The origin and metabolism of 5'-FDA in *Streptomyces cattleya*

University
of
St Andrews

A thesis presented for the degree of Doctor of Philosophy to the University of St. Andrews on the 10th January, 2005

by Steven L. Cobb

Declaration

I, Steven Lorimer Cobb hereby certify that this thesis, which is approximately 45, 000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date ...10/1/05.....

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Abstract

Fluorinated metabolites are extremely rare in nature and of the ~3500 halogenated natural products less than 20 of these have been shown to contain a fluorine atom. The bacterium *Streptomyces cattleya* has the ability to generate two fluorinated secondary metabolites, fluoroacetate (FAc) **15** and 4-fluorothreonine (4-FT) **16**. The biosynthetic pathway involved in the production of FAc **15** and 4-FT **16** has been investigated in detail using a combination of synthetic and analytical techniques.

The synthesis of the fluorinated nucleosides 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83** and 5'-fluoro-5'-deoxy-inosine (5'-FDI) **86** was achieved. 5'-FDA **83** was subsequently confirmed as the product of the fluorinase, the C-F bond forming enzyme in *S. cattleya*. The formation of 5-FDA **83** arises from the reaction between inorganic fluoride and *S*-adenosyl-L-methionine (SAM) **85**.

The fluorinase was found to operate in a reversible manner. Therefore it can be used to catalyse the production of SAM **85** from 5'-FDA **83** and L-methionine **84**. By exploiting this reaction it was possible to prepare a variety of SAM analogues. The fluorinase can also mediate a novel transhalogenation reaction in which a 5'-chloro-nucleoside is converted to 5'-fluoro-nucleoside. It was also found that the fluorinase will accept chloride ion as a substrate. However the formation of a carbon-chloride bond will only occur if an additional enzyme is added to drive the equilibrium to favour the chlorination reaction.

The enzyme responsible for the initial metabolism of 5'-FDA **83** was identified as a purine nucleoside phosphorylase (PNP). The PNP enzyme catalyses the formation of 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) **228**. ^{19}F NMR studies suggest that the bioconversion of 5-FDRP **228** to the known fluorinated intermediate fluoroacetaldyde **49** occurs *via* two further enzymes which have been tentatively assigned as an isomerase and an aldolase.

Acknowledgements

I would like to thank my supervisor Professor David O'Hagan for the encouragement, support and unusual golf shots that he has shown me over the last three years. I am also grateful to the EPSRC for financial support.

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Finally and most importantly I am completely indebted to my girlfriend and best friend Joanne Ritchie. Thank you for keeping me sane, smiling and well fed through out my PhD.

List of abbreviations

ATP	Adenosine tri-phosphate
ADA	Adenosine deaminase
AMPDA	5'-Adenylic acid deaminase
5'-BrDA	5'-Bromo-5'-deoxy-adenosine
5'-BrDI	5'-Bromo-5'-deoxy-inosine
bp	Boiling point
brs	Broad singlet
5'-ClDA	5'-Chloro-5'-deoxy-adenosine
5'-CDI	5'-Chloro-5'-deoxy-inosine
CFE	Cell free extract
CI	Chemical ionisation
CoA	Coenzyme A
conc.	Concentration
DAST	(Diethylamino)sulfurtrifluoride
DCM	Dichloromethane
DHAP	Dihydroxyacetone phosphate
DMSO	Dimethyl sulfoxide
d	Doublet
EI	Electron impact
ESMS	Electrospray mass spectrometry
eq.	Equivalent
FAc	Fluoroacetate
4-FT	4-Fluorothreonine
5'-FDA	5'-Fluoro-5'-deoxy-adenosine

5'-FDI	5'-Fluoro-5'-deoxy-inosine
5'-FDR	5'-Fluoro-5'-deoxy-D-ribose
5'-FDRP	5'-Fluoro-5'-deoxy-D-ribose-1-phosphate
GC-MS	Gas chromatography mass spectrometry
h	Hour
HPLC	High pressure liquid chromatography
Hz	Hertz
IR	Infrared spectroscopy
J	Coupling constant
LDA	Lithium diisopropylamide
mg	Milligram
ml	Millilitre
mp	Melting point
m	Multiplet
min	Minutes
mM	Millimolar
m/z	Mass over charge
MSTFA	*N*-Methyl-*N*-(trsimethylsilyl)trifluoroacetamide
N.D	Not detectable
NAD^+	Nicotinamide adenine dinucleotide (oxidised form)
NMR	Nuclear magnetic resonance
Nu	Nucleophile
PCC	Pyridinium chlorocromate
PDC	Pyridinium dichromate
PLP	Pyridoxal phosphate

ppm	Parts per million
PNP	Purine nucleoside phosphorylase
q	Quartet
rac	Racemic
rpm	Revolutions per minute
R.T	Room temperature
s	Singlet
SAH	*S*-Adenosyl-L-homocysteine
SAM	*S*-Adenosyl-L-methionine
TBAF	Tetrabutyl ammonium fluoride
THF	Tetrahydrofuran
TLC	Thin layer chromatography
t	Triplet
TMS	Trimethylsilyl
Tris	Tris(hydroxymethyl)aminoethane
UV	Ultra-violet

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1 Introduction

For thousands of years mankind has utilised the unique properties of plant extracts in a variety of ways that have consequently changed the way in which we live our lives.[1] The bioactive organic compounds that are contained in these plant extracts have been used for medicines **1-2**, stimulants **3-4**, poisons **5** and narcotics **6-7**. In more recent times organic compounds of enormous medicinal value have also been isolated from micro-organisms, a classic example being the antibiotic penicillin **8**.[2] These organic compounds, isolated from natural sources, are part of a substantial and ever increasing family of molecules known as natural products or secondary metabolites.

As our understanding of biochemistry and the analytical techniques used to explore the biosynthesis of natural products has advanced, the number of secondary metabolites that have been isolated and characterised has also increased dramatically.[3] In part the driving force behind the study of natural products has been led by the search for biologically active molecules, that may lead to new drug targets. In recent years the screening of secondary metabolites for bioactivity has resulted in the discovery of valuable molecules such as taxol **9**[4,5] ensuring that the study of these fascinating molecules continues well into the future.

