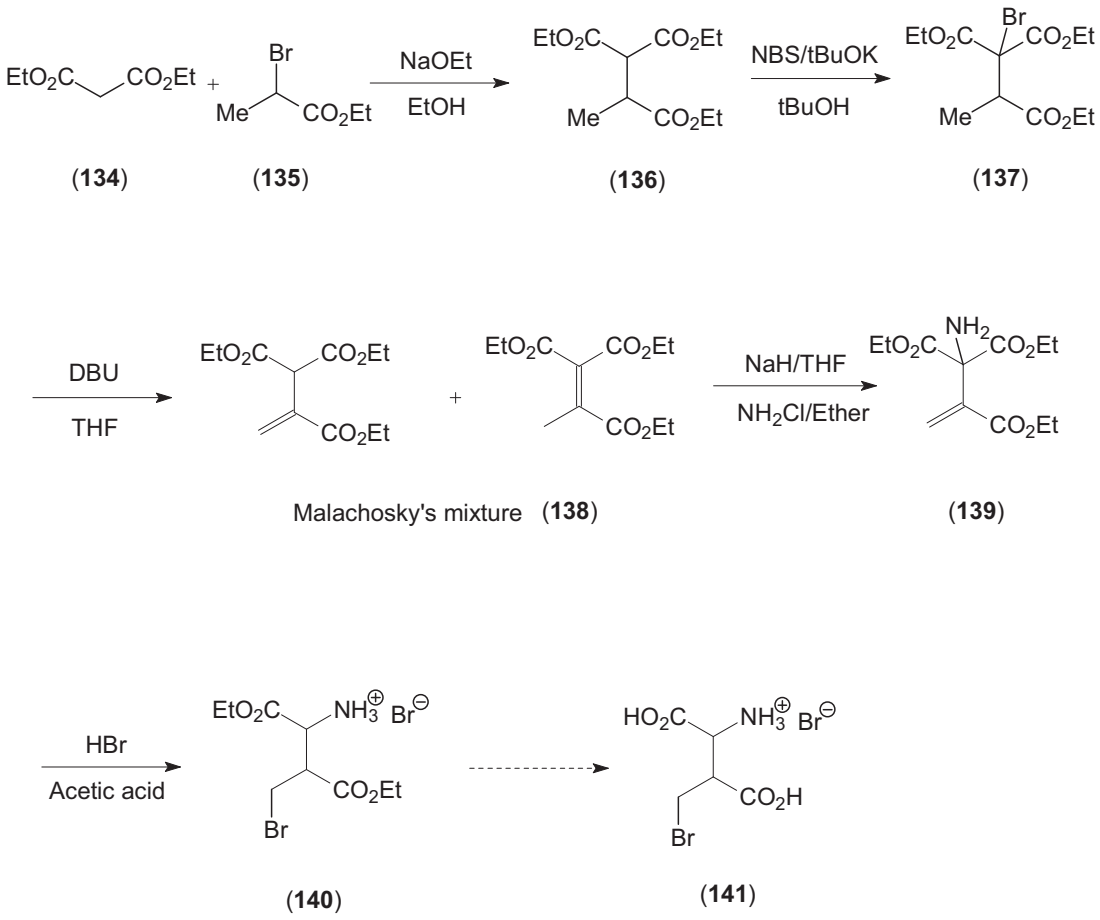
(**131**) (**132**) (**133**)

Scheme 38: Model study to synthesise Barton's ester

4.3.2 Synthetic approach *via* 1-amino-1,1,2-tricarbethoxyprop-2-ene intermediate.

A new synthetic route was explored via 1-amino-1,1,2-tricarbethoxyprop-2-ene intermediate. This was based on the work reported by Dowd *et al* [190]. β-Methyleneaspartic acid was treated with HBr in acetic acid to obtaining β-bromomethylaspartic acid hydrobromide salt. **Scheme 39** showed the synthetic pathway started with ethyl 2-bromopropionate and diethyl malonate.

The synthesis to Malachosky's mixture followed the method developed by C. Edwards.[191] The condensation of ethyl 2-bromopropionate (**135**) with the sodium salt of diethyl malonate (**134**) afforded triethyl propane-1,1,2-tricarboxylate (**136**). This reaction was performed with an excellent yield (92 %) on a large scale (*ca* 24 g of product). Sodium ethoxide was a very good base to react with diethyl malonate in order to obtain the required sodium salt. The triester was treated with *N*-bromosuccinimide (NBS) in the presence of a catalytic amount of potassium *tert*-butoxide in dry *t*-butanol at 35 °C to give triethyl 1-bromotriester (**137**) with 90 % yield. This reaction was quite reliable; it was carried out on about 15 g scale.



(**134**) (**135**) (**136**) (**137**)

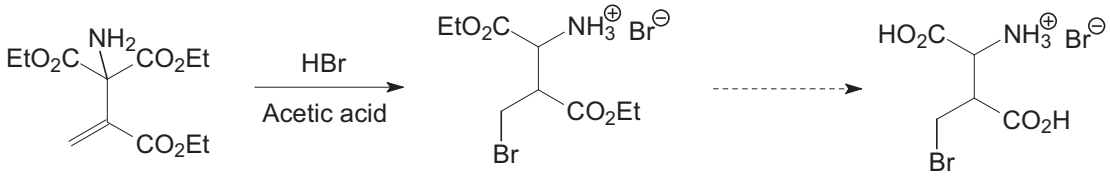
Malachosky's mixture (**138**) (**139**)

(**140**) (**141**)

Scheme 39: Synthesic pathway to diethyl 2-amino-3-(bromomethyl)succinate hydrobromide (**140**)

Using 1,8-diazabicyclo[5.4.0]undec-7-en (DBU) as a base, the dehydrobromination of triethyl 1-bromotriester (**137**) gave a 1:1 mixture of triethyl prop-1-ene-2,3,3-tricarboxylate and triethyl prop-1-ene-1,1,2-tricarboxylate. The isomeric mixture obtained is so-called Malachosky's mixture (**138**). The reaction could also give a by-product (**136**). In order to synthesise our target compound, Malachosky's mixture (**138**) was deprotonated with sodium hydride in anhydrous THF, followed by addition of an anhydrous solution of ethereal chloramine. The amination of the carbanion formed with excess of chloramines gave 1-amino-1,1,2-tricarbethoxyprop-2-ene

(**139**).[192] Chloramine was prepared using the procedure reported by Jaffari and Nunn.[193]



(**139**) (**140**) (**141**)

Scheme 40: Synthesis of radical precursor, 2-amino-3-(bromomethyl)succinic acid hydrobromide (**141**)

The final stage of the synthesis involved the deprotection and bromination of the amino tricarboxylic ester (**139**) was not completed due to lack of time. The preliminary study showed quite promising results. Diethyl 2-amino-3-(bromomethyl) succinate hydrobromide (**140**) was obtained from 1-amino-1,1,2-tricarbethoxyprop-2-ene (**139**) with hydrogen bromide in acetic acid at room temperature. (**Scheme 40**) Further works are required to complete the formation of diethyl 2-amino-3-(bromomethyl) succinate hydrobromide (**140**) and conversion of 2-amino-3-(bromomethyl)succinic acid hydrobromide (**141**).

4.4 CONCLUSIONS

The synthetic approach to a radical precursor for 3-methylaspartate has not been completed and further work is required. The first synthetic method utilised an intermediate aziridine. An acetyl group was used to protect the nitrogen of the aziridine. The key step of the synthetic route aimed to achieve nucleophilic ring opening of the aziridine dicarboxylate. No evidence of this chemistry was obtained. The result showed the electron withdrawing group (i.e. acetyl group) could not facilitate the ring opening of the aziridine dicarboxylate. Some examples in the literature demonstrated that electron releasing groups (e.g. benzyl group) could facilitate the ring opening. The second synthetic approach involved the β-alkylation of aspartic acid and bromodecarboxylation. The crystal structure of 2-acetylamino-3-ethoxycarbonyl-pentanedioic acid 1-ethyl ester (**123**) proved that the alkylation was in the favour of trans-alkylation with *t*-butyl acetate. The bromodecarboxylation was unsuccessful. This is possibly due to the decomposition of reaction intermediates. The third synthetic method was based on the work reported by Edwards and Dowd.[190, 191] 1-Amino-1,1,2-tricarbethoxyprop-2-ene (**139**) was synthesised successfully. The hydrolysis and deprotection of (**139**) was not completed. However, the preliminary study indicated that 2-amino-3-(bromomethyl) succinate hydrobromide (**140**) was formed.

Chapter 5

Summary and Future Work

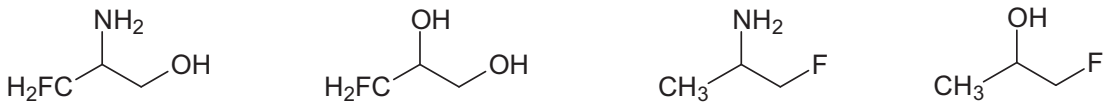
Coenzyme B_{12} (adenosylcobalamin, AdoCbl) assists certain enzymes in the catalysis of 1,2-shift interconversions involving radical intermediates, which are initiated by homolysis of the Co-C σ-bond. Despite many years of work carried out by scientists from all over the world, the mechanisms of the enzymatic radical rearrangements still remain unclear. In order to improve the understanding of the mechanism of AdoCbl-dependent glutamate mutase, diol dehydratase and EAL, three different strategies have been employed in this thesis to help us resolve the mysteries.

In Chapter 2, two biphenyl model receptors, (**36**) and (**37**) were successfully synthesised to investigate the binding property of the 'arginine claw' in glutamate muatse. Several solvent systems have been trialled under vapour diffusion to recrystallise both compounds. Unfortunately no X-ray crystal structures have been obtained for compounds (**36**) and (**37**). The binding property of the modified receptor model (**37**) to dicarboxylates (glutaric acid) in CD_3OD was investigated by ^1H NMR titration at fast host-guest exchange rate. The NMR result indicates that the binding constants are too small to be evaluated or the solvent system affects the complexation of the host and guest. So a new investigation method (such as isothermal titration calorimetry, or UV absoption) should be used to determine the binding constants in the future work.

A new methodology to insert a different conformation controlling group in the 'tritylase' model receptor, (**58**), has been explored, which could help the structure bending and closing the pocket, avoid the aggregation in solution forming micelles and perhaps to obtain a crystal structure of the molecule. Further experiments to insert guanidinium groups to the compound are required. The new model fits the original scaffold but may have the ability to mimic different enzymes (e.g. aldolase) not just B_{12}-dependent enzymes. UV techniques or isothermal calorimetry (ITC) for binding study with different dicarboxylates could be employed to investigate its binding properties. NOESY spectra could be used to show the distance of the residues to the active site and quality of the binding.

In order to elucidate the mechanisms of AdoCbl-dependent diol dehydratase and EAL, two trifluoro substrate derivatives (**68** and **69**) have been proposed and synthesised. 2-

Amino-3,3,3-trifluoropropanol (**69**) was found to be devoid of any activity against EAL according to a coupled assay with alcohol dehydrogenase. An EPR study has concluded that the CF_3-substrate did not lead to the significant formation of a paramagnetic intermediate. This may be due to the CF_3 group reducing the basicity of the amino group so that there is a diminished interaction with the active site in EAL. Or this may be caused by the significant steric effect introduced by the trifluoromethyl group. For future studies, a few potential substrates are suggested in **Figure 41**. The single-fluoro substituent could bring less inductive and steric effect to the enzyme substratesthan the trifluoromethyl group.

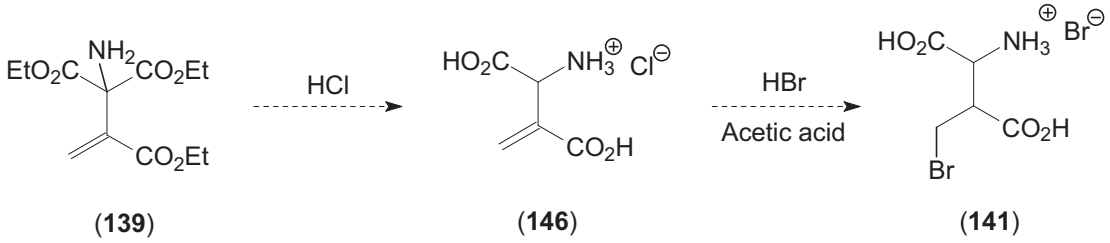


(**142**) (**143**) (**144**) (**145**)

Figure 41: Potential fluoro-substrates for EAL and diol dehydrotrase.

A model study in which the hydroxyl radical was used to attack the substrate analogue (**68**) of diol dehydratase showed that 1,1-difluoro-3-hydroxypropanone was formed by hydroxyl radical attack on 3,3,3-trifluoropropane-1,2-diol (**68**). Propanal and acetone were formed from the attack on propane-1,2-diol (**82**) (analysis by ^{1}H NMR, GC and GCMS, and by formation of the corresponding 2,4-dinitrophenylhydrazones). Pulse radiolysis is required to study the radical products and their relative yields in future works. Further product study under continuous γ-irradiation will be carried out for 3,3,3-trifluoropropane-1,2-diol (**68**) and propane-1,2-diol (**82**) using DNP derivatives as a probe. Authentic 1,1-difluoro-3-hydroxypropanone needs to be synthesised.

Chapter 4 describes the development of synthesis towards a radical precursor for 3-methylaspartate in glutamate mutase reaction. A variety of different synthetic routes have been attempted to the radical precursors (**100**) and (**101**). Various problems have been encountered in the synthetic approaches. Due to the lack of time, the work was incomplete and future works are required. Preliminary study indicated that 2-amino-3-(bromomethyl) succinate hydrobromide (**140**) was formed. Further acid hydrolysis may introduce an unwanted acid dehydration of bromo compound forming an alkene. (**Scheme 40**) Further work is required to study the formation of diethyl 2-amino-3-(bromomethyl) succinate hydrobromide (**140**) and conversion of 2-amino-3-(bromomethyl)succinic acid hydrobromide (**141**). A new strategy was proposed for the future work. (**Scheme 41)**. Tu [194] demonstrated that 3-methyleneaspartic acid hydrochloride (**146**) could be synthesised by deprotection and hydrolysation using hydrochloride acid. Then, the final compound (**141**) could be formed by bromination.



(**139**) (**146**) (**141**)

Scheme 41: Proposed synthesis of radical precursor, 2-amino-3-(bromomethyl) succinic acid hydrobromide (**141**)

Chapter 6

Experimental

6.1 MATERIALS AND METHODS

Chemicals were purchased primarily from Sigma-Aldrich, Fluka Chemicals, Alfa Aesar, Acros Organics or Lancaster Synthesis, and were used without further purification, unless indicated.

Anhydrous solvents (e.g. acetonitrile, *tert*-butanol, dichloromethane, diethyl ether, ethanol, methanol, tetrahydrofuran and trifluorotoluene) were purchased. Dichloromethane and ethyl acetate were distilled before use. Other solvents were AnalaR or laboratory grade, and were dried according to standard procedures when necessary. After drying, the solvent was stored with activated 4 Å molecular sieves under nitrogen. Ammonia gas was purchased in a cylinder from BOC and dried by calcium chloride and bubbling through sulphuric acid.

All moisture sensitive reactions were carried out in oven-dried glassware under nitrogen, which had been dried through an activated silica column.

Thin layer chromatography (TLC) was performed using aluminium-based plates pre-coated with silica gel (Kieselgel 60 F_{254}, 0.2 mm, Merck, Darmstadt, Germany). Column chromatography was carried out under medium pressure using Kieselgel silica gel 60 (40-63 microns) with eluting solvent system as indicated. Visualisation was by UV light (shortwave 254 nm) or by dipping in aqueous potassium permanganate, for amino compounds in ethanolic ninhydrin solution.

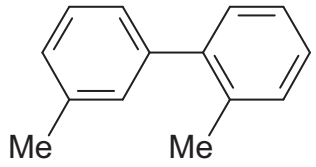
Melting points were determined using a Gallenkamp melting point apparatus in open capillaries and are uncorrected.

^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectra were obtained at the frequencies stated, using deuterated solvents as internal standards. 300 MHz ^{1}H spectra were obtained on a Bruker Avance BVT3200 spectrometer and 400 MHz ^{1}H spectra on a JEOL ECS400 spectrometer. ^{13}C spectra were obtained from the same instruments at 75 and 125 MHz, respectively. Chemical shifts are reported in parts per million (ppm), together with multiplicity, integration, assignment and coupling constants (Hz).

Infrared (IR) spectra were recorded on a Nicolet 20-PC FTIR spectrophotometer or a Varian 800 FTIR Scimitar Series spectrometer for samples mounted in potassium bromide discs or as a capillary film. Gas chromatography-mass spectra (GCMS) were recorded on a Varian Saturn 2200 GC-MS with various columns in positive or negative electrospray mode. LCMS analyses were performed on a Shimadzu LCMS-2010EV instrument operating in positive or negative electrospray mode. Other mass spectra and CHN analyses were recorded by Advanced Chemical and Material Analysis, Newcastle University.

6.2 EXPERIMENTAL CHAPTER 2

2,3-Dimethylbiphenyl (39)[195]



To a suspension of tolylboronic acid (2.65 g, 19.5 mmol), 3-iodotoluene (**38**) (3.93 g, 2.32 mL, 18 mmol) and CsF (5.68 g, 37.4 mmol) in dry 1,2-dimethoxyethane (DME, 50 mL), tetrakis(triphenylphosphine)palladium(0) (0.12 g, 0.12 mmol) was added under nitrogen. The mixture was heated at reflux for 3.5 h. The mixture was then cooled, water (50 mL) was added and the product was extracted into ethyl acetate (3 × 50 mL). The combined organic extracts were dried ($MgSO_4$). After removing the solvent under reduced pressure, the residue was purified by medium-pressure chromatography on silica eluting with petrol, to afford the title compound (3.14 g, 96 % yield) as a colourless oil. R_f 0.64 (petrol).

^{1}H NMR δ_H (300 MHz; $CDCl_3$): 2.44 (3H, s, Ar-CH_3), 2.55 (3H, s, Ar-CH_3), 7.27-7.50 (8H, m, Ar-H)

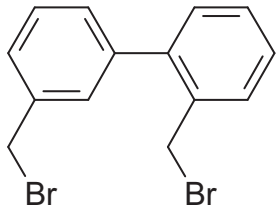
^{13}C NMR δ_c (75 MHz, $CDCl_3$): 20.7 (Ar-CH$_3$), 21.8 (Ar-CH$_3$), 126.1 (Ar), 126.7 (Ar), 127.5 (Ar), 127.9 (Ar), 128.3 (Ar), 128.4 (Ar), 130.1 (Ar), 130.3 (Ar), 130.6 (Ar), 135.7 (Ar), 138.0(Ar), 142.5 (Ar)

m/z (EI): 182 (M, 95 %), 167 (M-Me, 100 %), 152 (M-2 ×Me, 10 %), 115, 89

CHN: Found C, 91.86 %; H, 8.20 %; $C_{14}H_{14}$ requires C, 92.26 %; H, 7.74 %

υ_{max} (KBr)/cm^{-1}: 3056(Ar-H), 2923, 2857, 2734, 1586, 1476 (Ar-C), 1456, 755 (Ar)

2,3-bis-(Bromomethyl)-biphenyl (40)



A mixture of 2,3-dimethylbiphenyl (**39**) (0.84 g, 4.59 mmol), NBS (1.80 g, 10.10 mmol) and 1,1-azobis(cyanocyclohexane) (12 mg, 0.05 mmol) in dry trifluorotoluene (20 mL) was irradiated with visible light (100 W) while heating at reflux 90 °C for 5 h. TLC was used to monitor the reaction and after 2.5 h, 1,1-azobis (cyanocyclohexane) (12 mg) was added again. After cooling, the precipitated succinimide was filtered off and the solvent was removed under reduced pressure. The crude yellow oily residue was dissolved in trifluorotoluene (10 mL), and the solution was washed with saturated aqueous $Na_2S_2O_5$ (2 × 10 mL). The organic phase was dried ($MgSO_4$) and concentrated under reduced pressure to afford a yellow oily residue. The residue was purified by medium-pressure chromatography on silica eluting with a gradient of 1 → 10 % dichloromethane in petrol. The product-containing fractions were combined and the solvent was removed to afford a colourless oil (1.10 g, 70 % yield, with 10 % of the tribrominated product, identified by EIMS). R_f 0.3 (dichloromethane – petrol, 1 : 9 v/v)

m/z (EI) of tribromo-product: 418 ($[M+Br]^+$, 18 %), 339 (M, 20 %), 259 ($[M-Br]^+$, 45 %), 179 ($[M-2 \times Br]^+$, 100 %), 165 (35), 152 (15),139 (5), 115 (5), 89 (45), 76 (25), 63 (10), 39 (5)

After recrystallisation from petrol a white solid (1.00 g, 63 %) was collected.

m.p.: 44 - 46 °C from petrol.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 4.36 (2H, s, Ar-CH_2Br), 4.48 (2H, s, Ar-CH_2Br), 7.16-7.47 (8H, m, Ar-H)

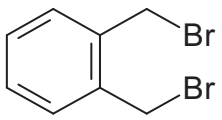
^{13}C NMR δ_C (75 MHz, $CDCl_3$): 32.1 (Ar-CH$_2$), 33.3 (Ar-CH$_2$), 128.4 (Ar), 128.6 (Ar), 128.9 (Ar), 129.1 (Ar), 129.4 (Ar), 130.0 (Ar), 130.7 (Ar), 131.3 (Ar), 135.7 (Ar), 138.4 (Ar), 141.3 (Ar), 141.9 (Ar)

m/z (EI): 338/340/342 (M, 1:2:1, 20 %), 259 ([M-Br]$^+$, 55 %), 179 ([M-2 × Br]$^+$, 100 %), 165 (35 %), 152 (10 %), 89 (27 %), 76 (15 %)

CHN: Found C, 49.28 %; H, 3.39 %; $C_{14}H_{12}Br_2$ requires C, 49.45 %, H, 3.56 %

υ_{max} (KBr)/cm^{-1}: 3050(Ar-H), 2980, 2850, 2650, 1580, 1493 (Ar-C), 1476, 916, 755 (Ar), 620

α,α-Dibromo-*o*-xylene (60)[196]



This was prepare from o-xylene by a similar procedure to that for compound **40**.