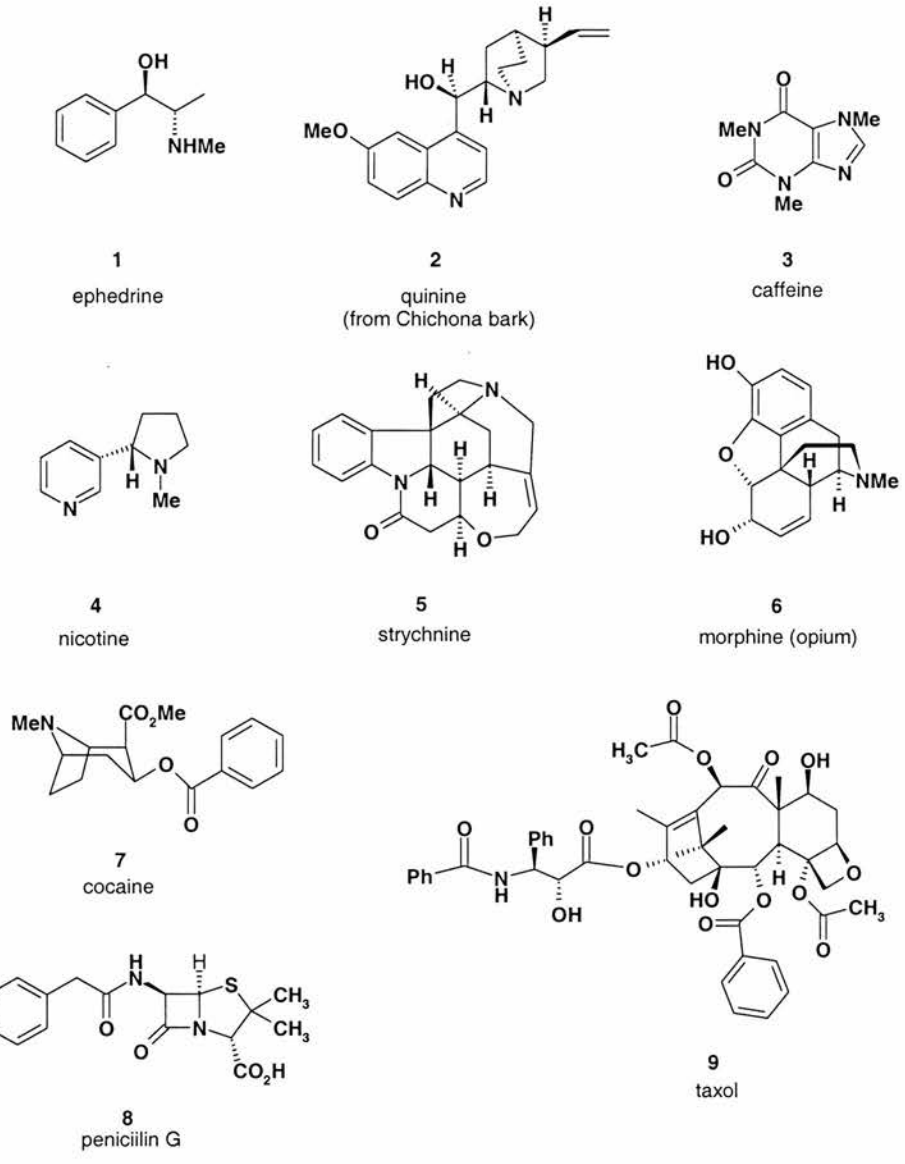


Figure 1.1 Biologically active secondary metabolites.

1.1 Biosynthesis of natural products

1.1.1 Primary and secondary metabolism

Natural products are metabolites of secondary metabolism. It is important to distinguish what is meant by primary and secondary metabolism. Primary metabolism is the term used to cover the metabolic pathways that are common to all living systems. These pathways are

similar but not always identical and can vary widely across genus. The primary metabolites such as sugars, amino acids and nucleic acids that are synthesised (anabolism) and degraded (catabolism) are essential for all living organisms to function[6] and are very much part of biochemical currency.

In addition to operating primary metabolic pathways most species have developed alternative more elaborate pathways that can convert primary metabolites into more structurally diverse and complex compounds. These natural products are not generally regarded as essential for the efficient functioning of a particular organism. Due to their non-essential role these molecules, which derive from pathways peripheral to the primary metabolic pathways, have become known as secondary metabolites. Although there is no universally accepted definition to distinguish between primary and secondary metabolites two main aspects tend to separate these molecules into distinct classes. In micro-organisms primary metabolites are normally biosynthesised in the first stage of cell growth (the trophosphase)[7] whereas secondary metabolites tend to be formed after this during the stationary phase of cell growth (the idiophase).[8] In addition secondary metabolites have a limited distribution which is normally constrained to a particular species.

1.1.2 Techniques to study biosynthesis[9,10]

1.1.2.1 Isotopic labelling studies

Once the structure of a secondary metabolite has been established it is often possible to hypothesise its biosynthetic origin by identifying structural motifs. However the biosynthesis of many secondary metabolites still remains less than obvious. In this thesis for example fluorinated natural products are considered. If this is the case the biosynthetic pathway needs to be determined *ab initio*. There are two general methods by which the

elucidation of biosynthetic pathways is achieved. The first involves the synthesis of suspected metabolic precursors that carry an isotopic label (either stable or radioactive). The candidate precursors are then fed to the organism and the way in which the isotopic label is incorporated into the secondary metabolite can be used to establish precursor relationships. In the case of radioisotopes the most commonly used isotopes are ^{14}C and ^{3}H. Both ^{14}C and ^{3}H are β-emitters and they have half lives of 5600 and 12.26 years respectively.

Radioisotopes offer a high level of sensitivity and have been widely used in the study of biosynthetic pathways since the early pioneering work carried out by Hevesy (Copenhagen) in the 1920's, but their use does also present several problems. There are obvious problems associated with radiation exposure monitoring and safety legislation. Also this approach offers little regio- and stereo-chemical information unless after precursor incorporation studies, careful and time consuming degradation studies are employed. The emergence of nuclear magnetic resonance (NMR) spectroscopy has significantly advanced the study of ^{13}C and ^{2}H incorporation into natural products. Incorporation of these stable isotopes represents an effective and attractive method for studying biochemical pathways. The deployment of stable isotopes avoids the problems associated with the handling and storage of radioactive isotopes and NMR analysis can provide information about levels of incorporation of isotope directly, without recourse to degradation studies. In the early stages of an investigation it is common to conduct straightforward labelling experiments with primary metabolites such as [1-^{13}C]acetate. Incorporation of the ^{13}C label into a natural product can be readily measured by ^{13}C NMR spectroscopy, where enhanced signals above the natural abundance in the ^{13}C NMR spectrum are observed.[9,11]

In addition to acetates that carry only a single [^{13}C] label, [1,2-^{13}C]acetate can also be used in biosynthetic studies. This is a particular powerful tool as it allows identification of carbon-carbon bonds that remain "intact" throughout the biosynthetic pathway. If the carbon-carbon bond is not broken then coupling between the two adjacent ^{13}C nuclei is retained and can be observed in the ^{13}C NMR spectrum. It is also possible to carry out biosynthetic investigations using molecules that contain a combination of ^{13}C and other stable isotopes. These include isotopes such as ^{18}O or ^{15}N which can used to search for the presence of intact ^{13}C-^{18}O or ^{13}C-^{15}N bonds respectively.

Deuterium (^{2}H) is widely used to probe pathways, and can be assayed by mass spectrometry or NMR spectroscopy either directly using ^{2}H NMR or indirectly *via* ^{13}C NMR. In the case of detection by ^{13}C NMR each heavy ^{2}H atom that is directly linked to a ^{13}C atom shifts the carbon resonance upfield by ~ 0.3-0.6 ppm. However coupling of deuterium (I=1) to ^{13}C reduces the intensity of the observed signal, but can still provide useful information.[12]

It is possible to overcome the problems of multiplicity and complex ^{13}C NMR spectra by incorporating the ^{2}H atom in the beta position to the ^{13}C nucleus. A shift in the carbon signal can still be observed but coupling between the two nuclei is now negligible (<1 Hz). The beta isotope effects are additive and are generally in the region of 0.01-0.1ppm per deuterium atom.[13] This particular technique can also be used to monitor two adjacent carbon bonds that remain intact through out the biosynthesis. The first examples of this technique was reported by Abell and Staunton in the early 1980's during their studies on the biosynthesis of the polyketide 6-methylsalicylic acid.[14]

1.1.2.2 Mutant studies

A more biochemically oriented strategy to study the biosynthesis of secondary metabolites involves blocking individual steps along the biosynthetic pathway to encourage the build up of potential intermediates. This can be achieved by selecting appropriate mutant strains after a mutagenesis programme, deficient in the expression of a particular enzyme on a biosynthetic pathway. In so doing the mutant strain may accumulate intermediates on the biosynthetic pathway. One of the major drawbacks with this technique is that it is often not very target specific. If the mutations are carried out using a random process (ie. irradiation with UV or X-rays) then it is possible that enzymes involved in primary metabolic pathways might also be affected. However with the advances in molecular biology it has become possible to eliminate the randomness of this process and it is now possible to target particular pathways at the genetic level. This type of directed mutagenesis has been used to great effect in polyketide biosynthesis where all of the genes that code for the biosynthesis of the molecule are clustered often in one open reading frame.[15] Another, approach to encourage the build up of potential intermediates on a biosynthetic pathway is to introduce chemical inhibitors. However without any prior knowledge of the type of enzyme that you wish to target on the pathway the use of "general" chemical inhibitors may not be specific enough to produce the desired results.

1.1.2.3 ^{19}F Nuclear magnetic resonance spectroscopy

As discussed in Section 1.1.2.1, nuclear magnetic resonance (NMR) spectroscopy can be used to great effect in the biosynthesis of secondary metabolites. An extension of this application is the use of ^{19}F NMR which offers an extremely powerful method by which biosynthetic pathways can be monitored in real time and in a non-invasive manner. The

fluorine atom which is the closest in size to hydrogen has little steric impact when introduced into a molecule. The inclusion of a fluorine as a pseudo isotope of hydrogen then allows the possibility to monitor a metabolic pathway or an enzymatic reaction by ^{19}F NMR spectroscopy.[16]

Several fungi are capable of sustaining growth using toluene as the sole carbon and energy source. De Bont *et al.* used ^{19}F NMR spectroscopy as an analytical technique to establish the initial biosynthetic steps involved in the catabolism of toluene by these fungi.[17] By using fluorinated isomers of toluene as substrate analogues it was possible to use ^{19}F NMR analysis to characterise the pattern of metabolite accumulation (Figure 1.2). The structural assignment of the various compounds (**10 – 14**) observed in the ^{19}F NMR spectra (Figure 1.2) was achieved by comparing the chemical shifts to reference compounds.

Because fluorine (^{19}F) couples to hydrogen (^{1}H) the chemical environment in which the fluorine is located can be established. This is exemplified in the ^{19}F NMR spectra for the known fluorinated secondary metabolites[18] fluoroacetate (FAc) **15** and 4-fluorothreoine (4-FT) **16**. For FAc **15** (Figure 1.3, spectrum A) the fluorine signal is a triplet (t) as the fluorine atom couples to two the geminal hydrogens (I=1/2). For 4-FT **16** (Figure 1.3, spectrum B) the signal appears as a doublet of triplets (d,t) due to the additional vicinal (J^2) coupling with a vincinal hydrogen.

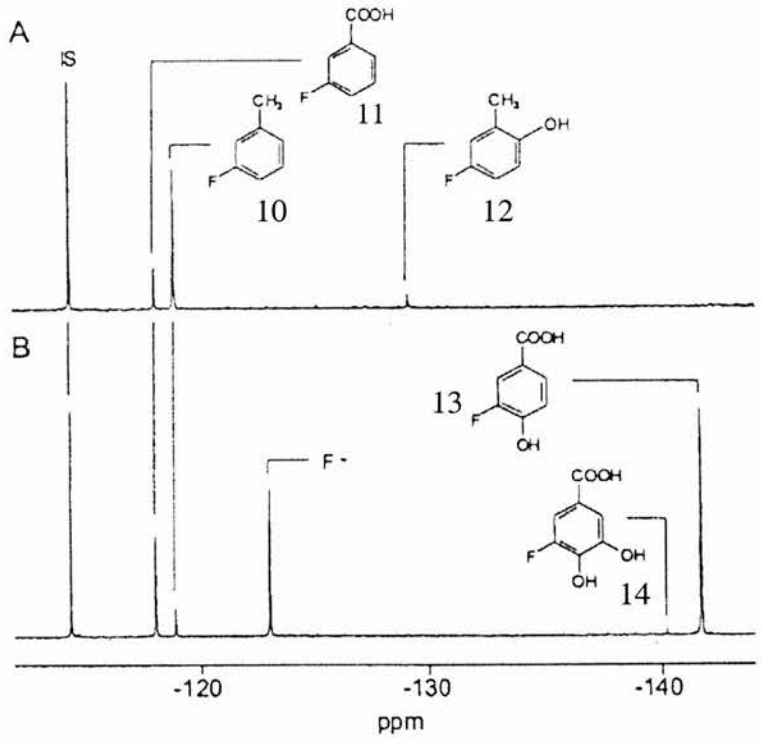


Figure 1.2 ^{19}F NMR spectra of the culture supernatant after incubation of *C. echinulata* CBS 596.68 (A) and *C. sphaeropermum* T0 (B) cells with 3-fluorotoluene. (IS) = 4-fluorobenzoate internal standard.