White solid, 54 % yield.

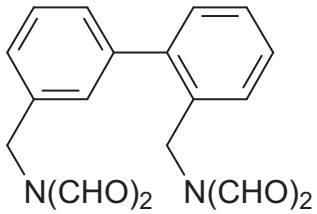
m.p. 88-90 °C from petrol (lit.[195] m.p. 92-93 °C), R_f 0.38 (petrol)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 4.59 (4H, s, 2 × CH_2Br), 7.18-7.31 (4H, m, Ar-H)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 30.1 (CH$_2$Br), 129.7 (Ar), 131.4 (Ar), 137.0 (Ar)

υ_{max} (KBr)/cm^{-1}: 3050 (Ar-H), 2730, 1494 (Ar-C), 1456, 764 (Ar), 737 (Br-C)

2,3-bis-(*N, N*-Diformylamidomethyl)-biphenyl (41)



A suspension of 2,3-bis-(bromomethyl)-biphenyl (**40**) (0.50 g, 1.47 mmol) and sodium diformylamide (0.34 g, 3.53 mmol) with 18-crown-6 (10 mg, 0.04 mmol) in dry acetonitrile (6 mL) was heated at reflux for 3.5 h. After cooling and filtration, the filter pad was washed with dry acetonitrile and the solvent was removed under reduced pressure from the combined filtrates. The residue was purified by medium pressure chromatography on silica eluting with petrol - ethyl acetate (1 : 2 v/v), to give the title compound (0.33 mg, 70 % yield) as a white solid. R_f 0.3 (petrol – ethyl acetate, 1 : 2 v/v)

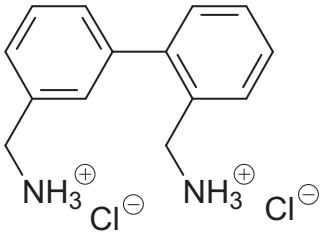
m.p.: 50-53 °C.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 4.60 (2H, s, Ar-CH_2-N), 4.74 (2H, s, Ar-CH_2-N), 7.09-7.29 (8H, m, Ar-H), 8.69 (2H, s, 2 × C*H*O), 8.77 (2H, s, 2 × C*H*O)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 39.0 (Ar-*C*H_2-N), 40.8 (Ar-*C*H_2-N), 125.7 (Ar), 126.3 (Ar), 127.0 (Ar), 127.5 (Ar), 128.0 (Ar), 128.4 (Ar), 129.6 (Ar), 132.8 (Ar), 136.1 (Ar), 140.4 (Ar), 165.7 (4 × CHO).

m/z (EI): 324 (M, 15%), 297 ([M-CHO]$^+$, 8%), 267 ([M- 2 × CHO]$^+$, 10%), 250 (12%), 194 (15%), 178 (100%), 165 (25%), 152 (10%)

2,3-bis-(Aminomethyl)-biphenyl dihydrochloride (42)



A mixture of 2,3-bis-(*N, N*-diformylamidomethyl)biphenyl (**41**) (130 mg, 0.40 mmol) and 5 % ethanolic hydrochloric acid (5 mL) was heated at reflux for 3.5 h and then evaporated to near dryness. The residue was dried *in vacuo* and the compound was collected as a white solid (118 mg, quantitative). The solid was dissolved in warm ethanol/ethyl acetate and the solution was allowed to cool slowly to room temperature, then stored at 4 °C. The resulting precipitate was collected by suction filtration and dried *in vacuo* at 50 °C, to afford the title product (90 mg, 70 % yield) as a white solid.

m.p.: 274-276 °C.

^{1}H NMR δ_H (300 MHz, D_2O): 4.06 (2H, s, Ar-CH_2-N), 4.12 (2H, s, Ar-CH_2-N), 7.26-7.50 (8H, m, Ar-H)

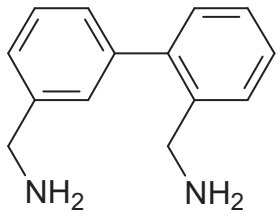
^{13}C NMR δ_c (75 MHz, D_2O): 41.8 (*C*H_2), 44.4 (*C*H_2), 129.2 (Ar), 129.8 (Ar), 129.9 (Ar),130.4 (Ar), 130.7 (Ar), 130.9 (Ar), 131.1 (Ar), 131.7 (Ar), 134.4 (Ar), 141.5 (Ar), 142.4 (Ar)

m/z (EI): 195 ($[M\text{-}HCl\text{-}NH_2]^+$, 100 %), 178 ($[M\text{-}2\times HCl\text{-}2\times NH_2]^+$, 35 %), 165 ($[M\text{-}2\times HCl\text{-}2\times NH_2\text{-}CH_3]^+$, 25 %), 152 ($[M\text{-}2\times HCl\text{-}2\times NH_2\text{-}2\times CH_3]^+$, 10 %), 122 (8), 105 (9), 77 (10)

υ_{max} (KBr)/cm^{-1}: 3000-2900 (Aryl C-H stretch), 2900-2600 (N-H stretch of amino bands), 1660, 1598, 1516 (Aromatic bands)

CHN: Found C, 57.87 %; H, 6.14 %; N, 9.61 %; $C_{14}H_{18}Cl_2N_2 \cdot 0.25H_2O$ requires C, 58.04 %; H, 6.44 %; N, 9.67 %

2,3-bis-(Aminomethyl)-biphenyl (43)

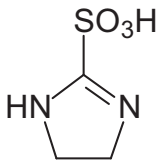


2,3-bis-(Aminomethyl)-biphenyl dihydrochloride (**42**) (0.85 g, 2.98 mmol) was suspended in dichloromethane (30 mL) and aqueous NaOH solution (0.25 M, 30 mL) was added. The mixture was stirred at room temperature for 1 h. The free amine was extracted with dichloromethane (3 × 20 mL). The combined organic layers were dried ($MgSO_4$) and the solvent was removed under reduced pressure to give a colourless oil (0.53 g, 85 % yield).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.27 (4H, br, 2 × NH_2), 3.71 (2H, s, Ar-CH_2-N), 3.82 (2H, s, Ar-CH_2-N), 7.14-7.38 (8H, m, Ar-H)

^{13}C NMR δ_c (75 MHz, $CDCl_3$): 44.3 (Ar-CH$_2$), 46.6 (Ar-CH$_2$), 127.0 (Ar), 127.1 (Ar), 128.0 (Ar), 129.1 (Ar), 129.3 (Ar), 129.4 (Ar), 129.6 (Ar), 130.1 (Ar), 130.3 (Ar), 132.1 (Ar), 132.2 (Ar), 132.6 (Ar)

4,5-Dihydro-1*H*-imidazole-2-sulfonic acid (44)[114, 197]

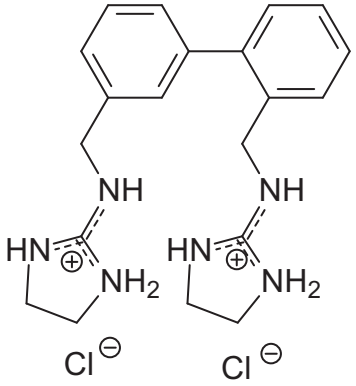


A suspension of imidazoline-2-thione (4.5 g, 45 mmol), Na_2MoO_4 (0.34 g, 1.4 mmol) and NaCl (1 g) in deionised water (21 mL) was cooled to -4 °C. H_2O_2 (37 mL, 30 % w/v, 0.3 mmol) was added dropwise to the mechanically stirred suspension. The suspension turned deep green. After the addition was complete, the green mixture was stored in fridge for 26 h. This resulted in a clear yellow solution with a white solid, which was collected *via* filtration, washed with cold water and dried *in vacuo*. (3.0 g, 44 % yield)

m.p.: 134-135 °C (lit.[196] value: 137-138 °C)

^{1}H NMR δ_H (300 MHz, D_2O): 3.85 (4H, s, 2 × C*H*$_2$), 10.35 (1H, br, SO_3H)

2,3-bis-{[(4,5-Dihydro-1*H*-imidazol-2-yl)-amino) methyl)-biphenyl dihydrochloride (36)



2,3-bis-(Aminomethyl)-biphenyl (**43**) (0.23 g, 1.12 mmol) was dissolved in Et_3N/H_2O/MeOH (12 mL) (1:1:1) with 4,5-dihydro-1H-imidazole-2-sulfonic acid (**44**) (0.36 g, 2.48 mmol) and the mixture was stirred for 24 h at room temperature. The solvent was removed and the residue was dried for 2 h *in vacuo.* Purification was carried out with an anion exchange resin Amberlite IR 400 set up with hydroxyl anions. Water was used as eluent system and the product was obtained as the free base. The detection method was TLC using ninhydrin dip (bright yellow colour). The fractions containing product were combined and the solvent was removed under reduced pressure (0.35 g, 82 % yield, free amine based calculation).

The free amine (0.35 g, mmol) was dissolved in water (8 mL) and conc. HCl (1 mL) was added dropwise and the mixture was stirred for 10 min. The solvent was removed under reduced pressure to afford a white hygroscopic solid. The title compound was obtained as a crystalline solid after freeze-drying.

m. p.: 123-125 °C

^{1}H NMR δ_H (300 MHz, D_2O): 3.44 (4H, s, 2 × CH_2), 3.61 (4H, s, 2 × CH_2), 4.27 (2H, s, CH_2N), 4.42 (2H, s, CH_2N), 7.20-7.45 (8H, m, Ar-H)

^{13}C NMR δ_C (75 MHz, D_2O): 43.4 (2 × *C*H_2), 43.5 (2 × *C*H_2), 45.7 (*C*H_2), 46.7 (*C*H_2), 126.9 (Ar), 127.0 (Ar), 128.0 (Ar), 129.1 (Ar), 129.2 (Ar), 129.3 (Ar), 129.4 (Ar), 129.7 (Ar), 130.0 (Ar), 131.1 (Ar), 131.2 (Ar)

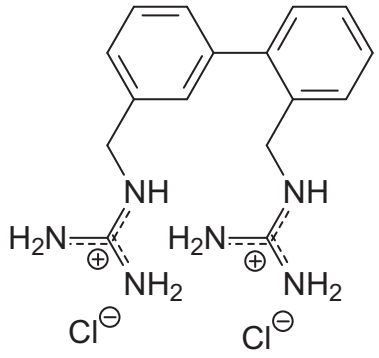
λ_{max} 285 (ε = 45,000 dm^3 mol^{-1} cm^{-1})

m/z (EI): 347 ([M- 2 × HCl]$^+$, 30 %), 280 (25 %), 265 (100 %), 194 (10), 179 (40)

CHN: Found C, 48.45 %; H, 6.00 %; N, 16.79 %; $C_{20}H_{26}Cl_2N_6$·4.2 H_2O requires C, 48.42 %, H, 6.94 %, N, 16.95 %

υ_{max} (KBr)/cm^{-1}: 3300-3200 (N-H stretch of guanidinium bands), 3060 (Aryl C-H stretch), 1668 (C=N, stretch), 1599 (Aromatic bands); (Ar), 134.1 (Ar), 137.44 (Ar), 141.7 (Ar), 142.0 (Ar), 142.3 (Ar), 159.8 (C-Guanidinium), 160.7 (C-Guanidinium)

2, 3-bis-Guanidinomethyl-biphenyl dihydrochloride (37)



2,3-bis-(Aminomethyl)-biphenyl (**43**) (0.53 g, 2.5 mmol) was dissolved in trifluoroethanol (12 mL) and 4-benzyl-3,5-dimethyl-1*H*-pyrazole-carboxamidine[108] (1.46 g, 5.54 mmol) was added. Once pyrazole was dissolved, triethylamine was added dropwise. The reaction mixture was warmed up to 60 °C and it was stirred overnight. The solvent was removed under reduced pressure to give an oily residue. Water (10 mL) was added and the solution was sonicated for 5 min. The suspension

was centrifuged for 10 min and the supernatant was separated. This step was repeated and the combined supernatants were washed with ethyl acetate (15 mL). The solvent was removed to give a white solid. The crude product was purified by chromatography on Amberlite IR 400 with hydroxyl groups. Water was used as eluent and the product was obtained as a free base. The detection method was TLC using ninhydrin dip (bright yellow colour). The fractions containing the product were combined and the solvent was removed under reduced pressure. The free amine (0.49 g, 2.33 mmol) was dissolved in water (5 mL) and conc. hydrochloric acid (0.08 mL, 2.42 mmol) was added dropwise. The mixture was stirred for 10 min and the solvent was removed under reduced pressure to afford the title compound as a white hydroscopic solid (0.76 g, 82 % yield) after freeze-drying.

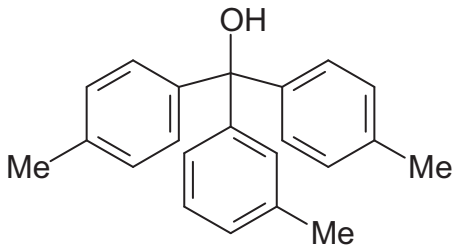
^{1}H NMR δ_H (300 MHz, D_2O): 4.16 (2H, s, C*H*$_2$), 4.34 (2H, s, C*H*$_2$), 7.14-7.39 (8H, m, Ar-H)

^{13}C NMR δ_C (75 MHz, D_2O): 45.3 (*C*H$_2$), 46.5 (*C*H$_2$), 128.1 (Ar), 129.5 (Ar), 129.7 (Ar), 129.8 (Ar), 130.1 (Ar), 130.3 (Ar), 130.6 (Ar), 131.9 (Ar), 134.8 (Ar), 138.4 (Ar), 142.9 (Ar), 143.6 (Ar)

m/z (EI): 397 ([M]$^+$), 255, 214, 149, 79

CHN: Found C, 47.12 %; H, 5.96 %; N, 21.44 %; $C_{16}H_{22}Cl_2N_6 \cdot 2H_2O$ requires C, 48.57 %, H, 6.95 %, N, 19.99 %

m-Tolyl-di-*p*-tolylmethanol (54)[198]



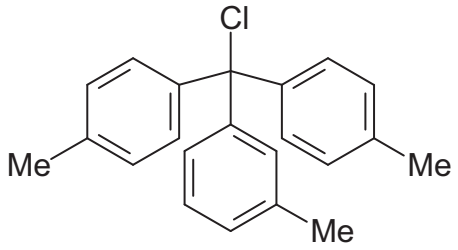
In a 3-necked round bottom flask, magnesium turnings (0.5 g, 20.8 mmol) and dry THF (10 mL) were placed and stirred vigorously for 10 min under nitrogen. 4-bromotoluene (**51**) (3.1 g, 18.2 mmol) in dry THF (5 mL) was added dropwise over 15 min. The reaction mixture was vigorously stirred at reflux for 3 h. Ethyl *m*-toluate (**53**) (1 mL, 1 g, 6.3 mmol) in dry THF (10 mL) was added to the reaction flask dropwise over 30 min and the mixture was heated at reflux for a further 4 h. The reaction was monitored by TLC using diethyl ether/petrol (1 : 9 v/v) as eluting system (R_f 0.8). When ethyl *m*-toluate had been consumed, the reaction mixture was poured over crushed ice (10 g) and water, and acidified with hydrochloric acid (2 M) to pH ≈ 2. The product was extracted with diethyl ether (3 × 20 mL) and the combined organics were washed with brine and dried ($MgSO_4$). The solvent was removed under reduced pressure. The title compound was obtained as a yellow oil. This was dissolved in diethyl ether and petrol at reflux and allowed to cool slowly to room temperature, then stored -20 °C overnight. The resulting crystals were collected by suction filtration and dried *in vacuo* at 30 °C to afford the title compound as a white microcrystalline solid. (1.56 g, 83 % yield)

m.p.: 95-97 °C (lit.[197] value: 95-96 °C)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 2.33 (3H, s, *m*-CH_3), 2.37 (6H, s, 2 × *p*-CH_3), 2.72 (1H, s, OH), 7.02-7.28 (12H, m, Ar-H)

υ_{max} (KBr)/cm^{-1}: 3600 (O-H stretch), 2900-3050 (C-H stretch of Ar), 1000-1500 (Ar bands)

***m*-Tolyl-*p*-ditolylchloromethane (55)[197]**

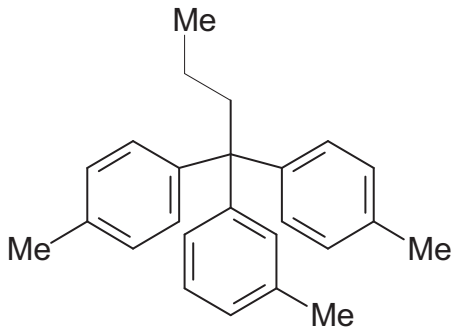


In a round bottom flask, *m*-tolyl-*p*-ditolylmethanol (**54**) (10.02 g, 33.1 mmol) was dissolved in the minimum amount of diethyl ether (10 mL). Conc. hydrochloric acid (40 mL) was added and there was a colour change from pale yellow to bright yellow/orange. The reaction mixture was stirred overnight at room temperature. IR analysis of an aliquot showed that the O-H stretch (3600 cm^{-1}) had disappeared. The mixture was diluted with ice cold water (10 mL) and extracted with diethyl ether (4 × 30 mL). The combined organic phases were dried ($MgSO_4$). The solvent was removed under reduced pressure to give the title compound as a yellow oil. (7.02 g, 66 % yield)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 2.23 (3H, s, *m*-CH_3), 2.28 (6H, s, 2 × *p*-CH_3), 6.92-7.10 (12H, m, Ar-H)

υ_{max} (KBr)/cm^{-1}: 2900-3050 (C-H stretch of Ar), 1000-1500 (Ar bands), 750 (C-Cl)

1-*m*-Tolyl-1,1-di-*p*-tolylbutane (56)



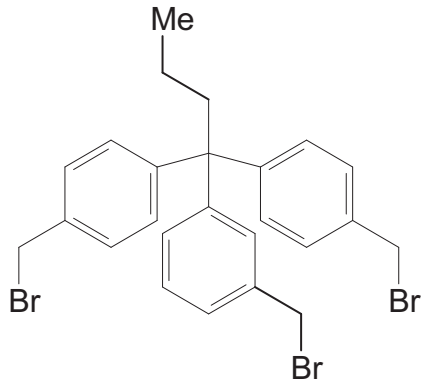
In a round bottom flask, a solution of *m*-tolyl-*p*-ditolylchloromethane (**55**) (3.69 g, 11.40 mmol) and dry THF (20 mL) was stirred vigorously for 10 min in a nitrogen atmosphere. Propyl magnesium chloride (10 mL, 30 mmol) was added dropwise over 30 min. A colour change was observed to deep red. The mixture was left to react in a nitrogen atmosphere at room temperature for 48 h. The reaction was monitored by TLC (R_f 0.8, dichloromethane – petrol, 1 : 4 v/v). The reaction mixture was poured over crushed ice (5 g) and acidified with hydrochloric acid (2 M). The crude product was extracted with diethyl ether (4 × 50 mL). The combined extracts were washed with $NaHCO_3$ and saturated brine and dried ($MgSO_4$). The solvent was removed under reduced pressure to give a yellow oil (2.94 g). The crude product was purified by medium-pressure column chromatography on silica eluting with dichloromethane – petrol (1 : 4 v/v). The product-containing fractions were combined and the solvent was removed to give the pure product as colourless oil. (1.64 g, 44 % yield)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 0.82 (3H, t, J = 7.4 Hz, CH_2CH_3), 0.93-1.03 (2H, m, $CH_2CH_2CH_3$), 2.19 (3H, s, *m*-CH_3), 2.22 (6H, s, *p*-CH_3), 2.37-2.42 (2H, t, J = 8.0 Hz, $CH_2CH_2CH_3$), 6.79-7.12 (12H, m, Ar-H)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 14.9 (*C*H_3), 19.5 (*C*H_2), 21.7 (2 × *p*-*C*H_3), 21.9 (*m*-*C*H_3), 43.6 (*C*H_2C), 56.6 (CH_2*C*), 126.6 (Ar), 126.7 (Ar), 126.8 (Ar), 127.2 (Ar), 127.8 (Ar), 128.4 (Ar), 128.7 (Ar), 129.2 (Ar), 129.5 (Ar), 129.7 (Ar), 130.3 (Ar), 130.5 (Ar), 135.3 (Ar), 135.9 (Ar), 137.3 (Ar), 141.7 (Ar), 145.4 (Ar), 148.5 (Ar)

m/z (EI): 327 ([M-C_3H_7]$^+$, 100%), 314 ([M-C_2H_5]$^+$, 75%), 286 ([M-H]$^+$, 55%), 285 ([M]$^+$, 100%)

1-[3-(Bromobenzyl)]-1,1-di[(4-bromobenzyl)]-butane (57)



In a 3-necked round bottom flask, *m*-tolyl-di-*p*-tolylbutane (**56**) (0.25 g, 0.76 mmol) and dry trifluorotoluene (30 mL) were placed under a nitrogen atmosphere. To the solution was added NBS (0.45 g, 2.5 mmol) and benzoyl peroxide (70 % pure, 0.03 g, 0.09 mmol). A condenser was fitted and a 150W tungsten lamp placed touching the flask to the side. The mixture was stirred for 3.5 h and the reaction was followed by TLC (R_f 0.3, dichloromethane - petrol, 1 : 4, v/v). The mixture was filtered through Celite and the solvent was removed under reduced pressure. The crude yellow oil was dissolved in trifluorotoluene (20 mL) and the organic phase was washed with saturated aqueous sodium metabisulfite (2 × 20 mL), and dried ($MgSO_4$). The solvent

was removed under reduced pressure to give a yellow oily residue. The crude product was purified by medium-pressure chromatography on silica eluting with dichloromethane – petrol (1 : 4 v/v). The title compound (0.13 g, 30 % yield) was obtained as a viscous oil from concentration of the product-containing fractions.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 0.82-0.86 (3H, t, J = 7.5 Hz, CH_2CH_3), 0.93-1.00 (2H, m, $CH_2CH_2CH_3$), 2.39–2.44 (2H, t, J = 8.1 and 7.5 Hz, CCH_2), 4.34 (2H, s, *m*-CH_2), 4.38 (4H, s, 2 × *p*-CH_2) 6.11-7.22 (12H, m, Ar-H)

Binding Experiments of 2, 3-bis-guanidinomethyl-biphenyl dihydrochloride (37) and bis-tetrabutylammonium glutarate in CD_3OD.