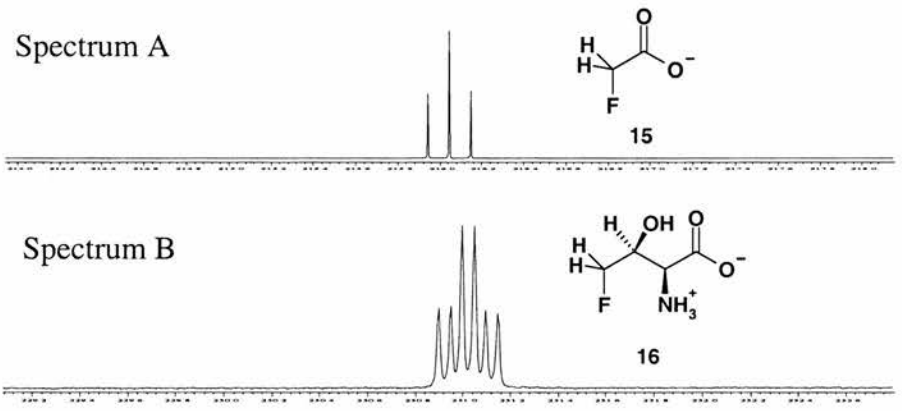


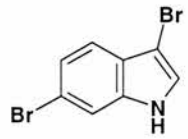
Figure 1.3 ^{19}F NMR spectra showing the fluorine signals of FAc **15** (spectrum A) and 4-FT. **16** (spectrum B).

It is also worth noting that the fluorine chemical shifts of **15** and **16** are quite distinct and are some 15 ppm apart at -216 ppm and -231 ppm respectively. The sweep width is broad in ^{19}F NMR spectroscopy with signals lying between -100 ppm and -300 ppm. This broad range means that it is possible to distinguish between several fluorinated species in a biosynthetic experiment with relative ease (see Figure 1.2). Coupling of fluorine to both ^{13}C and ^{2}H can also be exploited. Analysis of the ^{13}C NMR spectra of fluorine containing molecules will show J^1 coupling constants up to 190 Hz and a J^2 coupling constants of around 20 Hz. Deuterium atoms (^{2}H) can also induce a heavy isotope shifts (upfield) in the fluorine resonance of ~ 0.6 ppm for each geminal deuterium (eg. $CHF^{2}H$). The effect is additive and two geminal deuteriums induce an upfield shift of ~ 1.2 ppm and so on.[19]

1.2 Halogenated natural products

1.2.1 Introduction

The first review on halogenated secondary metabolites was published in 1968 and reported that only thirty such molecules had been identified.[20] By 1973 the number of halogenated natural products had increased to around 200.[21] A review by Gribble some 30 years later in 2003 revealed that the number of naturally produced organohalogens had increased dramatically to more than 3800.[22] This increase can be attributed to high throughput screening technologies and a growing interest in natural products as lead molecules for new pharmaceuticals. In addition there has been an increased focus on the marine environment as a source of bioactives, and halogenated natural products are disproportionately represented here.[23,24] In particular many of the brominated secondary metabolites such as 3,6-dibromoindole **17**[25] have been identified from marine sources.



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1.2.2 Chlorine, bromine and iodine containing natural products

In 2003 Gribble[22] reported that the majority of the 3800 naturally occurring organo-halogenated compounds identified contained either a chlorine (2200) or bromine (1950) atom. It is not surprising that chlorinated secondary metabolites make up the largest sub-group as the bioavailability of chloride is predominant over the other halides in sea water. The structural diversity of the chlorinated natural products is considerable, ranging from molecules as simple as the halo-alkanes[26] **18** and **19** to more complex compounds such as the important antibiotic vancomycin **20**.[27,28] The position and number of chlorine atoms contained within the molecule can also vary extensively. Consider for example the chlorinated natural products KS-504 **21**,[29] nordysidenin **22**[30,31] and the growth hormone 4-chloroindole-3-acetic acid **23**. Compound **21** is polychlorinated and contains approximately 70% chorine by weight, **22** contains a novel trichloromethyl group whereas **23** exhibits a chloro-aromatic motif, a motif that is particularly common among the chlorinated secondary metabolites.