The bis-tetrabutylammonium dicarboxylate salts were prepared from the commercially available glutaric acid and tetrabutylammonium hydroxide. Two equivalents of 2 M solution of Bu_4NOH in MeOH was added to glutaric acid in methanol. The solvent was removed under reduced pressure and the solid obtained was dried *in vacuo* at 60 ˚C for 24 hours.

Firstly, two stocks of solution were prepared in CD_3OD:

Host: biphenyl-diguanidinium chloride (**37**), 10 mL of a 1.5×10^{-3} M solution

Guest: bis-tetrabutylammonium glutarate. 2 mL of a 2.5×10^{-2} M solution

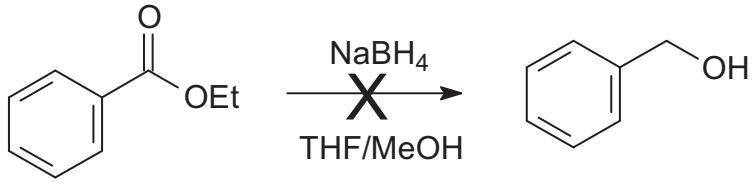
All the ^{1}H NMR results were collected from a 300 MHz Bruker Avance BVT3200 instrument at 293 K. A set of 10 titration samples were prepared in this experiment. In all samples the concentration of host was kept constant, whereas the concentration of the guest was varied in the range of 20-80 %.

Titrations were started with 0.4 mL of a known concentration (1.5×10^{-3} M) of the host, biphenyl-diguanidinium chloride (**37**), in CD_3OD into a NMR tube. A few μL of a known concentration solution of guest (2.5×10^{-2} M) was added into the NMR tube, the total volume of each sample was kept in 0.6 mL. (See following table) The mixture was shaken and immediately a ^1H NMR spectrum was measured. The difference in the chemical shift of benzylic protons was recorded. The results are presented in **Table 2** (page 62).

NMR tube No	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
Guest μL	0	3	14	25	48	72	96	120	144	168	192
CD_3OD μL	200	197	186	175	152	128	104	80	56	32	8

6.3 EXPERIMENTAL CHAPTER 3

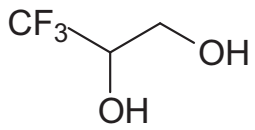
Attempted synthesis of benzyl alcohol (77)



Powered sodium borohydride (1.63 g, 43 mmol) was suspended in THF (20 mL) in presence of ethyl benzoate (**76**) (1.05 g, 1 mL, 7 mmol) during a period of 30 min under reflux (75 ˚C) and stirring. Methanol (6.33 g, 8 mL, 20 mmol) was added dropwise during a period of 15 min with bubbles formed. The mixture was stirred under reflux for a further 1 h. The reaction mixture was cooled to room temperature and quenched with a saturated solution of NH_4Cl (20 mL) for a further 1.5 h. The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 × 25 mL). The organic layers were combined and dried ($MgSO_4$). A pale yellow oil (0.95 g) was collected after removing the solvent under reduced pressure. ^{1}H NMR indicated that the yellow oil was ethyl benzoate (**76**), the starting material.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.27-1.32 (3H, t, *J* = 7.1 Hz, C*H*$_3$), 4.24-4.32 (2H, q, *J* = 7.1 Hz, C*H*$_2$), 7.31-7.36 (2H, t, *J* = 7.6 and 7.2 Hz, Ar-H), 7.43-7.47 (1H, t, *J* = 7.2 and 7.5 Hz, Ar-H), 7.94-7.97 (2H, d, *J* = 7.1 Hz, Ar-H)

3,3,3-Trifluopropane-1,2-diol (68)[159]



Ethyl trifluoropyruvate (4.25 g, 3.5 mL, 25 mmol) was dissolved in dry diethyl ether (30 mL) and added dropwise to a stirred suspension of lithium aluminium hydride (1 g, 26.3 mmol) in dry diethyl ether (30 mL), ice cold under nitrogen. The suspension was maintained at steady reflux for 3 hour. The suspension was cooled to 0 ˚C and 1 mL of cold water was added to the suspension. After addition of 2 M H_2SO_4 (20 mL), the mixture was stirred at room temperature for 1 h. The mixture was separated and the aqueous acidic phase was washed with ethyl acetate (4 × 20 mL). The combined organic phases were dried (Na_2SO_4). A yellow oil was obtained by removing the solvent under reduced pressure. The crude product was purified by medium-pressure chromatography on silica eluting with methanol – dichloromethane (1 : 19 v/v) to afford the title compound as a colourless viscous oil. R_f 0.2 (methanol – dichloromethane, 1 : 19 v/v). The final compound (2.74 g, 84 % yield) was obtained from kugelrohr distillation. (10-15 mmHg, 70 ˚C)

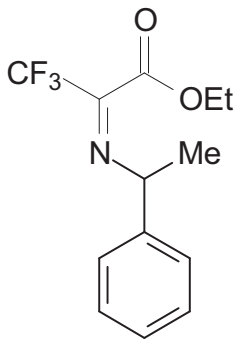
^{1}H NMR δ_H (300 MHz, D_2O): 3.60-3.67 (1H, dd, *J* = 4.5 and 6.9 Hz, C*H*H), 3.74-3.80 (1H, dd, *J* = 7.8 and 3.9 Hz, C*H*H), 4.03-4.14 (1H, m, C*H*)

^{1}H NMR δ_H (300 MHz, Methanol-d_4): 3.62-3.69 (1H, dd, *J* = 4.7 and 7.0 Hz, C*H*H), 3.93-4.04 (1H, dd, *J* = 7.8 and 4.0 Hz, C*H*H), 3.93-4.04 (1H, m, C*H*)

^{13}C NMR δ_C (75 MHz, Methanol-d_4): 62.2 (s, CH_2), 71.9-73.1 (q, *J* = 29.4 Hz, CF_3CH), 121.1-132.3 (q, *J* = 282 Hz, CF_3)

^{19}F NMR δ_F (400 MHz, D_2O): -79.2 (d, *J* = 7.3 Hz, CF_3)

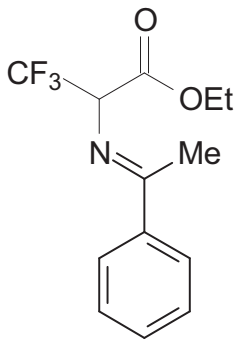
Ethyl 2-*N*-(1-phenylethyl)imino-3,3,3-trifluoropropionate (79)[162,163]



α-Methylbenzylamine (1.66 mL, 1.58 g, 13.0 mmol) in chloroform (10 mL) was added slowly to a solution of acetic acid (0.74 mL, 0.78 g, 13.0 mmol) in chloroform (5 mL) in a 2-necked round bottom flask fitted with a reflux condenser. The mixture was stirred for further 5 min. Ethyl trifluoropyruvate (1.42 mL, 2.0 g, 11.8 mmol) in chloroform (5 mL) was added to the mixture, which was stirred at 30 ˚C for 72 h. The solvent was removed under reduced pressure to give a yellow oil. The title compound was obtained as a pale yellow oil (2.51 g, 78 % yield) from filtration through a short-pad of silica gel eluting with diethyl ether – petrol (1 : 9 v/v).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.37 (3H, t, *J* = 7.2 Hz, CH_2CH_3), 1.48 (3H, d, *J* = 6.5 Hz, CH_3CHPh), 4.40 (2H, q, *J* = 7.2 Hz, CH_2CH_3), 4.88 (1H, q, *J* = 6.5 Hz, CH_3CHPh), 7.20-7.29 (5H, m, Ar-H)

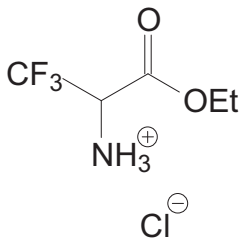
***N*-(1-Phenyl)ethylidene-3,3,3-trifluoroalanine ethyl ester (80)** [162,163]



Ethyl 2-*N*-(1-phenylethyl)imino-3,3,3-trifluoropropionate (**79**) was dissolved in 5 mL of triethylamine and the mixture was stirred at room temperature. Progress of the isomerisation was monitored by TLC and upon completion, any undissolved solid was removed by filtration and the triethylamine was removed *in vacuo*. The residual material was dried in high vacuum. The Schiff base (**80**) was used in the next experiment without further purification.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.33 (3H, t, *J* = 7.1 Hz, C*H*$_3$CH$_2$), 2.35 (3H, s, N=CC*H*$_3$), 4.30 (2H, q, *J* = 7.1 Hz, CH$_3$C*H*$_2$), 4.88 (1H, q, *J* = 7.2 Hz, CF$_3$C*H*), 7.40-7.48 (3H, m, H-Ar), 7.90-7.93 (2H, m, H-Ar)

3,3,3-Trifluoroalanine ethyl ester hydrochloride (81)[162]



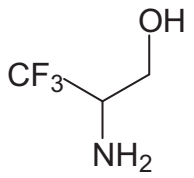
N-(1-Phenyl)ethylidene-3,3,3-trifluoroalanine ethyl ester (**80**) (1.48 g, 5.4 mmol) was dissolved in diethyl ether (12 mL) and 1 M hydrochloric acid (12 mL) was added dropwise. The mixture was stirred at room temperature for 2 h. The aqueous phase was separated and washed with diethyl ether (3 × 10 mL). The aqueous solution was evaporated *in vacuo* and freeze-dried to give a white solid. The solid was dissolved in acetonitrile at reflux and allowed to cool slowly to room remperature, then stored at -20 ˚C. The resulting crystals were collected by suction filtration and dried *in vacuo* at 40 ˚C to afford the title compound (0.76 g, 69 % yield) as an off-white crystalline solid.

m.p.: 154-156 °C (lit.[162] value: 153-157 °C)

^{1}H NMR δ_H (300 MHz, D_2O): 1.16 (3H, t, *J* = 7.3 Hz, CH_2CH_3), 3.05-3.12 (2H, q, *J* = 7.3 Hz, CH_2CH_3)

^{1}H NMR δ_H (300 MHz, Methanol-d_4):1.34 (3H, t, *J* = 7.3 Hz), 4.40-4.48 (2H, q, *J* = 7.2 Hz), 5.24-5,32 (1H, q, *J* = 7.8 Hz, CF_3CH)

2-Amino-3,3,3-trifluoropropanol (69)



Anhydrous diethyl ether (8 mL) was added dropwise to lithium aluminium hydride (97 mg, 2.56 mmol) in a dry, 25 mL, two-necked, round bottomed flask under nitrogen at 0 °C. The suspension was stirred for 5 min at room temperature and cooled to 0 °C again. 3,3,3-Trifluoroalanine ethyl ester hydrochloride (300 mg, 1.45 mmol) was added to the mixture slowly. (CAUTION: small portion of the solid were added very carefully under nitrogen). The mixture was warmed to room temperature and maintained at steady reflux for 1 hour. After the suspension was cooled to 0 °C, water (110 mg, 0.11 mL, 5.89 mmol) was added dropwise, and the mixture was stirred overnight at room temperature. The mixture was dried (Na_2SO_4), and the filtrate was filtered though a Celite bed. The solid was washed well with dichloromethane. The combined organic phases were concentrated under *vacuo*. The crude product was purified by sublimation (30-35 °C, 10-20 mmHg). The title compound was collected as white crystals. (0.48 g, 27 % yield)

m.p.: 40-42 °C

^{1}H NMR δ_H (300 MHz, D_2O): 1.65 (2H, br, N*H*$_2$), 3.23-3.29 (1H, m, CF_3C*H*CH_2), 3.50-3.56 (1H, dd, *J* = 3.0 and 8.0 Hz, CHC*H*H), 3.75-3.80 (1H, dd, *J* = 7.5 and 3.6 Hz, CHCH*H*)

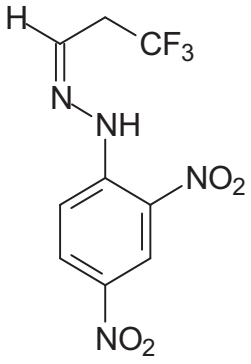
^{19}F NMR δ_F (400 MHz, D_2O): -76.7 (d, *J* = 7.3 Hz, C*F*$_3$)

Preparation of authentic trifluoro acetone-DNPs and trifluoro aldehyde-DNPs: general procedure

2,4-Dinitrophenylhydrazine solution was made up from 2,4-dinitrophenyhydrazine (2 g, 10.1 mmol) in concentrated H_2SO_4 (10 mL) cautiously diluted to 500 mL with distilled water. (5 mmol/l) The suspension obtained was stored in darkness and filtered immediately before use.

1,1,1-Trifluoroacetone or 3,3,3-trifluoropropionaldehyde (100 mg) was added to the prepared 2,4-dinitrophenylhydrazine solution (100 mL) in a flask at room temperature, causing an immediate yellow cloudiness, which rapidly coagulated to a precipitate. After standing for 30 min, with occasional shaking, the liquor was filtered. A yellow solid was obtained, which was dried in high vacuum.

1-(2,4-Dinitrophenyl)-2-(3,3,3-trifluoropropylidene)hydrazine[199]

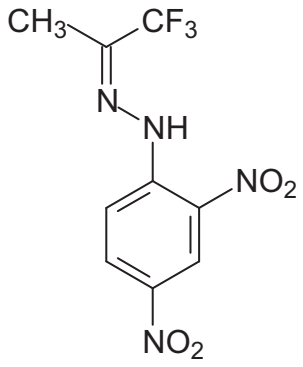


m.p.: 148-150 °C (lit.[198] value: 149.6-150.2 °C)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 3.19-3.25 (2H, m, CF_3CH_2), 7.40 (1H, t, *J* = 5.7 Hz, N=C*H*), 7.88 (1H, d, *J* = 9.5 Hz, H-6 Ar), 8.28 (1H, dd, *J* = 9.5 and 2.5 Hz, H-5 Ar), 9.06 (1H, d, *J* = 2.5 Hz, H-3 Ar), 11.15 (1H, br, N*H*)

^{19}F NMR δ_F (400 MHz, $CDCl_3$): -64.1 (t, *J* = 10.2 Hz, CF_3)

1-(2,4-dinitrophenyl)-2-(1,1,1-trifluoropropan-2-ylidene)hydrazine[200]



m.p.: 127-128 °C (lit.[199] value: 139 °C)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 2.16 (3H, s, CH_3), 7.97 (1H, d, *J* = 9.5 Hz, H-6 Ar), 8.34 (1H, dd, *J* = 9.5 and 2.5 Hz, H-5 Ar), 9.08 (1H, d, *J* = 2.5 Hz, H-3 Ar), 11.08 (1H, br, N*H*)

^{19}F NMR δ_F (400 MHz, $CDCl_3$): -71.0 (s, CF_3)

Preparation of EPR samples of 2-amino-3,3,3-trifluoropropanol and 2-amino-propanol

Conditions for the experiment:
10 mg/mL EAL (120 μM sites), 240 μM AdoCbl, 20 mM substrates (2-amino-3,3,3-trifluoropropanol and 2-amino- propanol), 10 mM potassium phosphate, pH 7.4.

Samples were prepared as a 100 mM stock solution in a darkened room at room temperature. The EAL had been prepared as a 48.3 mg/mL stock solution. The AdoCbl stock solution was prepared at 5.75 mM.

The experiments were carried out by mixing (in the following order) 165 μL potassium phosphate, 62 μL EAL and 60 μL substrate. After 13 μL AdoCbl was added, the total volume of the solution was made up to 300 μL. The solution was transferred to an EPR tube. Freezing times are as noted below.

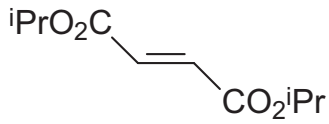
One sample was frozen in isopentane immediately after the addition of AdoCbl. Later this sample was thawed and allowed to incubate for 10 min at room temperature before freezing again. The second sample was frozen 1 min after the addition of AdoCbl. Later, this sample was thawed and incubated for 10 min and sonicated before refreezing. The EPR spectrum is shown in **Figure 31** (Page 94).

General procedure for radiolysis experiments

Continuous radiolysis experiments were performed on a ^{60}Co Gammacell apparatus. A solution of 3,3,3-trifluoropropane-1,2-diol or propane-1,2-diol (0.6-1 mmol) in D_2O (0.6-1 ml) was saturated with N_2O. The solution was γ-irradiated in the Gammacell with a dose of 1000 Gy to 3000 Gy. The crude irradiated sample was preliminarily analysed by ^1H-NMR. A few drops of 0.5 *N* hydrochloric acid was added to the crude solution. 1 mL of $CDCl_3$ was added to extract the mixture. The organic sample was analysed by ^1H-NMR, gas chromatography and gas chromatography mass spectrometry (ethyl acetate used as a solvent for the GC and GC-MS). 2,4-Dinitrophenylhydrazine solution (see last section) was added to the crude irradiated sample and the mixture was stirred overnight. After filtration, the liquor was extracted with dichloromethane (2 × 10 mL). The organic layer was washed by saturated $NaHCO_3$ and dried ($MgSO_4$). A red solid was obtained after removing the solvent under reduced pressure. The solid obtained was characterised by ^1H-NMR.