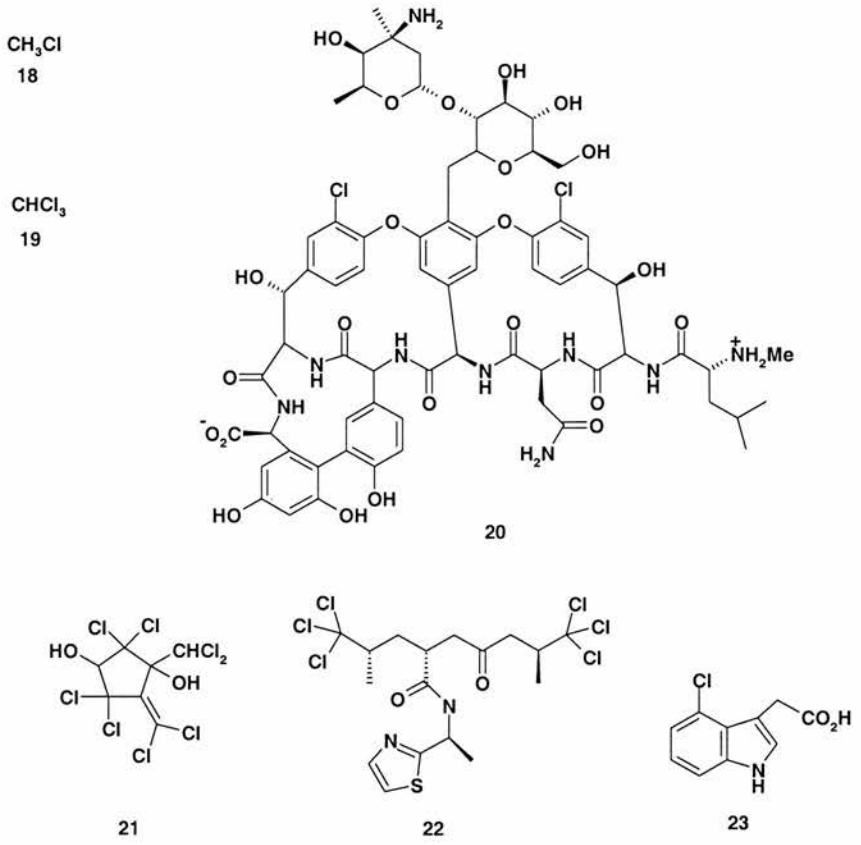


Figure 1.4 Examples of chlorine containing secondary metabolites.

Brominated natural products are also extensive in their diversity and structural complexity. The bromine atom(s) can be located in a variety of positions and chemical environments as illustrated by compounds **24-26** and poly-brominated secondary metabolites are not uncommon e.g. **27** and **28**.

Both chlorinated and brominated natural products have been found to originate from a variety of sources (i.e. plant, bacterial) but again the majority of molecules have been isolated from marine sources.[22,26]

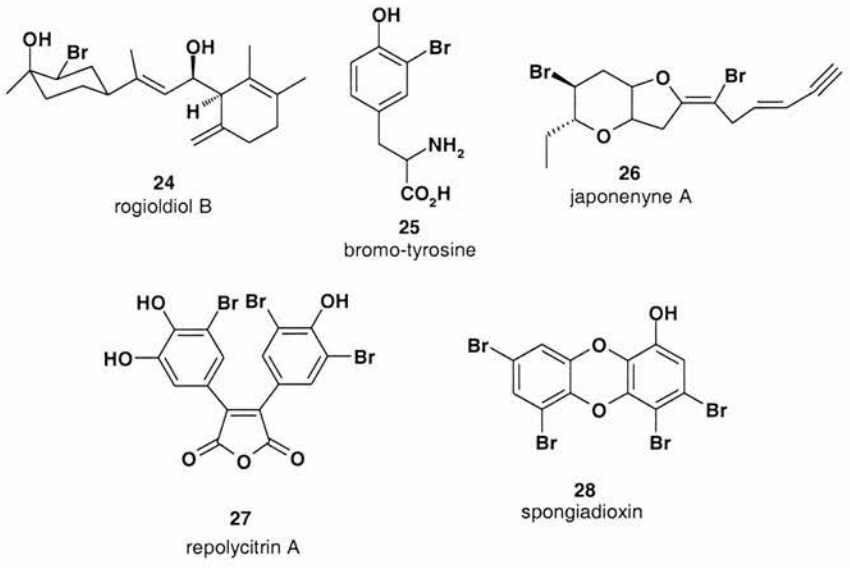


Figure 1.5 Examples of bromine containing secondary metabolites.[22]

Natural products containing iodine are relatively rare. The few organo-iodine natural products can be grouped into five main classes. These are i) volatile compounds such as iodomethane,[32] ii) nucleoside derivatives,[33] iii) tyrosine derivatives including the human thyroid hormone, thyroxine **29**,[6] iv) fatty acid derivatives[34] and v) terpene derivatives.[35] In 2004 a new class of tryptophan based iodinated compounds were isolated from the marine sponge *Plakortis simplex* and are the first reported examples of naturally occurring iodoindoles **30** and **31**.[36]

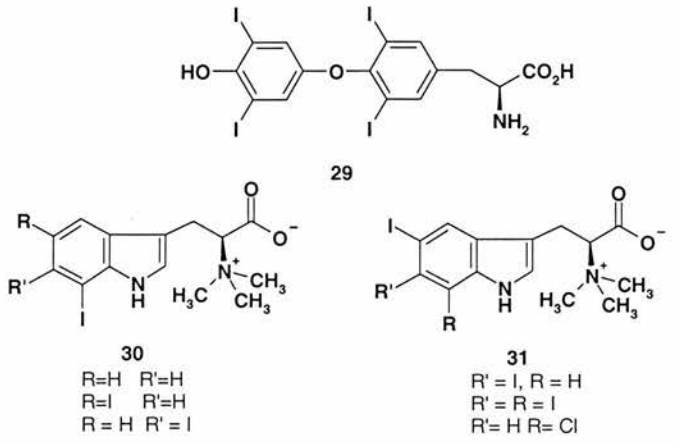
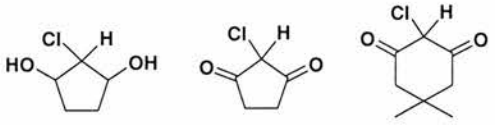


Figure 1.6 Examples of iodine containing secondary metabolites.

1.3 Enzymatic bio-halogenation

1.3.1 General overview of enzymatic bio-halogenation[37]

The first halogenating enzyme was detected in 1959 from the fungus *Calariomyces fumago*, an organism which had been shown to produce the chlorinated secondary metabolite caldariomycin **32**.[38] The enzyme required a halide ion and hydrogen peroxide which led to it being termed a chloroperoxidase. The enzyme accepted chloride, bromide or iodide but not fluoride as a substrate. During the characterisation of the enzyme an assay was developed to detect the halogenation of mono-chlorodimedone **34** a synthetic molecule similar in structure to 2-chloro-1,3-cyclopentadione **33**, an intermediate on the biosynthetic pathway to **32**.[39]

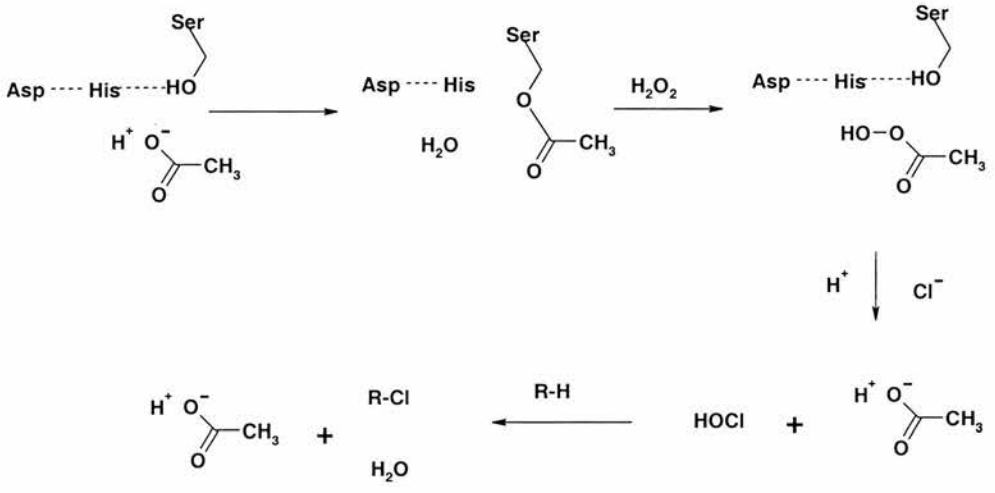


32 33 34

The dimedone assay has become the standard method of analysis for detecting haloperoxidase activity and it was using this assay that a variety of other haloperoxidase enzymes were isolated in the proceeding years.[40] Chloroperoxidases have the ability to process chloride, bromide and iodide, but bromoperoxidases can only oxidise bromide and iodide. Iodoperoxidases can only oxidise iodide. The chloroperoxidase isolated from *C. fumago* was shown to be a heme dependent haloperoxidase. Haloperoxidases have since been reported from a variety of species that require vanadium instead of iron as a metal co-factor.[41,42] Both of these metal dependent haloperoxidases have been shown to utilise hypohalous acid (XOH) as the halogenating agent.[43,44]

A further class of halogenating enzymes has been isolated from *Pseudomonas* and

Streptomyces bacterial strains.[45] Although these enzymes require peroxide they are devoid of a heme or metal co-factor. The three dimensional structure of these enzymes has revealed a catalytic triad consisting of serine, aspartate and histidine residues.[47] The enzyme actually acts as a perhydrolase in the presence of peroxide and the acyl-enzyme intermediate that is initially formed is broken down to form a peracid. The peracids that are generated can oxidise a halide ion to give hypohalous acids which can then act as halogenating agents (Scheme 1.1).[47,48]

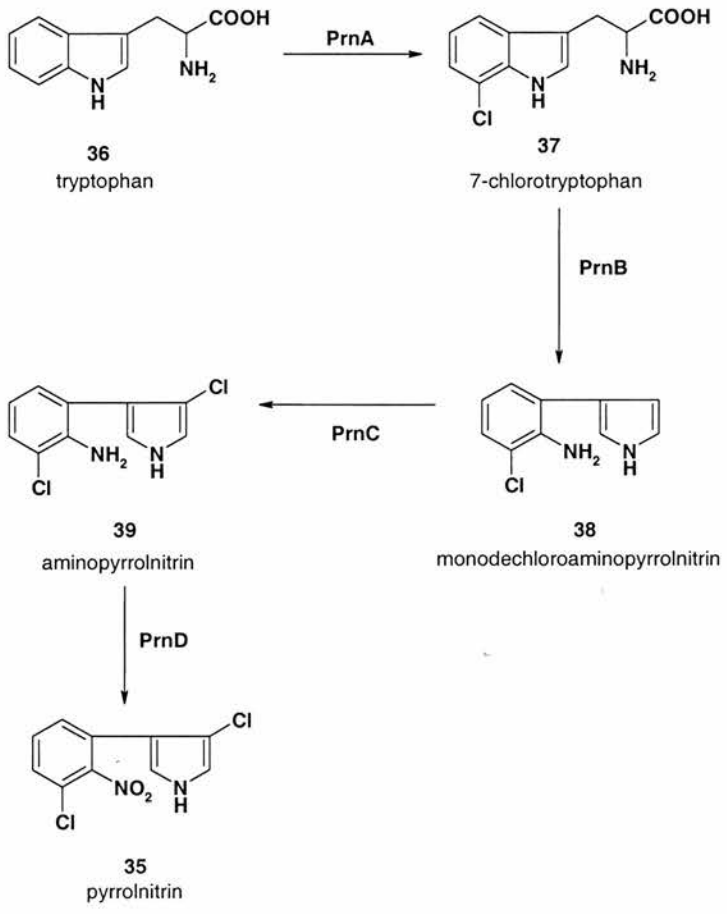


Scheme 1.1 Enzymatic reaction sequence of the perhydrolases.[47,48]

The fact that the majority of the peroxide dependent halogenating enzymes have been isolated using the same dimedone assay highlights their lack of substrate specificity and regioselectivity.

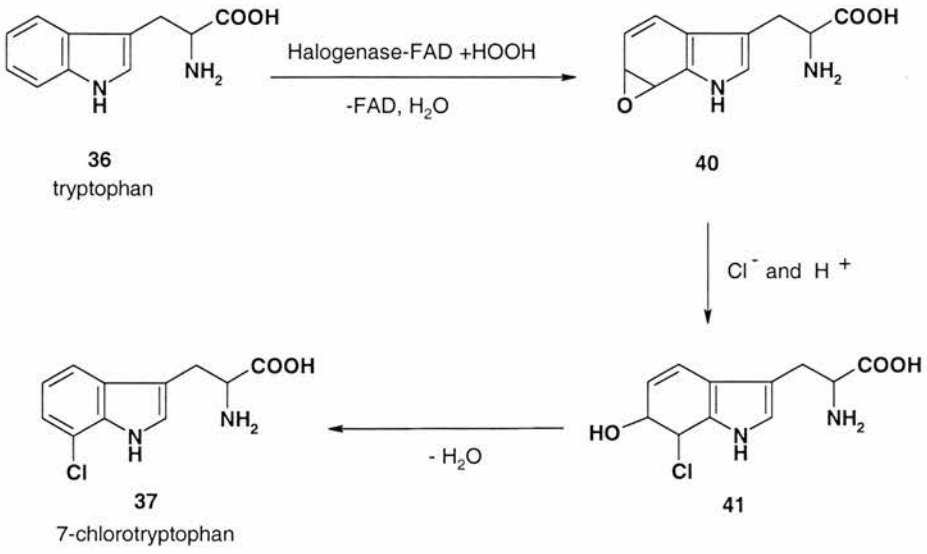
Recently the biosynthetic gene cluster for the biosynthesis of pyrrolnitrin **35** was cloned and sequenced (Scheme 1.2).[49] It was interesting that of the four genes identified, two of them (PrnA) and (PrnC) were shown to code for two different halogenating enzymes that had no sequence homology with any known haloperoxidase or perhydrolase.[50] During the purification of the enzymes it was demonstrated that a further enzyme, a NADPH

dependent flavin reductase, that was not coded for in the pyrrolnitrin biosynthetic gene cluster, was also required for halogenation activity.