6.4 EXPERIMENTAL CHAPTER 4

But-2-enedioic acid diisopropyl ester (104)[201]

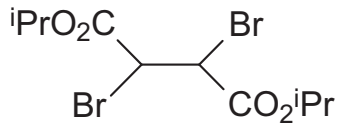


Fumaric acid (**103**) (1.0 g, 8.62 mmol) was dissolved in dry isopropanol (6 mL, 34.46 mmol) under an atmosphere of nitrogen. Sulfuric acid (1 mL, 0.86 mmol) was added and the reaction mixture was heated at reflux (90 °C) overnight. After cooling, the solvent was removed and the crude product was redissolved in ethyl acetate (20 mL), washed with aqueous $NaHCO_3$ (3 × 20 mL) and dried ($MgSO_4$). The solvent was removed to afford the title compound as colourless oil (1.27 g, 74 % yield).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.24-1.26 (12H, d, *J* = 6.3 Hz, 4 × C*H*$_3$), 4.03-5.11 (2H, septet, *J* = 6.3 Hz, 2 ×C*H*CH$_3$CH$_3$), 6.77 (2H, s, C*H*=C*H*)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 22.1 (4 × *C*H$_3$), 69.3 (2 × *C*H), 134.3 (2 × *C*=*C*), 164.9 (2 ×*C*=O)

rac-2, 3-Dibromo-succinic acid diisopropyl ester (105)



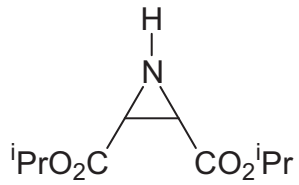
But-2-enedioic acid diisopropyl ester (**104**) (1.27 g, 6.35 mmol) was dissolved in dry dichloromethane (30 mL). Bromine (0.78 g, 0.25 mL, 4.88 mmol) was added dropwise over 30 min and the reaction mixture was stirred at room temperature for 2 days. The reaction was followed by TLC eluting with dichloromethane – petrol (2 : 5 v/v). The reaction mixture was washed with aqueous sodium metabisulfite, dried ($MgSO_4$) and the solvent was removed. The crude compound was purified by medium pressure chromatography eluting with dichloromethane – petrol (2 : 5, v/v). The product containing fractions were collected and the solvent was removed to give the title compound (1.86 g, 81 % yield) as a white solid. R_f 0.50 (dichloromethane – petrol, 2 : 5 v/v)

m.p.: 51-52 °C

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.29-1.32 (12H, dd, *J* = 6.3 Hz and 1.8 Hz, 4 × C*H*$_3$), 4.62 (2H, s, C*H*Br), 5.09-5.17 (2H, septet, *J* = 6.3 Hz, 2 ×C*H*CH_3CH_3)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 21.6 (4C, *C*H_3), 42.9 (2C, *C*H), 70.9 (2C, *C*Br), 166.1 (2C, *C*OOCH)

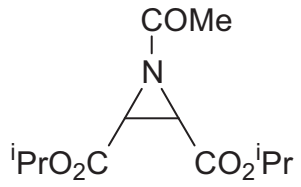
Aziridine-2,3-dicarboxylic acid diisopropyl ester (106)[202]



Into a 3-necked round bottomed flask, dry actonitrile (20 mL) was cooled in a acetone/dry ice bath to -20 °C under an atmosphere of nitrogen. Ammonia gas was bubbled into the solution for 25 min at this temperature. 2,3-Dibromo-succinic acid diisopropyl ester (**105**) (1.5 g, 4.13 mmol) in dry acetonitrile (20 mL) was added dropwise to the solution. The reaction mixture was allowed to warm up to room temperature and was stirred overnight. The reaction mixture was filtered through Celite and the solvent was removed. The crude product was purified by medium pressure chromatography on silica eluting with petrol - diethyl ether (3 : 7 v/v). The product containing fractions were collected and the solvent was removed to afford the title compound (0.48 g, 54 % yield) as yellow oil. R_f 0.65 (petrol - diethyl ether, 3 : 7 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.21 (12H, d, J = 6.3 Hz, 4 × CH_3), 1.70 (1H, br, NH), 2.75 (2H, s, C*H*NHC*H*), 4.97-5.05 (2H, septet, J = 6.3 Hz, $CO_2CHCH_3CH_3$)

1-Acetyl-aziridine-2,3-dicarboxylic acid diisopropyl ester (107)

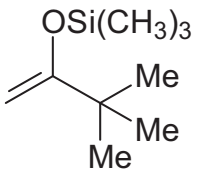


Aziridine-2,3-dicarboxylic acid diisopropyl ester (**106**) (0.87 g, 4.05 mmol) was dissolved in pyridine (5 mL) and the mixture was stirred for 15 min. Acetic anhydride (1.21 mL, 1.12 mL, 11.86 mmol) was added dropwise to the solution and the reaction mixture was warmed up at 60 °C for 1 h. The reaction mixture was allowed to cool down and water (5 mL) was added. The mixture was extracted with dichloromethane (4 × 10 mL). The combined organic fractions were washed with 2 M hydrochloric acid (4 × 10 mL), dried ($MgSO_4$) and the solvent was removed to afford a yellow oil. The crude product was purified by medium pressure chromatography on silica eluting with ethyl acetate – petrol (3 : 7 v/v) to afford the title compound (0.48 g, 46 % yield) as a colourless oil. R_f 0.7 (ethyl acetate – petrol 3 : 7 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.25-1.28 (12H, dd, *J* = 6.3 and 2.7 Hz, 4 × C*H*$_3$), 2.09 (3H, s, COC*H*$_3$), 3.35 (2H, s, C*H*NC*H*), 5.00-5.09 (2H, septet, *J* = 6.3 Hz, CO_2CH)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 21.9 (4C, *C*H$_3$), 24.3 (1C, COC*C*H$_3$), 40.7 (2C, CO_2CH), 70.7 (2C, *C*HNC*C*H), 166.2 (2C, *C*=O), 177.5 (1C, NC=O)

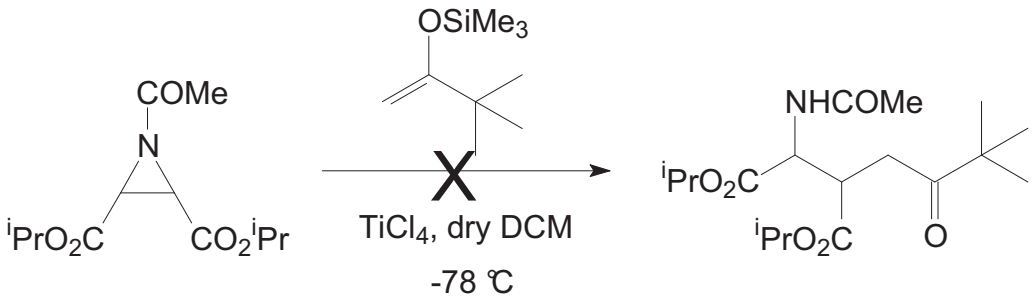
(2,2-Dimethyl-1-methylene-propoxy)-trimethyl-silane (108)[181]



Into a 3-necked round-bottomed flask, pinacolone (**111**) (4.00 g, 5.0 mL, 39.8 mmol) was added to triethylamine (4.84 g, 6.69 mL, 47.83 mmol) followed by the addition of trimethylchlorosilane (5.20 g, 6.07 mL, 47.86 mmol). Sodium iodide (7.20 g, 48.03 mmol) in dry acetonitrile (40 mL) was added dropwise to the reaction mixture. The reaction was stirred at room temperature for 25 min. Cold pentane (40 mL) was added followed by ice-water (40 mL). The organic phase was separated and the product was extracted with pentane (3 × 40 mL). The combined organic fractions were washed with saturated aqueous ammonium chloride (30 mL), dried (Na_2SO_4) and the solvent was removed. The crude was distilled under vacuum (b.p. 68 °C at 80 mmHg) and the product was obtained pure as a colourless oil (3.66 g, 53 % yield).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 0.0 (9H, s, Si(CH_3)$_3$), 0.84 (9H, s, 3 × CH_3), 3.72 (1H, d, *J* = 1.3 Hz, C=C*H*H), 3.88 (1H, d, *J* = 1.3 Hz, C=CH*H*)

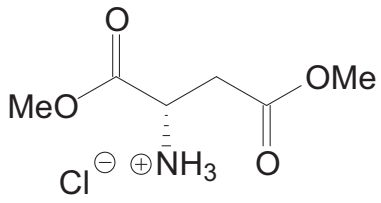
Attempted synthesis of diisopropyl 2-acetamido-3-(3,3-dimethyl-2-oxobutyl) succinate (109)



1-Acetyl-aziridine-2,3-dicarboxylic acid diisopropyl ester (**107**) (0.66 g, 2.57 mL) in dry dichloromethane (10 mL) was added dropwise to titanium tetrachloride (1 M, 2.82 mL, 2.82 mmol) in a round-bottom flask at -78 °C under nitrogen. (2,2-Dimethyl-1-methylene-propoxy)-trimethyl-silane (**108**) (0.49 g, 2.82 mmol) in dichloromethane (10 mL) was added dropwise into the mixture. The mixture was stirred for 4 h and warmed to room temperature. Water was slowly added into the reaction mixture. The organic layer was separated and the aqueous layer was washed with diethyl ether (3 × 20 mL). The combined organic layers were dried ($MgSO_4$) and filtered through Celite bed. A pale yellow oil (0.65 g) was obtained after removing the solvent under reduced pressure. ^{1}H NMR indicated that this was starting material (**107**).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.25-1.28 (12H, dd, *J* = 6.3 and 2.7 Hz, 4 × C*H*$_3$), 2.09 (3H, s, COC*H*$_3$), 3.35 (2H, s, C*H*NC*H*), 5.00-5.09 (2H, septet, *J* = 6.3 Hz, CO_2C*H*)

Dimethyl (*S*)-aspartate hydrochloride (113)[203]

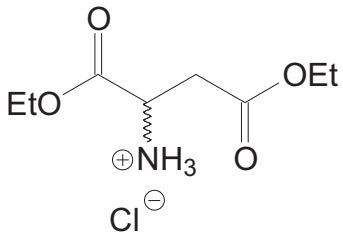


Thionyl chloride (16.7 g, 10 mL, 0.14 mmol) was added dropwise to a suspension of (*S*)-aspartic acid (13.3 g, 0.10 mmol) in methanol (75 mL) at 0 °C. The bath was removed, the solution was stirred at room temperature for 45 h and the solvent was removed under reduced pressure. The residue was triturated with diethyl ether. The solid was collected by suction filtration and dried *in vacuo* at 50 °C to afford the title compound as a white solid. (19.4 g, 98 % yield)

m.p. 114-115 °C (lit.[22] mp 116-117 °C)

^{1}H NMR δ_H (300 MHz, D_2O): 3.18 (2H, t, *J* = 4.6 and 6.3 Hz, CH_2), 3.56 (3H, s, β-OCH_3), 3.65 (3H, s, α-OCH_3), 4.51 (1H, t, J = 4.6 and 5.2 Hz, C*H*)

Diethyl rac-aspartate hydrochloride (120)[204]



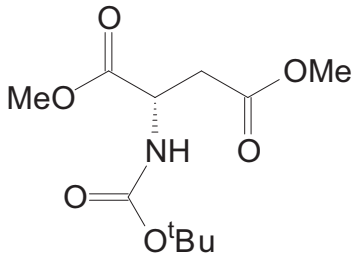
The title compound was obtained as a white solid with 94 % yield from a similar procedure to that of (**113**).

m.p. 96-98 °C (lit.[205] mp 105-106 °C)

^{1}H NMR δ_H (300 MHz, Methanol-d4): 1.28-1.33 (6H, t, *J* = 7.2 Hz, 2 × CH_2CH_3), 3.03 (2H, d, *J* = 6.1 Hz, CH_2CO), 4.20-4.28 (4H, q, *J* = 7.1 Hz, 2 × CH_2CH_3), 4.29-4.33 (1H, t, *J* = 5.5 Hz, $CHCH_2$)

^{13}C NMR δ_C (75 MHz, Methanol-d4): 14.0 (CH_3), 14.1 (CH_3), 34.9 (CH_2CO), 50.4 ($COCHNH_2$), 62.5 (OCH_2), 62.6 (OCH_2), 168.8 (*C*=O), 170.8 (*C*=O)

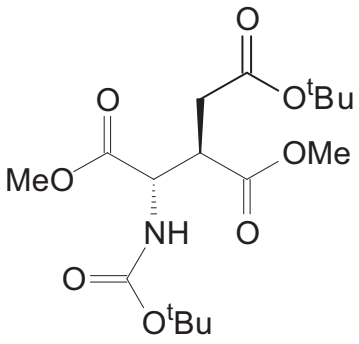
Dimethyl (*S*)-2-(*tert*-butoxycarbonylamino)succinate (114)[206]



Triethylamine (16.26 g, 22.40 mL, 160.69 mmol) and di-*tert*-butyl dicarbonate (18.37 g, 84.10 mmol) were added sequentially to a suspension of dimethyl-(*S*)-aspartate hydrochloride (**113**) (14.80 g, 74.90 mmol) in THF (120 mL) at room temperature under nitrogen. The mixture was stirred for 72 h, and the solvent was removed under reduced pressure. Ethyl acetate (80 mL) was added to extract the residue. The suspension was filtrated and the filtrate was dried ($MgSO_4$). A pale yellow residue was collected after removing the solvent under reduced pressure. The product was purified by medium-pressure chromatography eluting with ethyl acetate – petrol (3 : 7 v/v), to afford the title compound (12.34 g, 63 % yield) as a white solid. R_f 0.4 (ethyl acetate – petrol, 3 : 7 v/v).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.43 (9H, s, 3 × CC*H*$_3$), 2.77-2.84 (1H, dd, *J* = 4.7 and 17.0 Hz, C*H*HCO), 2.96-3.03 (1H, dd, *J* = 4.3 and 17.1 Hz, CH*H*CO), 3.68 (3H, s, COOC*H*$_3$), 3.74 (3H, s, COOC*H*$_3$), 4.54-4.59 (1H, pentet, *J* = 4.2 and 8.6 Hz, C*H*), 5.48-5.50 (1H, d, *J* = 8.3 Hz, N*H*)

(1*S*,2*R*)-3-*tert*-Butyl-1,2-dimethyl-1-(*tert*-butoxycarbonylamino)propane-1,2,3-tricarboxylate (115)[207]

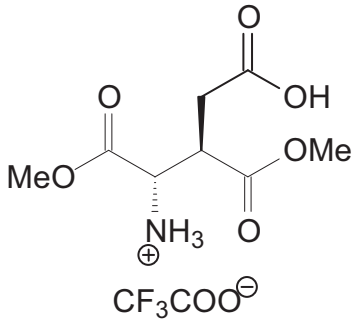


To a stirred solution of hexamethyldisilazane (2.73 g, 3.60 mL, 16.9 mmol) in dry THF (30 mL) was added 2.5 M of *n*-butyllithium in hexane (2.5 M, 6.80 mL, 16.9 mmol) at 0 °C under a nitrogen atmosphere. The mixture was stirred for 20 min and then cooled to -78 °C. A solution of compound (**114**) (2.00 g, 7.70 mmol) in THF (10 mL) was added slowly to the mixture. The reaction mixture was stirred for 2 h at the same temperature. After the addition of *tert*-butyl bromoacetate (1.85 g, 1.4 mL, 9.48 mmol), the reaction mixture was stirred for an additional 3 h and quenched by addition of 3 M hydrochloric acid to pH ≈ 3. The aqueous layer was saturated with sodium chloride and extracted with ethyl acetate (2 × 20 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure to give an oily residue. The crude product was purified by medium-pressure chromatography on

silica eluting with ethyl acetate – petrol (3 : 7 v/v) to afford the title compound (1.0 g, 35 % yield) as an oil. R_f 0.65 (ethyl acetate – petrol, 3 : 7 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.40 (18H, s, 6 × CC*H*$_3$), 2.42-2.49 (1H, dd, *J* = 6.8 and 17.0 Hz, C*H*HCO), 2.60-2.68 (1H, dd, *J* = 7.7 and 17.0 Hz, CH*H*CO), 3.52-3.58 (1H, m, CHC*H*CH$_2$), 3.64 (3H, s, COOC*H*$_3$), 3.70 (3H, s, COOC*H*$_3$), 4.56-4.60 (1H, dd, *J* = 3.6 and 9.5 Hz, NHC*H*CH), 5.26 (1H, br s, N*H*)

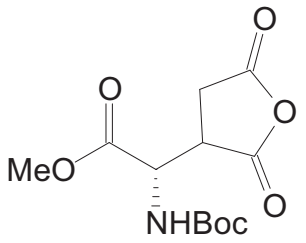
(3*R*,4*S*)-Amino-5-methoxy-3-(methoxycarbonyl)-5-oxopentanoic acid (116)



Compound (**115**) (0.70 g, 1.86 mmol) in trifluoroacetic acid (8 mL) was stirred at room temperature for 20 min. The mixture was evaporated to dryness and residual trifluoroacetic acid was removed azeotropically with chloroform (5 × 20 mL) to afford the title compound as an oil that was used without further purification.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 3.03-3.24 (2H, m, C*H*$_2$CO), 3.76 (3H, s, COOC*H*$_3$), 3.84 (3H, s, COOC*H*$_3$), 4.12-4.26(1H, m, CHC*H*CH$_2$), 4.41-4.50 (1H, m, C*H*CH)

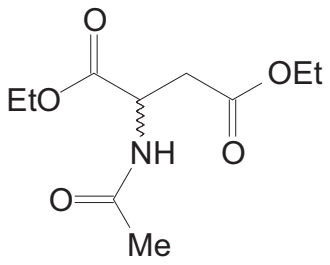
(2*S*)-Methyl-2-(*tert*-butoxycarbonylamino)-2-(2,5-dioxotetrahydrofuran-3-yl)acetate (118)



Triethylamine (3.63 g, 5 mL, 35.87 mmol) and di-*tert*-butyl dicarbonate (1.80 g, 8 mmol) were added sequentially to a solution of crude acid **116** (1.48 g, 7.49 mmol) in THF (20 mL) at room temperature. The mixture was stirred overnight and the solvent was removed under reduced pressure. The residue was dissolved in ethyl acetate (20 mL) and the solution was extracted with saturated brine (15 mL). The aqueous layer was washed with ethyl acetae (3 × 15 mL), and the combined organic phases were dried ($MgSO_4$). The solvent was removed under reduced pressure. The crude product was purified by medium-pressure chromatography on silica eluting with ethyl acetate – petrol (3 : 7 v/v), to afford the title compound (0.25 g, 46 % yield) as a colourless oil. R_f 0.7 (ethyl acetate – petrol, 3 : 7 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.44 (9H, s, 3 × CC*H*$_3$), 2.77-2.84 (1H, dd, *J* = 4.6 and 17.0 Hz, C*H*HCO), 2.96-3.03 (1H, dd, *J* = 3.8 and 17.0 Hz, CH*H*CO), 3.75 (3H, s, COOC*H*$_3$), 4.07-4.11 (1H, m, CHC*H*CH$_2$), 4.53-4.58 (1H, dd, *J* = 4.1 and 8.0 Hz, NHC*H*CH), 5.47-5.50 (1H, d, *J* = 7.9 Hz, N*H*)

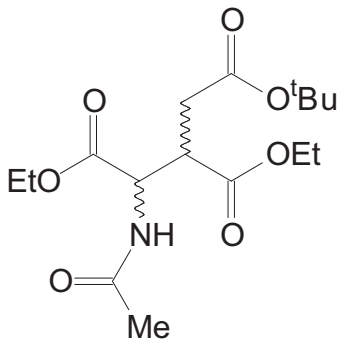
Diethyl rac-2-acetamidosuccinate (121)[208]



Triethylamine (4.04 g, 5.56 mL, 40 mmol) was added to a suspension of racemic diethyl aspartate hydrochloride (**120**) (1.73 g, 10 mmol) in dichloromethane (50 mL) at room temperature. The suspension was dissolved after triethylamine was added. Acetic anhydride (1.22 g, 1.13 mL, 12 mmol) was added sequentially to the mixture which was stirred for 1 h, and the solvent was removed under reduced pressure. Ethyl acetate (20 mL) was added to dissolve the residue. The solvent was filtrated and dried ($MgSO_4$). A yellow residue was collected after removing the solvent under reduced pressure. The product was purified by medium-pressure chromatography eluting with ethanol – dichloromethane (1 : 10 v/v) to afford the title compound (1.56 g, 68 % yield) as a colourless oil. R_f 0.6 (ethanol – dichloromethane, 1 : 10 v/v).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.17-1.23 (6H, m, 2 × OCH_2CH_3), 1.97 (3H, s, CH_3CO), 2.73-2.80 (1H, dd, J = 4.5 and 17.2 Hz, C*H*HCO), 2.92-2.99 (1H, dd, J = 4.3 and 17.2 Hz, CH*H*CO), 4.04-4.20 (4H, m, 2 × OCH_2CH_3), 4.74-4.79 (1H, m, $NHCHCH_2$), 6.46 (1H, br, N*H*)

3-*t*-Butyl 1,2-diethyl rac-1-acetamidopropane-1,2,3-tricarboxylate (122)



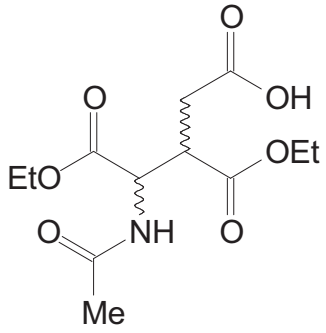
To a stirred solution of hexamethydisilazane (0.85 g, 1.12 mL, 5.27 mmol) in THF anhydrous (10 mL) was added 2.5 M of n-butyllithium in hexane (2.5 M, 5.30 mmol, 2.12 mL) at 0 °C under nitrogen atmosphere. The mixture was stirred for 10 min and cooled to -78 °C. A solution of diethyl 2-acetamidosuccinate (**121**) (0.56 g, 2.4 mmol) in THF (10 mL) was added dropwise to the mixture. The reaction mixture was stirred for 20 min at the same temperature. After dropwise addition of tert-butyl bromoacetate (0.63 g, 0.48 mL, 3.23 mmol), the reaction mixture was stirred for a further 2 h and quenched by addition of 1 N HCl solution to pH ≈ 3. The aqueous layer was saturated with NaCl and extracted with ethyl acetate (2 × 15 mL). The combined organic layers were dried ($MgSO_4$) and evaporated under reduced pressure to give a yellow oil (1.87g). The crude product was purified by medium pressure chromatography on silica eluting with ethyl acetate – petrol (4 : 6 v/v). The product-containing fractions were collected and the solvent was removed to afford the title compound as pale yellow oil (0.72 g, 87 % yield). R_f 0.2 (ethyl acetate – petrol, 4 : 6 v/v).

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.27 (6H, t, J = 7.2 Hz, 2 × OCH_2CH_3), 1.44 (9H, s, $C(CH_3)_3$), 2.04 (3H, s, CH_3CO), 2.46-2.54 (1H, dd, J = 6.5 and 17.2 Hz, C*H*HCO),

2.62-2.71 (1H, dd, J = 7.8 and 17.2 Hz, CH*H*CO), 3.57-3.63 (1H, m, CHC*H*CH_2), 4.14-4.24 (4H, q, J = 7.2 Hz, 2 × OC*H*$_2$$CH_3$), 4.92-4.96 (1H, dd, J = 3.5 and 9.1 Hz, NHC*H*CH), 6.23-6.26 (1H, d, J = 8.9, N*H*)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 14.3 (OCH$_2$*C*H$_3$), 21.2 (*C*H$_3$CO), 28.4 (C(*C*H$_3$)$_3$), 34.9 (*C*H$_2$CO), 43.8 (*C*HCO), 52.9 (O*C*H$_2$), 61.5 (O*C*H$_2$), 62.1 (*C*HNH), 81.6 (O*C*(CH$_3$)$_3$), 170.5 (*C*=O), 170.7 (*C*=O), 172.5 (*C*=O)

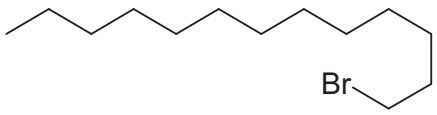
ras-4-Acetamido-5-ethoxy-3-(ethoxycarbonyl)-5-oxopentanoic acid (123)



3-*t*-Butyl 1,2-dimethyl 1-acetamidopropane-1,2,3-tricarboxylate (**122**) (0.32 g, 0.9 mmol) in trifluoroacetic acid (5 mL) was stirred at room temperature for 20 min. The mixture was evaporated to dryness and residual trifluoroacetic acid was removed azeotropically with chloroform (5 × 10 mL) to afford the product as a viscous oil. The compound was dissolved in acetonitrile at reflux and allowed to cool slowly to room temperature, then stored at -20 °C overnight. The resulting crystals were collected by suction filtration and dried *in vacuo* at 50 °C to afford the title compound (0.23 g, 89 % yield) as a white crystalline solid. X-ray crystal structure analysis of the crystals indicated the compound was mixture of (2*S*,3*R*)/(2*R*,3*S*) isomer. (See appendix 2 for X-ray crystal structure)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.22-1.30 (6H, t, *J* = 8.0 and 15.2 Hz, 2 × OCH_2CH_3), 2.00 (3H, s, CH_3CO), 2.56-2.64, (1H, dd, *J* = 7.9 and 17.5 Hz, C*H*HCO), 2.77-2.85 (1H, dd, *J* = 6.4 and 17.5 Hz, CH*H*CO), 3.64-3.69 (1H, m, CHC*H*CH_2), 4.12-4.25 (4H, m, 2 × OCH_2CH_3), 5.00-5.04 (1H, dd, *J* = 3.2 and 8.9 Hz, NHC*H*CH), 6.68-6.71 (1H, d, *J* = 8.9, N*H*)

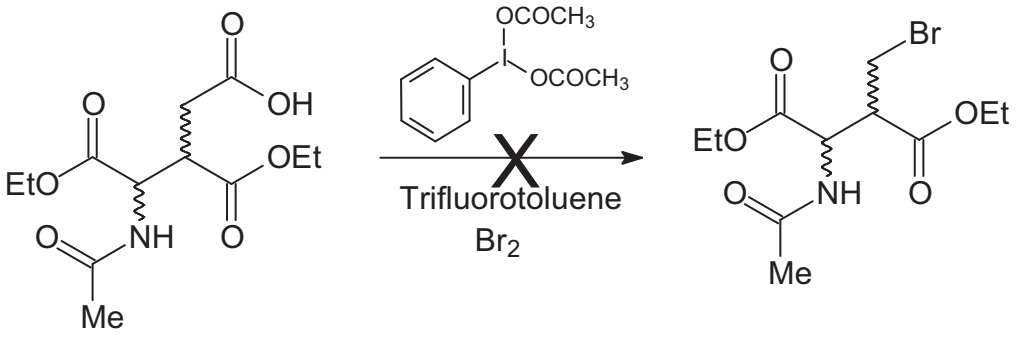
Bromotridecane (126)[209]



A mixture of the myristic acid (684 mg, 3 mmol), iodobenzene diacetate (1.45 g, 4.5 mmol) and bromine (0.77 g, 0.25 mL, 4.5 mmol) in trifluorotoluene (20 mL) under nitrogen was irradiated at reflux with a 150 W tungsten lamp. The mixture was stirred for 4 h and cooled to room temperature. Iodobenzene diacetate (1.45 g, 4.5 mmol) and bromine (0.77 g, 0.25 mL, 4.5 mmol) were added and irradiation under the same conditions was continued for 18 h. The solution obtained was cooled to room temperature. It was washed with saturated aqueous sodium metabisulfite (3 × 20 mL), saturated aqueous $NaHCO_3$ (3 × 20 mL) and brine (3 × 20 mL) and dried (Na_2SO_4). A pale yellow residue (0.69 g) was obtained after removing the solvent under reduced pressure.

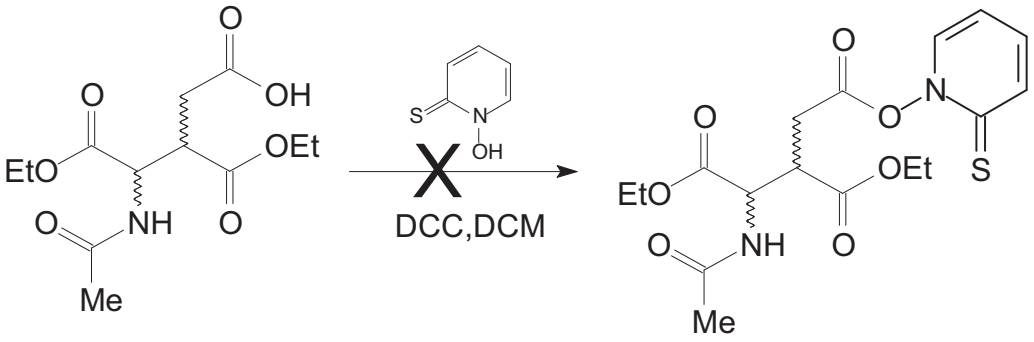
^{1}H NMR δ_H (300 MHz, $CDCl_3$): 0.90-0.94 (3H, t, *J* = 6.8 and 6.4 Hz, CH_3), 1.30 (20H, b, 10 × CH_2), 1.83-1.90 (2H, m, CH_2CH_2Br), 3.41-3.45 (2H, t, *J* = 6.9 Hz CH_2Br)

Attempted synthesis of diethyl rac-2-acetamido-3-(bromomethyl)succinate (124)



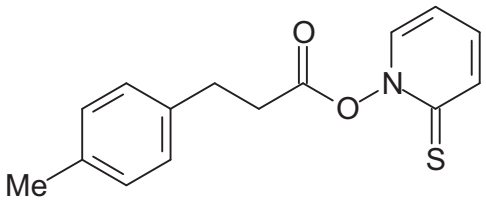
A similar procedure to last experiment was undertaken to synthesis diethyl 2-acetamido-3-(bromomethyl)succinate from 4-acetamido-5-ethoxy-3-(ethoxycarbonyl) -5-oxopentanoic acid (**123**) (500 mg, 1.92 mmol). A pale yellow residue (0.46 g) was obtained after removing the solvent under reduced pressure. The residue was purified by medium pressure chromatography on silica eluting with petrol - ethyl acetate – methanol (7 : 2 : 1 v/v). All the fractions were collected and the solvent was removed. No starting material or desired product was observed by ^{1}H NMR analysis.

Attempted synthesis of 1,2-diethyl 3-(2-thioxopyridin-1(2H)-yl) 1-acetamidopropa-ne-1,2,3-tricarboxylate (130)



N-Hydroxythiopyridone (**132**) (1.5 mg, 0.9 mmol) was added to dicyclohexylcarbodiimide (200 mg, 0.98 mmol) in dichloromethane (10 mL). The solution was cooled to 0 °C and 4-acetamido-5-ethoxy-3-(ethoxycarbonyl)-5-oxopentanoic acid (**123**) (240 mg, 0.8 mmol) in dichloromethane (5 mL) was added dropwise to the mixture. The reaction was allowed to warm to room temperature and stirred for further 16 h. The bright green suspension was filtered through a bed of silica gel (2 cm) eluting with dichloromethane. A green residue (68 mg) was collected after removing the solvent under reduced pressure. ^{1}H NMR indicated that no starting material (**123**) or desired product (**124**) was obtained.

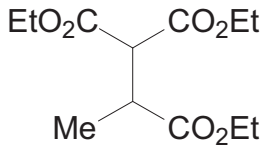
2-Thioxopyridin-1(2H)-yl 3-*p*-tolylpropanoate (133)



A similar procedure to last experiment was carried out to synthesise 2-thioxopyridin-1(2H)-yl 3-*p*-tolylpropanoate (**133**) from 3-*p*-tolylpropanoic acid (**131**) (164 mg, 1 mmol). A green solid (220 mg) was obtained after removing the solvent under reduced pressure.

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 2.26 (1H, s, C*H*$_3$), 2.92-3.06 (4H, m, 2 × C*H*$_2$), 6.51-6.56 (1H, m, NC*H*), 7.07-7.19 (1H, m, 5 × C*H*), 7.34-7.37 (1H, d, *J* = 7.0 Hz, C*H*), 7.60-7.63 (1H, d, *J* = 8.8 Hz, C*H*)

Triethyl propane-1,1,2-tricarboxylate (136)[191]



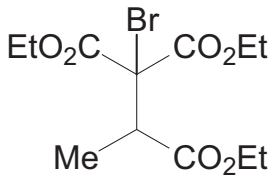
Diethyl malonate (**134**) (16.1 g, 15.3 mL, 100 mmol) was added to an ethanolic solution of sodium ethoxide (1.4 M, 82 mL, 115 mmol) [made by the reaction of sodium (3.55 g) with dry ethanol (100 mL)]. The resulting sodium salt was cooled in an ice-bath and ethyl 2-bromopropionate (**135**) (18 g, 13 mL, 100 mmol) was added dropwise. The resulting mixture was heated under reflux for 3 h. After cooling, first to room temperature and then in an ice-bath, the solution was filtered to remove sodium bromide by using a Celite bed. The solvent from the filtrate was removed *in vacuo* and the resulting residue was distilled in vacuum affording the title compound as a colourless oil (24 g, 92 % yield).

b.p.: 96-98 °C/0.5 mmHg

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.20-1.28 (12H, m, 4 × CH_3), 3.13 (1H, dq, J_q = 7.2 Hz, J_d = 2.3 Hz, CH_3CH), 3.70 (1H, d, J = 9.6 Hz, CH), 4.10-4.23 (6H, m, 3×CH_2CH$_3$)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 14.2 (*C*H$_3$), 14.3 (2 × *C*H$_3$), 15.2 (*C*H$_3$), 39.5 (CH_3*C*HCO$_2$Et), 55.1 (EtO$_2$C*C*HCO$_2$Et), 61.1 (*C*H$_2$O), 61.7 (2 × *C*H$_2$O), 168.1 (2 × *C*=O), 174.1 (*C*=O)

Triethyl 1-bromopropane-1,1,2-tricarboxylate (137)[191]

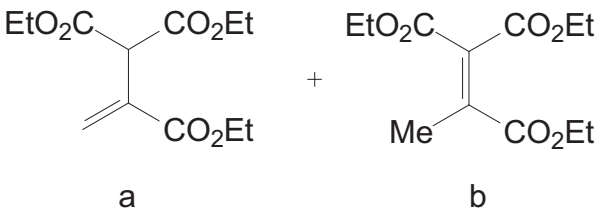


N-Bromosuccinimide (NBS, 8.22 g, 46.2 mmol) was added to a stirred solution of triethyl propane-1,1,2-tricarboxylate (**136**) (12 g, 46.2 mmol) in dry *tert*-butanol (250 mL). After the reaction mixture was stirred rapidly for 15 min, a catalytic amount of potassium *tert*-butoxide (260 mg, 2.32 mmol) was added. The reaction was stirred for a further 64 h at 35 °C. The colour of the solution changed from white to a dark brown. The solvent was removed under reduced pressure and the residue was dissolved in water and diethyl ether. After separation, the aqueous layer was extracted with diethyl ether (3 × 70 mL). The combined ethereal solution was washed with saturated brine (50 mL) and dried ($MgSO_4$).The pale yellow oil was purified by medium-pressure chromatography on silica eluting with dichoromethane to afford the title compound (14.04 g, 90 % yield) as a pale yellow oil. R_f 0.38 (dichloromethane)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.19 (3H, t, *J* = 7.2, C*H*$_3$), 1.24 (3H, t, *J* = 7.2, C*H*$_3$), 1.25 (3H, t, *J* = 7.2, C*H*$_3$), 1.41 (3H, d, *J* = 7.2, CHC*H*$_3$), 3.46 (1H, q, *J* = 6.9 and 7.2 Hz, CH_3CH), 4.10 (2H, q, *J* = 7.2 Hz, C*H*$_2$CH$_3$), 4.21 (2H, q, *J* = 7.2 Hz, C*H*$_2$CH$_3$), 4.23 (2H, q, *J* = 7.2 Hz, C*H*$_2$CH$_3$)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 14.0 (*C*H$_3$), 14.3 (2 × *C*H$_3$), 15.1 (*C*H$_3$), 47.7 (CH_3CHCO_2Et), 61.4 (*C*H$_2$O), 63.4 (2 × *C*H$_2$O), 64.3 (*C*Br), 166.1 (*C*=O), 166.4 (*C*=O) 171.4, (*C*=O)

Triethyl prop-2-ene-1,1,2-tricarboxylate(a) and triethyl prop-1-ene-1,1,2-tricarboxylate (b), (Malachosky's mixture) (138)[191]

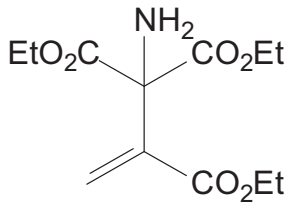


a b

1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU, 1.19 g, 7.8 mmol) was added to an ice-cold stirred solution of triethyl 1-bromopropane-1,2-2-tricarboxylate (**137**) (2.4 g, 7.1 mmol) in anhydrous THF (40 mL). The reaction was kept in an ice bath for 3 h. The reaction was quenched with acetic acid until pH ≈ 6 and the hydrobromide salt of DBU was filtered off through Celite bed. The solvent was removed under reduced pressure to yield a brown residue. The unstable mixture was purified immediately by medium-pressure chromatography on silica eluting with ethyl acetate - petrol (1 : 9 v/v) to obtain the title compounds (1:1) (0.70 g, 38 % yield) as a colourless oil. R_f 0.30 (ethyl acetate – petrol, 1 : 9 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.15-1.27 (18H, m, 6 × CH_2CH_3)[a,b], 2.13 (3H, s, CH_3)[b], 4.09-4.26 (12H, m, 6 × CH_2CH_3)[a,b], 4.54 (1H, s, EtO_2CCHCO_2Et)[a], 5.79 (1H, s, CH*H*)[a], 6.44 (1H, s, C*H*H)[a].

1-Amino-1,1,2-tricarbethoxyprop-2-ene (139)[192]



Malachosky's mixture (**138**) (1.8 g, 7 mmol) was dissolved in 15 mL of anhydrous THF under nitrogen and stirred in ice bath. Sodium hydride (60 % w/w NaH in mineral oil, 0.36 g, 9 mmol) dissolved in anhydrous THF (15 mL) was added dropwise to the solution of Malachosky's mixture. The reaction mixture was stirred at 0 °C for 1 h. The solvent was evaporated *in vacuo*, yielding a solid salt. The anionic solid was suspended in diethyl ether (14 mL) and added to an ice-cold, stirred solution of chloramine in anhydrous diethyl ether (0.105 M, 100 mL) by a dropping funnel. (CAUTION: Chloramine is a highly unstable compound; this procedure must be carried out behind protecting shield) The solution was stirred at 0 °C for 1 h and then 2 h at room temperature. The resulting mixture was filtered and extracted with 2 M HCl (4 × 6 mL). The combined aqueous phase was basified by K_2CO_3 solution, extracted with diethyl ether (4 × 10 mL) and dried ($MgSO_4$). The ethereal solution was concentrated and purified by medium-pressure chromatography on silica eluting with ethyl acetate - petrol (1 : 4 v/v) to obtain the title compound (0.81 g, 43 % yield) as a pale yellow oil. R_f 0.30 (ethyl acetate – petrol, 1 : 4 v/v)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.20-1.26 (9H, m, 3 × CH_3), 2.34 (2H, br, NH_2), 4.13-4.23 (6H, m, 3×CH_2CH$_3$), 5.81 (1H, s, C*H*CH), 6.31 (1H, s, CHC*H*)

^{13}C NMR δ_C (75 MHz, $CDCl_3$): 14.2 (2 × *C*H$_3$), 14.4 (CH$_3$), 61.5 (*C*H$_2$O), 62.6 (2 ×

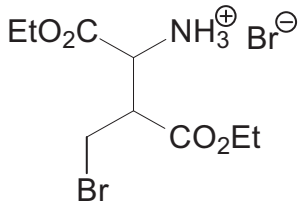
CH_2O), 68.5 (*C*NH_2), 126.2 (C*C*H_2), 140.4 (*C*CH_2), 165.9 (*C*=O), 170.2 (2 × *C*=O)

Preparation of chloramine[193]

CAUTION: Chloramine is a highly unstable compound; this experiment must be carried out behind protecting shield.

Powdered ammonium chloride (2.97 g, 55 mmol) was suspended in diethyl ether and cooled to -10 °C (ice bath with NaCl), followed by addition of concentrated aqueous NH_3 (30% w/w NH_3 in water, 5.4 mL, 82 mmol). Aqueous sodium hypochloride (3 M, 18.5 mL, 55 mmol) was added in small portions over 10 min with vigorous stirring and the internal temperature was kept around -10 °C. The mixture was transferred to a precooled separating funnel and the aqueous phase was separated. The organic phase was washed with cooled saturated brine and dried over granulated $CaCl_2$ at -10 °C for 1 h. The resulting solution was stored for 7 h at -10 °C. The concentration was measured by titration of a test solution containing chloramine with standard sodium thiosulfate after addition of 10 % aqueous potassium iodide. The observed concentration was 0.105 M.

Synthesis of diethyl 2-amino-3-(bromomethyl)succinate hydrobromide (140)[210]

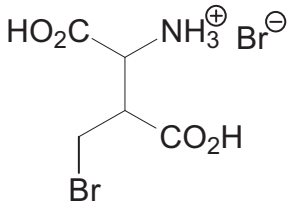


45 % (w/v) Hydrogen bromide in acetic acid (4 mL) was added to a flask containing 1-amino-1,1,2-tricarbethoxyprop-2-ene (**139**) (239 mg, 0.87 mmol) at ice bath. The

mixture was warmed to room temperature and stirred for 20 h. A yellow oil was obtained after removing the solvent *in vacuo*. (368 mg, 97 %)

^{1}H NMR δ_H (300 MHz, $CDCl_3$): 1.27-1.32 (6H, t, *J* = 7.1 Hz, 2 × C*H*$_3$), 3.80-3.88 (4H, q, *J* = 7.1 Hz, 2 × C*H*$_2$CH$_3$), 3.94-4.14 (1H, m, C*H*NH$_2$), 4.27-4.46 (3H, m, BrC*H*$_2$C*H*)

Attempted synthesis of 2-amino-3-(bromomethyl)succinic acid hydrobromide (141)[190]



45 % (w/v) Hydrogen bromide in acetic acid (4 mL) was added to a flask containing the crude product of last experiment (368 mg) at room temperature. The mixture was stirred at 70 °C for 65 h. A brown yellow solid (234 mg) was obtained after removing the solvent *in vacuo* and freeze-drying the residue.