Scheme 1.2 Pyrrolnitrin **35** biosynthetic pathway.

These halogenation enzymes required $FADH_2$ and this was recycled by the flavin reductase (Scheme 1.3). These novel enzymes were then classed as $FADH_2$-dependent halogenases. The mechanism that was initially proposed for the enzymatic halogenation of tryptophan **36** to give 7-chlorotryptophan **37** is shown in Scheme 1.3. This involves a nucleophilc mechanism with chloride ion attacking an arene oxide **40**.



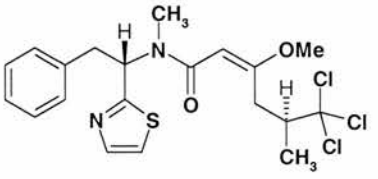
Scheme 1.3 Proposed mechanism for the chlorination of the $FADH_2$-dependent halogenase.[37]

An alternative mechanism for the chlorination of tryptophan **36** involving hypochlorous acid has also been proposed. In this case the chlorination would proceed by a more conventional electrophilic aromatic substitution reaction. Despite the recent publication of the X-ray crystal data for the PrnA (tryptophan 7-halogenase) enzyme[51] the exact mechanism of chlorination remains unresolved.

This type of halogenation is becoming more widely implicated as having a major role in the biosynthesis of halogenated secondary metabolites certainly in bacteria. So far genes coding for $FADH_2$-dependent halogenases have been found in the biosynthetic gene cluster for every halogenated metabolite that has been cloned and sequenced (up to 2003). [37]

It is clear that an unusual and probably quite separate mechanism of halogenation must exist during the biosynthesis of the secondary metabolite barbamide **42**, which has a novel trichoro methyl functionality. Halogenation by a $FADH_2$-dependent halogenase does not occur as labelling studies have ruled out a double bond intermediate.[31] Currently it is believed that the halogenation process proceeds by a radical mechanism and involves a

direct and sequential series of chlorinations on the methyl group.[52]



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1.4 Fluorinated natural products

1.4.1 Fluorine in nature

Fluorine is the most abundant halogen in the Earth's crust but of the ~3800 naturally occurring organo-halogen compounds[31] only 13 organo-fluorine compounds have been identified to date.[53] The principal reason for this is that seawater typically contains only 1.3 ppm fluoride in comparison to 1900 ppm of chloride.[54] Another equally important factor that limits the participation of fluorine in biochemical processes is the high heat of hydration of the fluoride ion (Table 1.1).

Halogen, X^-	Heat of hydration, X^- [KJ mol^{-1}]	Standard redox potential (E^0)
F^-	490	-3.06
Cl^-	351	-1.36
Br^-	326	-1.07
I^-	285	-0.54

Table 1.1 Heat of hydration and standard redox potential for the halogens.

The large heat of hydration associated with fluorine means that in water fluoride ion is heavily hydrated rendering it a poor nucleophile. This hinders its ability to participate as a nucleophile in displacement reactions. In addition the haloperoxidase (Section 1.3.1) reaction which plays a significant role in the formation of natural organohalogen compounds does not extend to fluorination due to the high redox potential that is necessary

to generate F^+ from F^-. This oxidation is not compensated by hydrogen peroxide reduction.

1.4.2 Fluorinated natural products from plants

1.4.2.1 Fluoroacetate

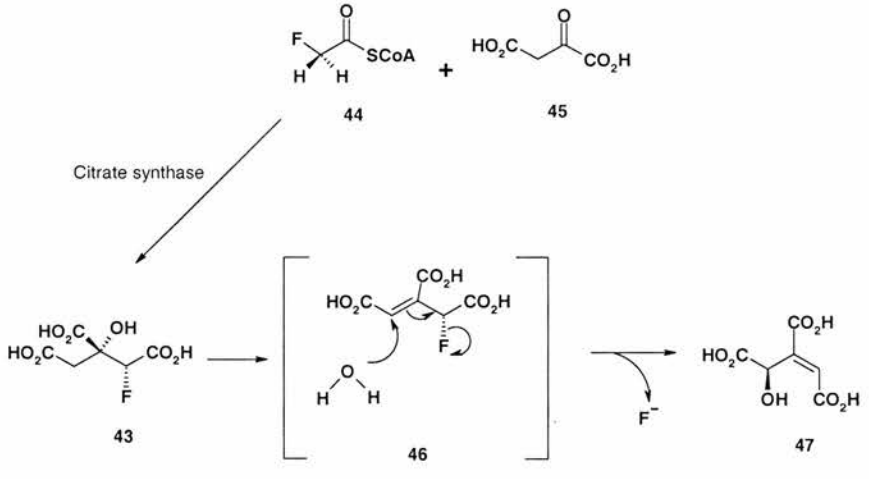
In 1943 Marais isolated and identified the first organofluorine natural product, the toxin FAc **15** from the South African shrub *Dichapetalum cymosum*.[55,56] It had long been recognised by farmers around Pretoria that this shrub was very toxic to their livestock. A fact underlined by the name given to the plant by the locals, "gifblaar" or "poison leaf". Gifblaar can contain up to 250 ppm FAc **15** in its leaves in early spring. FAc **15** has subsequently been found at varying concentrations in a variety of plants throughout the world. Many of the species that produce fluoroacetate can be found in Australia[57] where over 40 plant species have been identified. The large majority of these species (33 from 40) belong to the genus *Gastrolobium* and *Oxylobium* and are confined to the south western corner of Australia. In addition to Australia and Africa, fluoroacetate producing plants have been found in the Indian subcontinent[58] and in South America.[59]

Several plant species have developed as fluoroacetate accumulators and are capable of accumulating FAc **15** in high concentrations, presumably as a means of defence. To date the most toxic plant reported belongs to the genus *Dichapetalum*. *D. braunii* and has been shown to contain a staggering 8000 ppm (8 mg /g) of fluoroacetate within its seeds.[60]