^{1}H NMR δ_H (300 MHz, D_2O): 6.20 (1H, s, C*H*H=C), 6.60 (1H, s, CH*H*=C)

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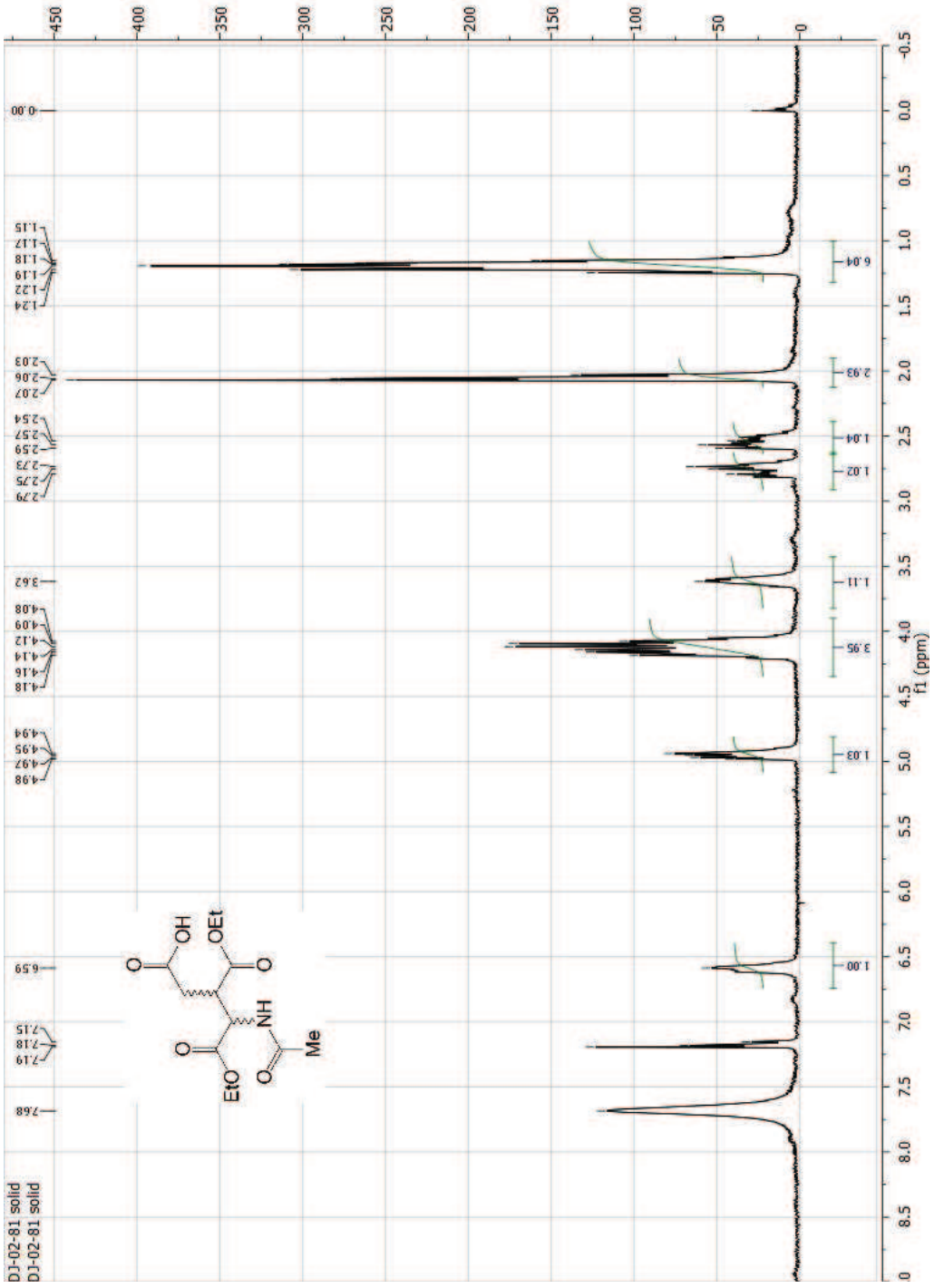
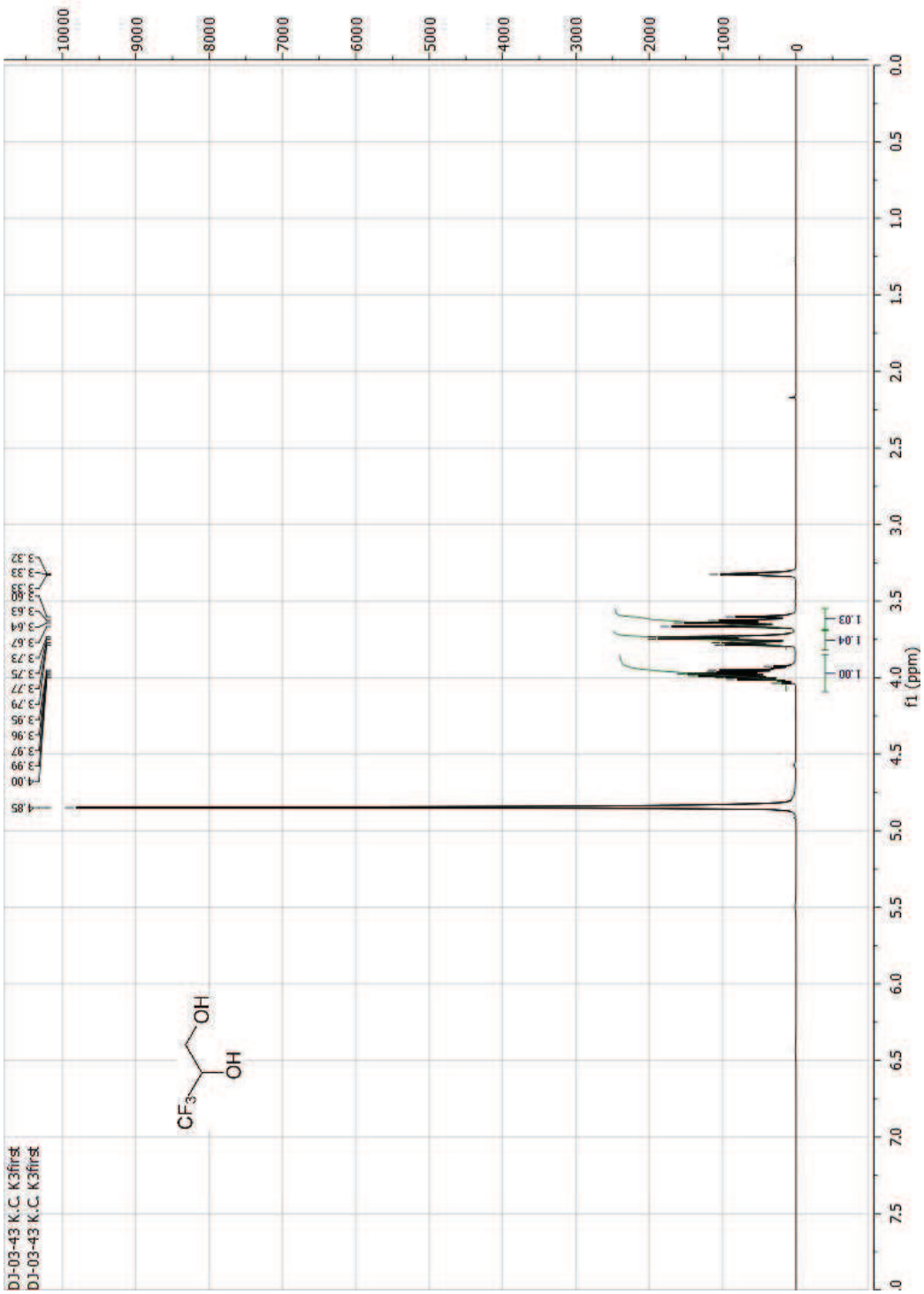
Appendix 1: Selected NMR spectra

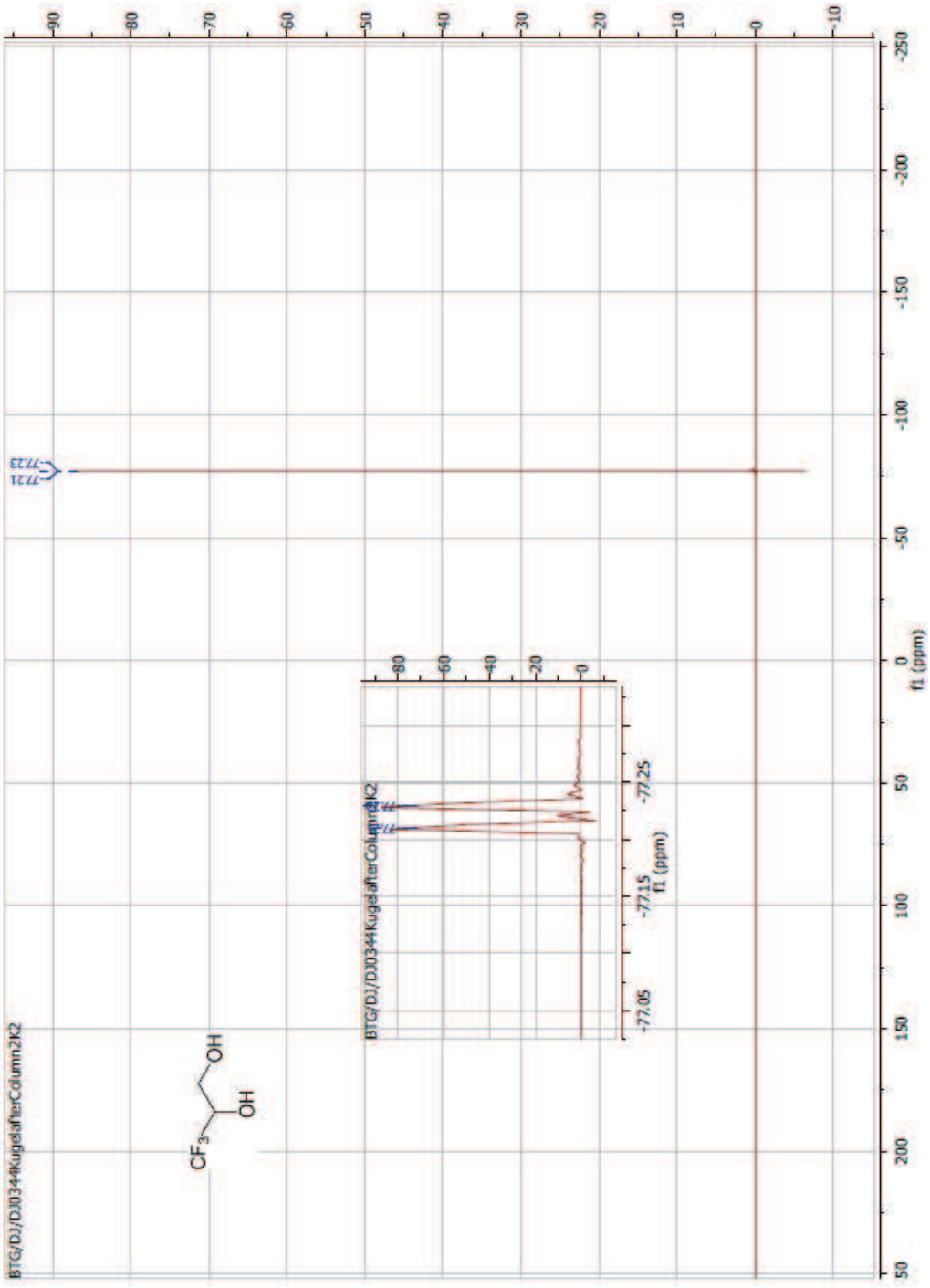
4-Acetamido-5-ethoxy-3-(ethoxycarbonyl)-5-oxopentanoic acid (123)



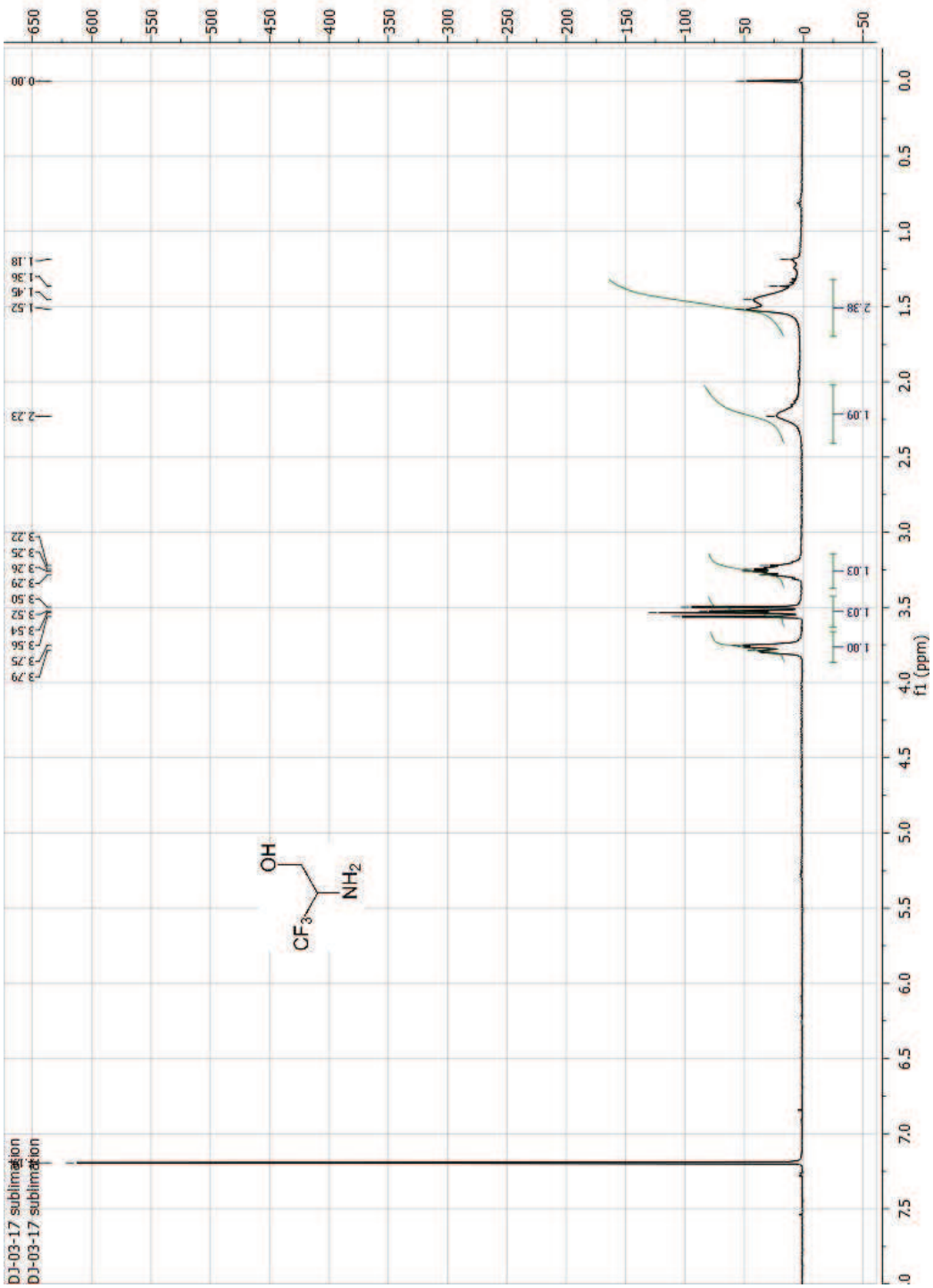
3,3,3-Trifluopropane-1,2-diol (68)



3,3,3-Trifluopropane-1,2-diol (68)



2-Amino-3,3,3-trifluoropropanol (69)



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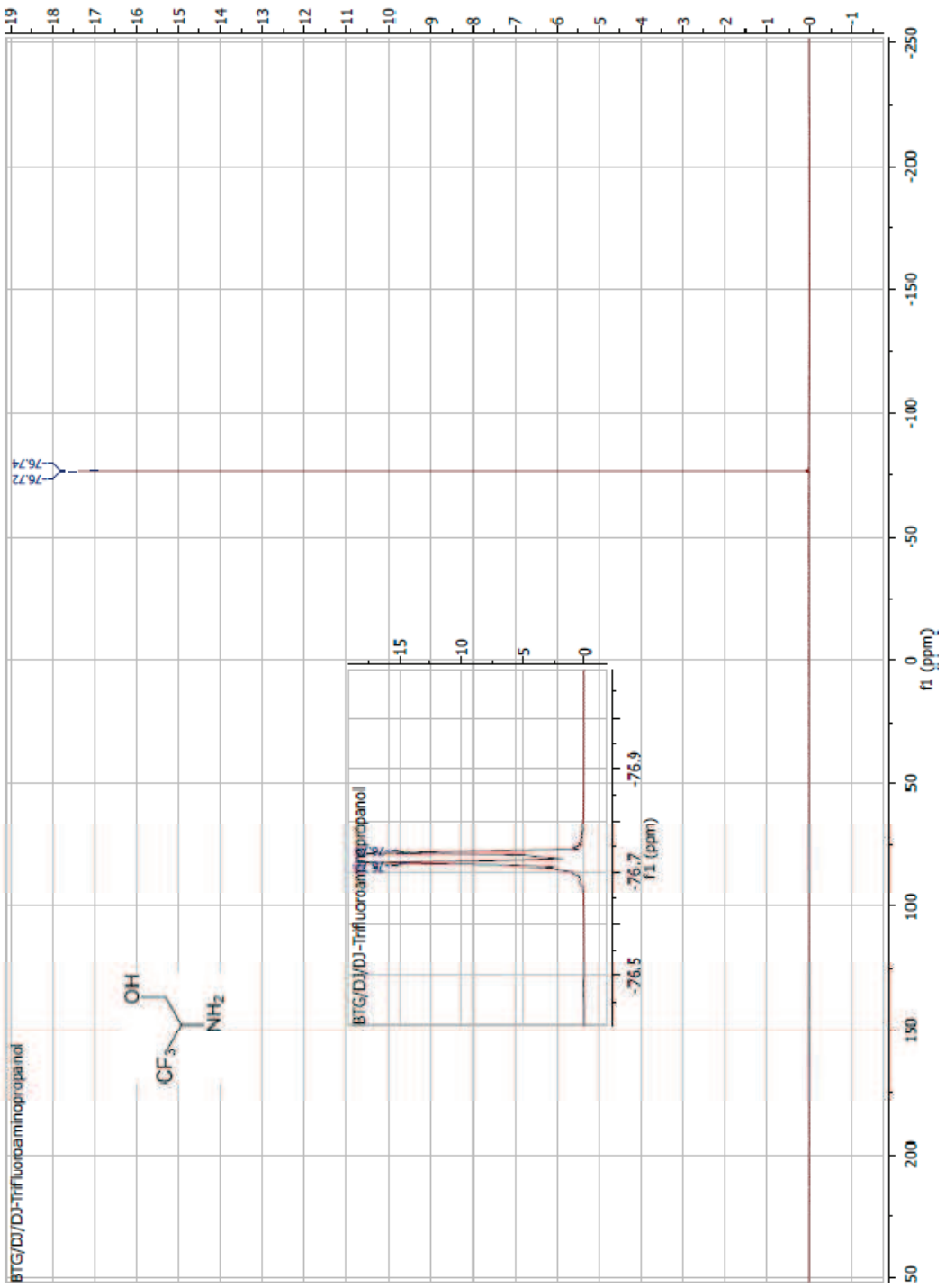


Appendix 2: Crystal structure of ras-4-Acetamido-5-ethoxy-3-(ethoxycarbonyl)-5-oxopentanoic acid (123)

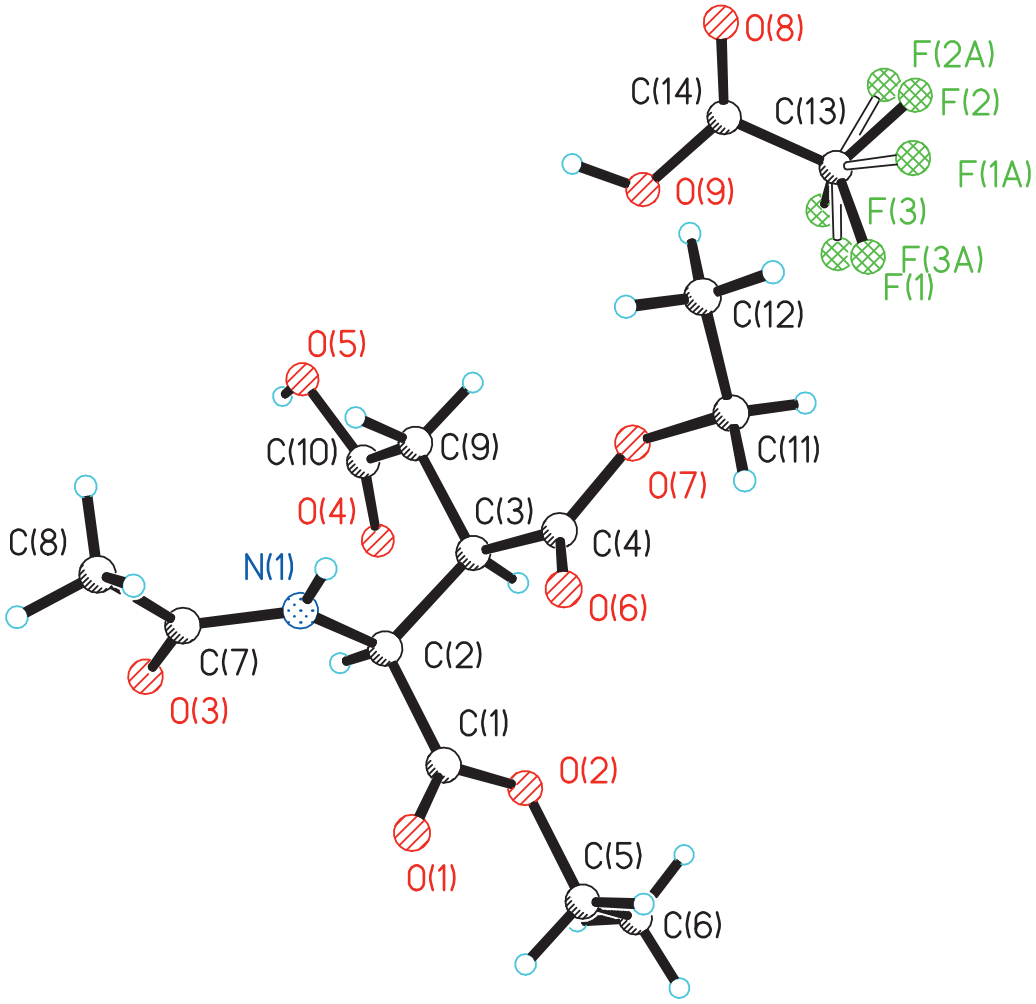


Table 1. Crystal data and structure refinement for btg78.

Identification code	btg78	
Chemical formula (moiety)	$C_{12}H_{19}NO_7 \cdot C_2HF_3O_2$	
Chemical formula (total)	$C_{14}H_{20}F_3NO_9$	
Formula weight	403.31	
Temperature	150(2) K	
Radiation, wavelength	MoKα, 0.71073 Å	
Crystal system, space group	monoclinic, Pc	
Unit cell parameters	a = 5.7171(5) Å	α = 90°
	b = 13.241(2) Å	β = 98.131(14)°
	c = 12.493(3) Å	γ = 90°
Cell volume	936.2(3) $Å^3$	
Z	2	
Calculated density	1.431 g/cm^3	
Absorption coefficient μ	0.137 mm^{-1}	
F(000)	420	
Crystal colour and size	colourless, 0.43 × 0.40 × 0.10 mm^3	
Reflections for cell refinement	122 (θ range 2.5 to 27.5°)	
Data collection method	Nonius KappaCCD diffractometer	
	ϕ and ω scans	
θ range for data collection	4.1 to 27.5°	
Index ranges	h −7 to 6, k −17 to 17, l −16 to 16	
Completeness to θ = 26.0°	99.1 %	
Reflections collected	9936	
Independent reflections	4009 (R_{int} = 0.0327)	
Reflections with $F^2>2\sigma$	3562	
Absorption correction	semi-empirical from equivalents	
Min. and max. transmission	0.9435 and 0.9865	
Structure solution	direct methods	
Refinement method	Full-matrix least-squares on F^2	
Weighting parameters a, b	0.0486, 0.2384	
Data / restraints / parameters	4009 / 32 / 287	
Final R indices [$F^2>2\sigma$]	R1 = 0.0386, wR2 = 0.0923	
R indices (all data)	R1 = 0.0489, wR2 = 0.0991	
Goodness-of-fit on F^2	1.064	
Absolute structure parameter	1.0(8)	
Largest and mean shift/su	0.001 and 0.000	
Largest diff. peak and hole	0.25 and −0.17 e $Å^{-3}$	

Table 2. Atomic coordinates and equivalent isotropic displacement parameters ($Å^2$) for btg78. U_{eq} is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U_{eq}
O(1)	0.2676(2)	0.40330(11)	0.02553(11)	0.0269(3)
O(2)	0.5788(2)	0.30602(10)	0.09005(10)	0.0248(3)
O(3)	0.4659(3)	0.63343(11)	0.19087(15)	0.0363(4)
O(4)	0.9479(3)	0.43605(14)	0.37205(13)	0.0407(4)
O(5)	0.8405(3)	0.50188(12)	0.52135(12)	0.0317(3)
O(6)	0.1387(3)	0.27250(12)	0.22411(12)	0.0358(4)
O(7)	0.3303(3)	0.20743(12)	0.37879(12)	0.0398(4)
O(8)	0.3541(5)	0.11768(15)	0.77670(17)	0.0603(5)
O(9)	0.5666(4)	0.18571(14)	0.65663(16)	0.0488(5)
F(1)	0.4968(15)	–0.0107(5)	0.5743(5)	0.119(4)
F(2)	0.5495(14)	–0.0626(4)	0.7399(7)	0.094(2)
F(3)	0.8184(7)	0.0121(4)	0.6758(4)	0.0739(14)
F(1A)	0.372(3)	–0.0468(8)	0.6462(17)	0.148(6)
F(2A)	0.630(4)	–0.0396(15)	0.766(2)	0.183(11)
F(3A)	0.686(6)	0.0063(8)	0.609(2)	0.199(12)
N(1)	0.2690(3)	0.49347(13)	0.22578(13)	0.0233(3)
C(1)	0.4198(3)	0.37637(14)	0.09863(15)	0.0210(4)
C(2)	0.4615(3)	0.42509(13)	0.21101(14)	0.0198(4)
C(3)	0.5080(3)	0.34663(14)	0.30333(14)	0.0204(4)
C(4)	0.3012(3)	0.27280(15)	0.29631(15)	0.0239(4)
C(5)	0.5766(4)	0.25840(18)	–0.01642(16)	0.0314(5)
C(6)	0.8121(4)	0.20774(17)	–0.01637(18)	0.0340(5)
C(7)	0.2835(3)	0.59316(14)	0.21657(15)	0.0228(4)
C(8)	0.0728(4)	0.65383(17)	0.23923(19)	0.0333(5)
C(9)	0.5629(3)	0.39576(15)	0.41571(15)	0.0229(4)
C(10)	0.8017(3)	0.44616(14)	0.43215(14)	0.0215(4)
C(11)	0.1514(6)	0.1284(2)	0.3787(2)	0.0530(7)
C(12)	–0.0072(6)	0.1541(3)	0.4588(3)	0.0611(8)
C(13)	0.5783(8)	0.0102(2)	0.6761(3)	0.0661(9)
C(14)	0.4840(5)	0.11307(18)	0.7103(2)	0.0405(5)

Table 3. Bond lengths [Å] and angles [°] for btg78.

O(1)–C(1)	1.222(2)	O(2)–C(1)	1.316(2)
O(2)–C(5)	1.471(2)	O(3)–C(7)	1.253(3)
O(4)–C(10)	1.208(2)	O(5)–C(10)	1.329(2)
O(5)–H(5)	0.89(3)	O(6)–C(4)	1.200(2)
O(7)–C(4)	1.338(2)	O(7)–C(11)	1.463(3)
O(8)–C(14)	1.191(3)	O(9)–C(14)	1.299(3)
O(9)–H(9)	0.94(3)	F(1)–C(13)	1.321(5)
F(2)–C(13)	1.275(7)	F(3)–C(13)	1.374(6)
F(1A)–C(13)	1.405(14)	F(2A)–C(13)	1.30(2)
F(3A)–C(13)	1.106(10)	N(1)–C(2)	1.457(2)
N(1)–C(7)	1.329(3)	N(1)–H(1)	0.85(2)
C(1)–C(2)	1.533(2)	C(2)–H(2A)	1.0000
C(2)–C(3)	1.547(2)	C(3)–H(3A)	1.0000
C(3)–C(4)	1.527(3)	C(3)–C(9)	1.539(2)
C(5)–H(5A)	0.9900	C(5)–H(5B)	0.9900
C(5)–C(6)	1.504(3)	C(6)–H(6A)	0.9800

C(6)–H(6B)	0.9800	C(6)–H(6C)	0.9800
C(7)–C(8)	1.508(3)	C(8)–H(8A)	0.9800
C(8)–H(8B)	0.9800	C(8)–H(8C)	0.9800
C(9)–H(9A)	0.9900	C(9)–H(9B)	0.9900
C(9)–C(10)	1.507(3)	C(11)–H(11A)	0.9900
C(11)–H(11B)	0.9900	C(11)–C(12)	1.482(5)
C(12)–H(12A)	0.9800	C(12)–H(12B)	0.9800
C(12)–H(12C)	0.9800	C(13)–C(14)	1.547(4)

C(1)–O(2)–C(5)	117.36(14)	C(10)–O(5)–H(5)	106.8(15)
C(4)–O(7)–C(11)	116.82(18)	C(14)–O(9)–H(9)	113.4(17)
C(2)–N(1)–C(7)	123.29(17)	C(2)–N(1)–H(1)	118.1(14)
C(7)–N(1)–H(1)	118.3(14)	O(1)–C(1)–O(2)	124.92(17)
O(1)–C(1)–C(2)	124.32(17)	O(2)–C(1)–C(2)	110.65(15)
N(1)–C(2)–C(1)	110.80(14)	N(1)–C(2)–H(2A)	106.9
N(1)–C(2)–C(3)	112.09(15)	C(1)–C(2)–H(2A)	106.9
C(1)–C(2)–C(3)	112.85(14)	H(2A)–C(2)–C(3)	106.9
C(2)–C(3)–H(3A)	107.2	C(2)–C(3)–C(4)	109.70(15)
C(2)–C(3)–C(9)	112.81(15)	H(3A)–C(3)–C(4)	107.2
H(3A)–C(3)–C(9)	107.2	C(4)–C(3)–C(9)	112.28(15)
O(6)–C(4)–O(7)	125.13(19)	O(6)–C(4)–C(3)	123.78(17)
O(7)–C(4)–C(3)	111.05(16)	O(2)–C(5)–H(5A)	110.2
O(2)–C(5)–H(5B)	110.2	O(2)–C(5)–C(6)	107.33(16)
H(5A)–C(5)–H(5B)	108.5	H(5A)–C(5)–C(6)	110.2
H(5B)–C(5)–C(6)	110.2	C(5)–C(6)–H(6A)	109.5
C(5)–C(6)–H(6B)	109.5	C(5)–C(6)–H(6C)	109.5
H(6A)–C(6)–H(6B)	109.5	H(6A)–C(6)–H(6C)	109.5
H(6B)–C(6)–H(6C)	109.5	O(3)–C(7)–N(1)	120.61(17)
O(3)–C(7)–C(8)	122.52(18)	N(1)–C(7)–C(8)	116.86(18)
C(7)–C(8)–H(8A)	109.5	C(7)–C(8)–H(8B)	109.5
C(7)–C(8)–H(8C)	109.5	H(8A)–C(8)–H(8B)	109.5
H(8A)–C(8)–H(8C)	109.5	H(8B)–C(8)–H(8C)	109.5
C(3)–C(9)–H(9A)	109.2	C(3)–C(9)–H(9B)	109.2
C(3)–C(9)–C(10)	112.03(15)	H(9A)–C(9)–H(9B)	107.9
H(9A)–C(9)–C(10)	109.2	H(9B)–C(9)–C(10)	109.2
O(4)–C(10)–O(5)	122.31(17)	O(4)–C(10)–C(9)	124.03(16)
O(5)–C(10)–C(9)	113.63(16)	O(7)–C(11)–H(11A)	109.8
O(7)–C(11)–H(11B)	109.8	O(7)–C(11)–C(12)	109.3(2)
H(11A)–C(11)–H(11B)	108.3	H(11A)–C(11)–C(12)	109.8
H(11B)–C(11)–C(12)	109.8	C(11)–C(12)–H(12A)	109.5
C(11)–C(12)–H(12B)	109.5	C(11)–C(12)–H(12C)	109.5
H(12A)–C(12)–H(12B)	109.5	H(12A)–C(12)–H(12C)	109.5
H(12B)–C(12)–H(12C)	109.5	F(1)–C(13)–F(2)	112.6(5)
F(1)–C(13)–F(3)	102.6(5)	F(1)–C(13)–F(1A)	57.5(7)
F(1)–C(13)–F(2A)	137.2(12)	F(1)–C(13)–F(3A)	54.5(19)
F(1)–C(13)–C(14)	111.1(3)	F(2)–C(13)–F(3)	103.5(5)
F(2)–C(13)–F(1A)	65.3(8)	F(2)–C(13)–F(2A)	27.3(13)
F(2)–C(13)–F(3A)	124.8(7)	F(2)–C(13)–C(14)	114.4(4)
F(3)–C(13)–F(1A)	144.1(6)	F(3)–C(13)–F(2A)	84.6(10)
F(3)–C(13)–F(3A)	48(2)	F(3)–C(13)–C(14)	111.8(3)
F(1A)–C(13)–F(2A)	92.2(12)	F(1A)–C(13)–F(3A)	107.9(17)

F(1A)–C(13)–C(14)	103.6(6)	F(2A)–C(13)–F(3A)	123.2(17)
F(2A)–C(13)–C(14)	104.6(10)	F(3A)–C(13)–C(14)	120.1(6)
O(8)–C(14)–O(9)	129.0(2)	O(8)–C(14)–C(13)	120.9(3)
O(9)–C(14)–C(13)	110.1(3)		

Table 4. Anisotropic displacement parameters ($Å^2$) for btg78. The anisotropic displacement factor exponent takes the form: $-2\pi^2[h^2a^{*2}U^{11} + ...+ 2hka^*b^*U^{12}]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
O(1)	0.0225(7)	0.0370(8)	0.0201(6)	0.0048(5)	–0.0004(5)	0.0046(6)
O(2)	0.0282(7)	0.0309(7)	0.0149(6)	–0.0012(5)	0.0011(5)	0.0081(6)
O(3)	0.0297(8)	0.0268(7)	0.0547(10)	0.0009(7)	0.0142(7)	–0.0003(6)
O(4)	0.0255(8)	0.0667(11)	0.0324(8)	–0.0196(8)	0.0130(6)	–0.0159(7)
O(5)	0.0229(7)	0.0473(9)	0.0251(7)	–0.0122(6)	0.0044(5)	–0.0098(6)
O(6)	0.0317(8)	0.0418(8)	0.0308(8)	0.0032(6)	–0.0065(6)	–0.0140(7)
O(7)	0.0547(10)	0.0371(8)	0.0256(7)	0.0082(6)	–0.0012(7)	–0.0178(7)
O(8)	0.0887(16)	0.0452(10)	0.0521(11)	0.0029(9)	0.0268(11)	
–0.0017(10)						
O(9)	0.0610(12)	0.0312(8)	0.0577(11)	0.0012(7)	0.0207(9)	0.0079(8)
F(1)	0.158(6)	0.084(4)	0.094(4)	–0.060(3)	–0.056(4)	0.059(4)
F(2)	0.114(5)	0.0419(19)	0.140(5)	0.022(2)	0.069(4)	0.008(2)
F(3)	0.059(2)	0.058(2)	0.104(3)	0.0098(19)	0.014(2)	0.0238(14)
F(1A)	0.215(15)	0.060(5)	0.186(13)	–0.076(7)	0.089(13)	–0.044(8)
F(2A)	0.152(15)	0.116(13)	0.28(2)	0.110(14)	0.028(13)	0.097(12)
F(3A)	0.35(3)	0.050(6)	0.27(2)	0.046(11)	0.27(2)	0.058(13)
N(1)	0.0155(8)	0.0304(9)	0.0249(8)	0.0003(6)	0.0055(6)	0.0012(6)
C(1)	0.0199(9)	0.0252(9)	0.0184(8)	0.0031(7)	0.0039(7)	–0.0004(7)
C(2)	0.0164(8)	0.0247(9)	0.0182(8)	0.0006(7)	0.0019(6)	–0.0008(7)
C(3)	0.0198(9)	0.0259(9)	0.0152(8)	–0.0014(7)	0.0011(6)	–0.0003(7)
C(4)	0.0279(10)	0.0261(9)	0.0182(8)	–0.0022(7)	0.0047(7)	–0.0031(7)
C(5)	0.0346(11)	0.0398(12)	0.0193(9)	–0.0077(8)	0.0019(8)	0.0041(9)
C(6)	0.0367(12)	0.0370(11)	0.0294(10)	–0.0074(9)	0.0082(8)	0.0030(9)
C(7)	0.0215(9)	0.0275(10)	0.0188(8)	–0.0017(7)	0.0010(7)	0.0026(7)
C(8)	0.0256(11)	0.0338(11)	0.0402(11)	–0.0028(9)	0.0035(9)	0.0071(8)
C(9)	0.0196(9)	0.0347(11)	0.0145(7)	–0.0032(7)	0.0029(7)	–0.0044(7)
C(10)	0.0191(9)	0.0270(9)	0.0177(8)	0.0012(7)	0.0004(7)	–0.0003(7)
C(11)	0.077(2)	0.0428(14)	0.0402(13)	0.0042(11)	0.0112(13)	
–0.0315(14)						
C(12)	0.0475(16)	0.070(2)	0.0659(19)	0.0144(15)	0.0071(14)	
–0.0051(14)						
C(13)	0.093(3)	0.0368(15)	0.069(2)	0.0044(14)	0.013(2)	0.0161(17)
C(14)	0.0481(14)	0.0340(12)	0.0368(12)	–0.0023(9)	–0.0023(10)	0.0006(10)

Table 5. Hydrogen coordinates and isotropic displacement parameters ($Å^2$)for btg78.

	x	y	z	U
H(2A)	0.6076	0.4672	0.2141	0.024
H(3A)	0.6507	0.3070	0.2911	0.024
H(5A)	0.5514	0.3100	–0.0743	0.038
H(5B)	0.4476	0.2081	–0.0293	0.038
H(6A)	0.8117	0.1702	–0.0840	0.051
H(6B)	0.8409	0.1611	0.0450	0.051

H(6C)	0.9370	0.2590	–0.0101	0.051
H(8A)	0.0740	0.7198	0.2038	0.050
H(8B)	0.0801	0.6634	0.3174	0.050
H(8C)	–0.0727	0.6177	0.2112	0.050
H(9A)	0.4397	0.4465	0.4242	0.027
H(9B)	0.5580	0.3434	0.4719	0.027
H(11A)	0.2290	0.0627	0.3973	0.064
H(11B)	0.0586	0.1227	0.3058	0.064
H(12A)	–0.1263	0.1010	0.4595	0.092
H(12B)	–0.0857	0.2186	0.4392	0.092
H(12C)	0.0857	0.1597	0.5308	0.092
H(5)	0.988(5)	0.5246(18)	0.5261(19)	0.025(6)
H(1)	0.147(4)	0.4687(15)	0.2481(17)	0.016(5)
H(9)	0.513(5)	0.250(2)	0.674(2)	0.044(7)

Table 6. Torsion angles [°] for btg78.

C(5)–O(2)–C(1)–O(1)	1.8(3)	C(5)–O(2)–C(1)–C(2)	−174.49(16)
C(7)–N(1)–C(2)–C(1)	−100.87(19)	C(7)–N(1)–C(2)–C(3)	132.08(17)
O(1)–C(1)–C(2)–N(1)	11.1(2)	O(1)–C(1)–C(2)–C(3)	137.73(18)
O(2)–C(1)–C(2)–N(1)	−172.60(15)	O(2)–C(1)–C(2)–C(3)	−46.0(2)
N(1)–C(2)–C(3)–C(4)	68.88(19)	N(1)–C(2)–C(3)–C(9)	−57.1(2)
C(1)–C(2)–C(3)–C(4)	−57.1(2)	C(1)–C(2)–C(3)–C(9)	176.97(15)
C(11)–O(7)–C(4)–O(6)	1.4(3)	C(11)–O(7)–C(4)–C(3)	−176.5(2)
C(2)–C(3)–C(4)–O(6)	5.5(3)	C(2)–C(3)–C(4)–O(7)	−176.52(16)
C(9)–C(3)–C(4)–O(6)	131.8(2)	C(9)–C(3)–C(4)–O(7)	−50.2(2)
C(1)–O(2)–C(5)–C(6)	162.14(17)	C(2)–N(1)–C(7)–O(3)	2.2(3)
C(2)–N(1)–C(7)–C(8)	−177.31(17)	C(2)–C(3)–C(9)–C(10)	−70.6(2)
C(4)–C(3)–C(9)–C(10)	164.86(16)	C(3)–C(9)–C(10)–O(4)	−10.9(3)
C(3)–C(9)–C(10)–O(5)	170.61(16)	C(4)–O(7)–C(11)–C(12)	−103.8(3)
F(1)–C(13)–C(14)–O(8)	114.1(6)	F(1)–C(13)–C(14)–O(9)	−65.6(6)
F(2)–C(13)–C(14)–O(8)	−14.7(7)	F(2)–C(13)–C(14)–O(9)	165.6(5)
F(3)–C(13)–C(14)–O(8)	−131.9(4)	F(3)–C(13)–C(14)–O(9)	48.4(4)
F(1A)–C(13)–C(14)–O(8)	54.0(10)	F(1A)–C(13)–C(14)–O(9)	−125.7(10)
F(2A)–C(13)–C(14)–O(8)	−41.9(12)	F(2A)–C(13)–C(14)–O(9)	138.4(12)
F(3A)–C(13)–C(14)–O(8)	174(2)	F(3A)–C(13)–C(14)–O(9)	−5(3)

Table 7. Hydrogen bonds for btg78 [Å and °].

D–H...A	d(D–H)	d(H...A)	d(D...A)	<(DHA)
O(5)–H(5)...O(1A)	0.89(3)	1.86(3)	2.740(2)	168(2)
N(1)–H(1)...O(4B)	0.85(2)	2.09(2)	2.870(2)	151(2)
N(1)–H(1)...O(6)	0.85(2)	2.61(2)	3.018(2)	110(2)
O(9)–H(9)...O(3C)	0.94(3)	1.59(3)	2.514(2)	168(3)

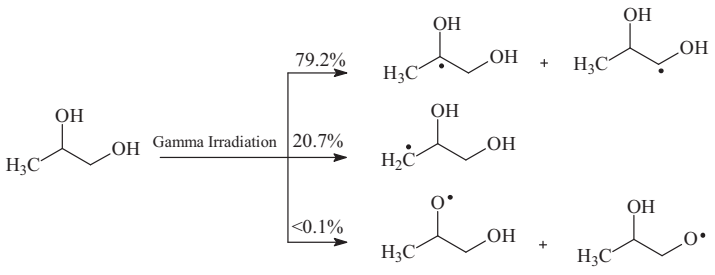
Symmetry operations for equivalent atoms
A x+1,–y+1,z+1/2 B x–1,y,z C x,–y+1,z+1/2

Appendix 3: Addendum to Section 3.6

Effect of the Trifluoromethyl Group in a Substrate for Coenzyme B_{12}-dependent Diol Dehydratase

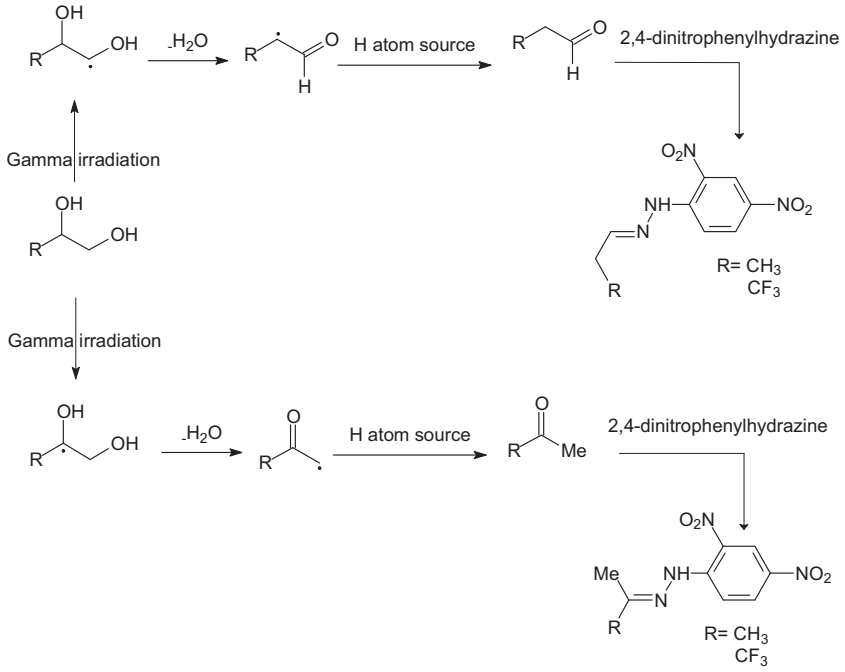
Previous research (See Section 3.6) by the author in Bologna funded by COST (October-December 2008) indicated that propionaldehyde and acetone were formed by the continuous γ-irradiation of propane-1,2-diol ($CH_3CHOHCH_2OH$), whilst 1,1-difluoro-3-hydroxypropanone was formed from 3,3,3-trifluoropropane-1,2-diol ($CF_3CHOHCH_2OH$). The expected 3,3,3-trifluoropropionaldehyde and 1,1,1-trifluoroacetone could not be determined from the ^1H NMR analysis of the irradiation mixture derived from $CF_3CHOHCH_2OH$. However, this work was limited by the availability of only small quantities of $CF_3CHOHCH_2OH$. The diol has now been synthesised in gram quantities at Newcastle and further experiments have been conducted in Bologna since submission of the thesis. The products from continuous irradiation experiments with the above diols were investigated by HPLC analysis. 2,4-Dinitrophenylhydrazine was used as a probe for the aldehyde and ketone products.

The irradiation study was carried out by the author and Sebastian Barata-Vallejo at CNR, Bologna supervised by Dr. Chryssostomos Chatgilialoglu. Radicals were generated by continuous γ-irradiation of the 1,2-diols in water saturated with nitrous oxide. The reaction of hydroxyl radicals with propane-1,2-diol gave a mixture of radicals (**Scheme 1**).[1]



Scheme 1: Relative yields of radicals from hydrogen radical abstraction for propane-1,2-diol[1]

2,4-Dinitrophenylhydrazine has been used as a probe to analyse for carbonyl groups associated with aldehydes and ketones.(**Scheme 2**)



Scheme 2: Potential products from the radicals derived from ethane-1,2-diol, 3,3,3-trifluoropropane-1,2-diol and propane-1,2-diol.

Results and discussion

HPLC analysis of the DNP derivatives of irradiated mixture showed that formaldehyde, acetaldehyde, acetone and propionaldehyde were formed by the continuous γ-irradiation of propane-1,2-diol; formaldehyde and acetaldehyde were formed by the continuous γ-irradiation of ethane-1,2-diol. The products were confirmed by the HPLC-MS and comparisons with authentic compounds. Further experiment was required to remove carbonyl impurities from the starting material, 3,3,3-trifluoropropane-1,2-diol. A preliminary experiment indicated that 3,3,3-trifluoropropionaldehyde was formed from γ – irradiation of $CF_3CHOHCH_2OH$.

HPLC analysis of the DNP derivatives of irradiated propane-1,2-diol

A solution of propane-1,2-diol in H_2O at natural pH was γ – irradiated with a dose rate of ca. 5.9 Gy/min in the present of N_2O. 2,4-Dinitrophenylhydrazine was added to convert the products to 2,4-DNP derivatives. HPLC analysis of the mixture of 2,4-DNP derivatives indicated the formation of formaldehyde, acetaldehyde, acetone and propionaldehyde. (**Figure 1 and 2**) For recognition and quantification of the 2,4-dinitrophenylhydrazone of the carbonyl compounds, a published protocol[2] was employed in our experiments. The comparison was obtained from the same derivatisation procedure performed with the commercially available compounds followed by HPLC analysis.

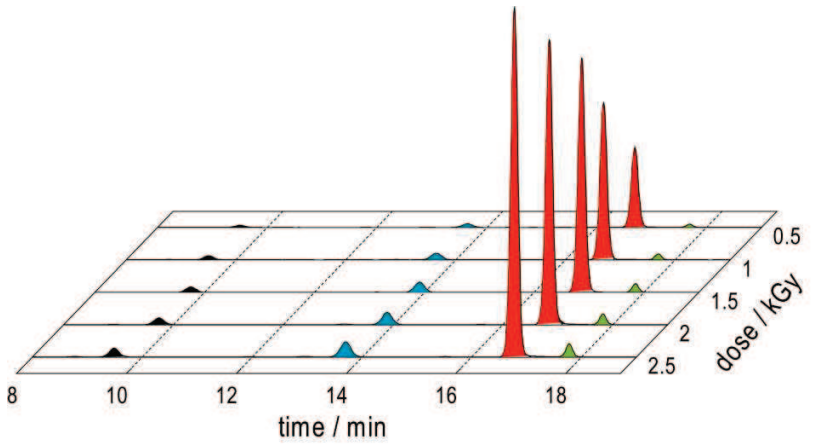
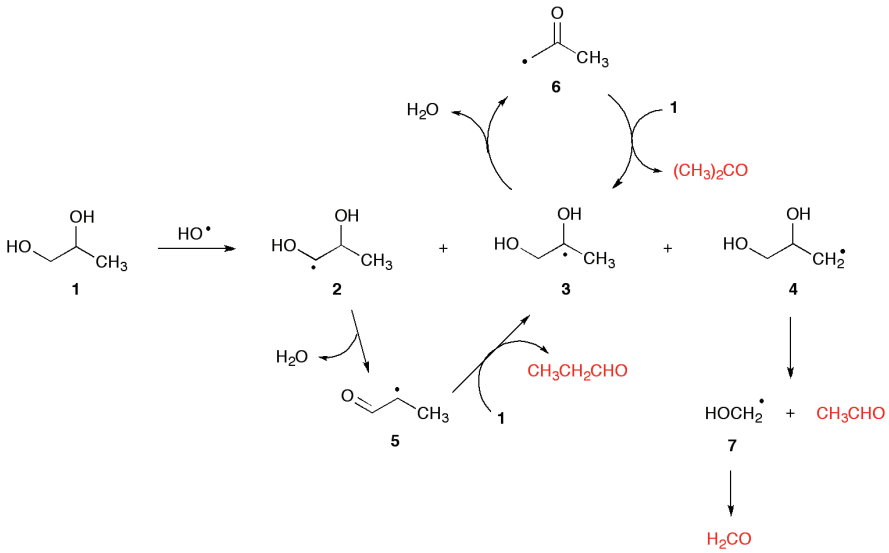


Figure 1: HPLC analyses of γ-irradiation of N_2O-saturated solutions of 2 M propane-1,2-diol at natural pH (dose rate of ~5.9 Gy/min) after 2,4-DNP derivatisation of the carbonyl compounds. The black and blue peaks correspond to formaldehyde and acetaldehyde, whereas the red and green peaks correspond to acetone and propionaldehyde, respectively.

The product yield (mol/kg) divided by the absorbed dose (1 Gy = 1 J/kg) gave the radiation chemical yield. Analysis of the data from **Figure 1** in terms of radiation chemical yield (G) gave G(Formaldehyde) = 0.08, G(Acetaldehyde) = 0.17, G(Acetone) = 3.05, and G(Propionaldehyde) = 0.09 μmol/J when the lines are extrapolated to zero dose. On the basis of the known rate constants for the reactions of H• atom and HO• radical with propane-1,2-diol, taking into account that G(HO•) + G(H•) = 0.62 μmol/J, G(Acetone) = 3.05 μmol/J suggested that there were chain reactions which lead to the radical intermediate forming acetone. The reaction mechanism for the γ-irradiation of N_2O-saturated solution of propane-1,2-diol (**Scheme 3**) was proposed based on the G values obtained from the γ-irradiation of N_2O-saturated solutions of 2 M propane-1,2-diol.

This mechanism also supported by the results obtained from the γ-irradiation of N_2O-saturated solutions of 0.2 M propane-1,2-diol. (**Figure 2**)



Scheme 3: Reaction mechanism for the γ-irradiation of N_2O-saturated solution of propane-1,2-diol

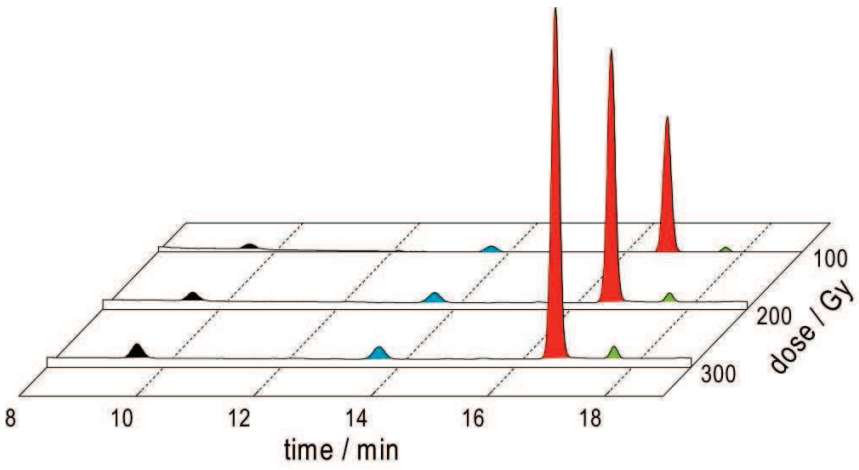


Figure 2: HPLC analyses of γ-irradiation of N_2O-saturated solutions of 0.2 M propane-1,2-diol at natural pH (dose rate of ~5.9 Gy/min) after 2,4-DNP derivatisation of the

carbonyl compounds. The black and blue peaks correspond to formaldehyde and acetaldehyde, whereas the red and green peaks correspond to acetone and propionaldehyde, respectively.

HPLC analysis of the DNP derivatives of irradiated ethane-1,2-diol

In order to understand the reaction mechanism proposed in **Scheme 3**, the same experiments was carried out using ethane-1,2-diol as starting material. HPLC analysis of the 2,4-DNP derivatives of irradiated mixtures indicated the formation of formaldehyde and acetaldehyde. (**Figure 3 and 4**) The data obtained from the experiment of 2 M ethane-1,2-diol gave G(Formaldehyde) = 0.26 and G(Acetaldehyde) = 1.87 μmol/J when the lines are extrapolated to zero dose. This result indicated the chain reaction to form acetaldehyde. The data obtained from the experiment of 0.2 M ethane-1,2-diol gave G(Formaldehyde) = 0.17 and G(Acetaldehyde) = 0.42 μmol/J. This indicated no chain reaction exists in this experiment.

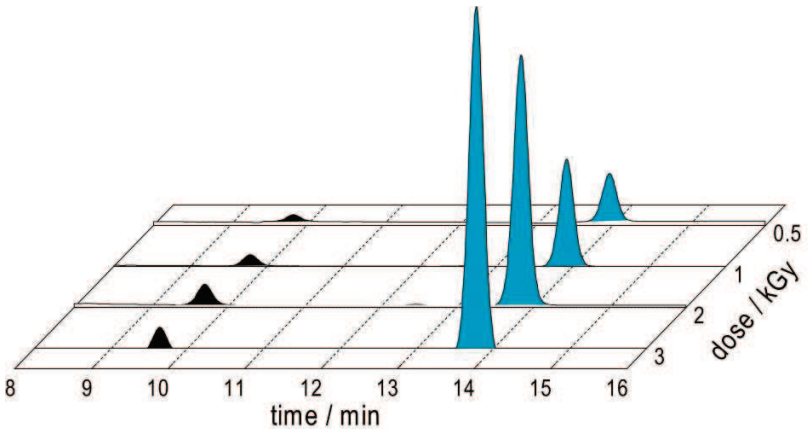


Figure 3: HPLC analyses of γ-irradiation of N_2O-saturated solutions of 2 M ethane-1,2-diol at natural pH (dose rate of ~5.9 Gy/min) after 2,4-DNP derivatisation of the carbonyl compounds. The black and blue peaks correspond to formaldehyde and acetaldehyde, respectively.

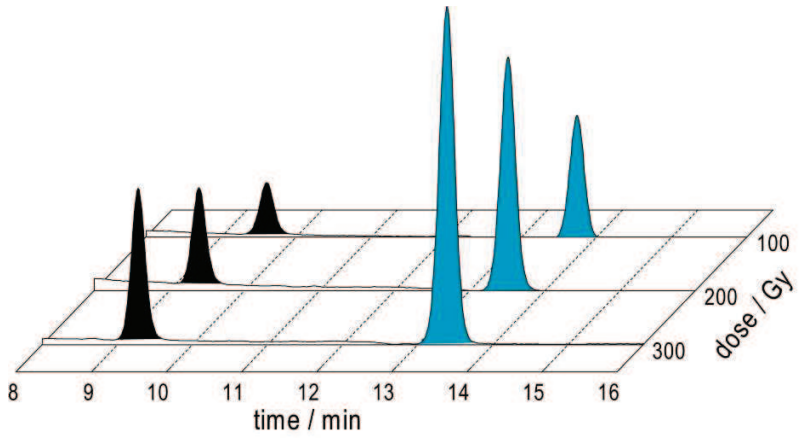
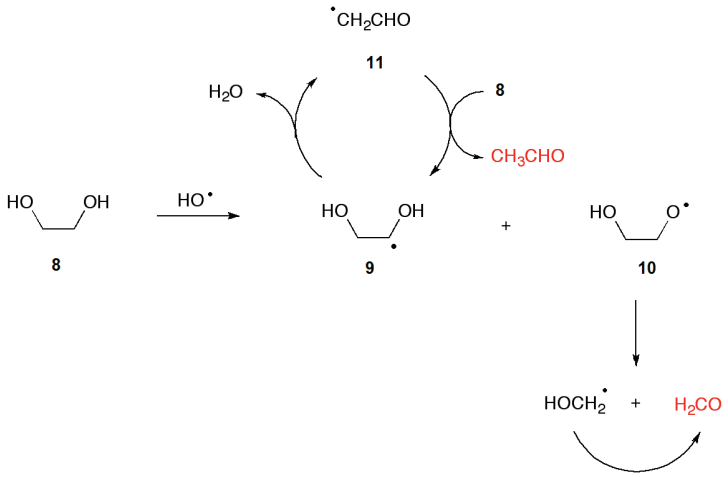


Figure 4: HPLC analyses of γ-irradiation of N_2O-saturated solutions of 0.2 M ethane-1,2-diol at natural pH (dose rate of ~5.9 Gy/min) after 2,4-DNP derivatisation of the carbonyl compounds. The black and blue peaks correspond to formaldehyde and acetaldehyde, respectively.

On the basis of the data obtained from the experiments of 0.2 M and 2 M ethane-1,2-diol, the reaction mechanism (**Scheme 4**) of radical attack on ethane-1,2-diol was proposed.



Scheme 4: Reaction mechanism for the γ-irradiation of N_2O-saturated solution of ethane-1,2-diol

HPLC analysis of the DNP derivatives of irradiated 3,3,3-trifluoropropane-1,2-diol

HPLC quantification of DNP derivatives of 3,3,3-trifluoropropane-1,2-diol indicated that there is 0.32 mM carbonyl impurities in the 0.2 M 3,3,3-trifluoropropane-1,2-diol aqueous solution (≈ 0.16 % impurities). Therefore, further experiment was required to remove the carbonyl impurities from the starting material, 3,3,3-trifluoropropane-1,2-diol. The preliminary result indicated that only 3,3,3-trifluoropropionaldehyde was formed in the radical reaction, no 1,1,1-trifluoroacetone was identified from the HPLC analysis of the irradiated sample (300 Gy, 0.2 M 3,3,3-trifluoropropane-1,2-diol).

Experimentals

The quantitations were performed as the corresponding 2,4-dinitrophenylhyddrazone derivative, following a published protocol[2] adapted to our case. 1 mL of the irradiated sample was diluted with 0.5 mL 0.4 % v/v conc. H_3PO_4 (in 9/1 acetonitrile/water, v/v). and 0.5 mL of 2,4-dinitrophenylhydrazine 40 mM in acetonitrile is added and solution was vortexed for 5 mins and left for 16 hours. After derivatisation, the reaction was diluted to 1/10 ratio in acetonitrile/water (1/1 v/v) and 20 μL were injected for HPLC analysis using a LiChroCART RP 18 5μ column (250 mm × 4 mm) with detection at λ = 360 nm.

Mobile phase A was water (0.1% TFA) and mobile phase B was acetonitrile (0.1% TFA). The separation was obtained at a flow rate of 1 mL/min with a gradient program as follows: 10 min at 40 % B, followed by a 30.0 min step to increase eluent B to100 %. Then washing at 100 % B and equilibration at 40 % B. Total time was 40.0 min.

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[1] Asmus K., Mockel H., and Henglein A., *J. Phys. Chem.*, **1973**, *77*, 1218-1221.

[2] Barata-Vallejo S., Ferreri C., Postigo A., and Chatgilialoglu C., *Chem. Res. Toxicol.*, **2010**, *23*, 258-263.