1.4.2.2 Fluorocitrate

The mechanism of FAc **15** toxicity was explored in the early 1950's by the British biochemist, Sir Rudolph Peters.[53] He proposed that its toxicity arises from the *in vivo* conversion to fluorocitrate **43** by the action of citrate synthase.[61] Many of the plants that

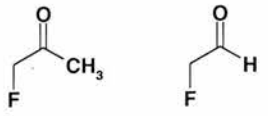
have the ability to accumulate fluoroacetate have been shown also to contain trace amounts of fluorocitrate.[62,63] Condensation of fluoroacetyl-CoA **44** with oxaloacetate **45** by citrate synthase, an enzyme which normally processes acetyl-CoA, generates fluorocitrate **43**. This reaction is completely stereoselective and ironically only produces the toxic (*2R,3R*)-stereoisomer of fluorocitrate.[64,65] The other three streoisomers are not toxic. It has recently been reported that aconitase converts fluorocitrate **43** to fluoro-*cis*-aconitate **46** which is subsequently attacked by hydroxide to generate 4-hydroxy-*trans*-aconitate **47** (Scheme 1.4). The acute toxicity then arises due to the fact that compound **47** is a potent competitive inhibitor of aconitase, the enzyme after citrate synthase on the citric acid cycle.[66] Additionally it has been suggested that fluorocitrate **43** covalently binds to proteins involved in citrate transport across the mitrochondrial membrane.[67] The toxicity attributed to this latter process has been estimated to be 10^4 times more significant than aconitase inhibition alone.



Scheme 1.4 Metabolism of FAc-SCoA **44** and its subsequent conversion to 4-hydroxy-*trans*-aconitate **47**.

1.4.2.3 Fluoroacetone

Fluoroacetone **48** has been reported as a metabolite of FAc **15** in the plant *Acacia georginae* by Peters and Shorthouse.[68,69]

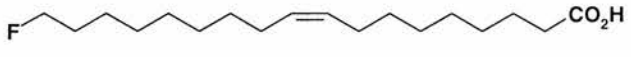


48 **49**

However the authors themselves conceded that there may be a question surrounding the validity of **48** as a fluorinated metabolite. They indicated that the 2,4-dinitrophenylhyrazone derivatisation method used to detect **48** could not distinguish between fluoroacetone **48** and fluoroacetaldehyde **49**. With the discovery that **49** plays a key role in the biosynthesis of the fluorinated metabolites produced by *S. cattleya* (section 1.5) it is possible that the 2,4-dinitrophenylhyrazone derivative detected was that of fluoroacetaldehyde **49** and not fluoroacetone **48**.

1.4.2.4 Fluorinated fatty acids

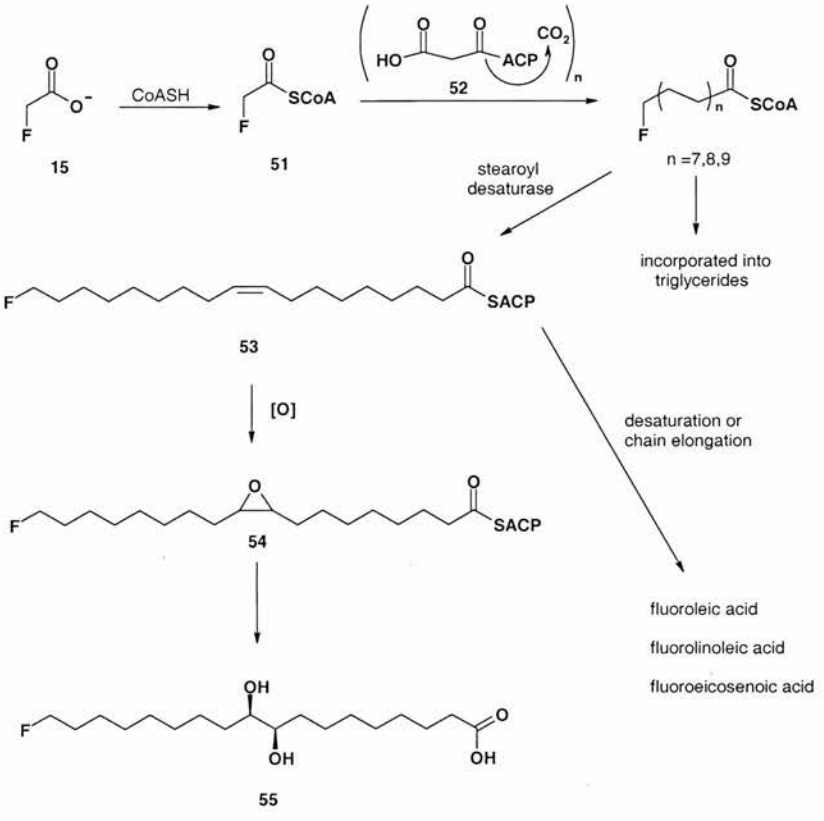
Sir Rudolph Peters also identified ω-fluorooleic acid **50** from the seeds of the shrub *Dichapetalum toxicarium*.[70,71] As well as the isolation of **50** which accounted for 80% of the total organic fluorine present, trace amounts of ω-fluoropalmitic acid were also identified.



50

Re-analysis of the seed oils of *D. toxicarium* by GC-MS has resulted in the identification of a further five ω-substituted fluoro acids ($C_{16:0F}$, $C_{18:0F}$, $C_{18:2F}$, $C_{20:0F}$ and $C_{20:1F}$).[72] A

hypothetical biosynthetic pathway to these fluorinated fatty acids is summarised in Scheme 1.5.[73] If the plant has the ability to generate and utilise fluoroacetyl-CoA **51** then it is envisaged that the biosynthetic pathway would follow along similar lines to conventional fatty acid biosynthesis. It is worth noting that the fluorine atom is only ever located on the terminal carbon (ω) implying that a fluorinated analogue of malonyl acyl carrier protein **52** cannot be biosynthesised in this system.



Scheme 1.5 Putative biosynthetic pathway to ω-fluorofatty acids in *D. toxicarium*.[73]

1.4.3 Fluorinated natural products from marine sources

1.4.3.1 5'-Fluorouracil based alkaloids from the marine sponge *Phakellia fusca*

Although the majority of the chlorine and bromine containing natural products originate from marine sources, the first fluorinated natural product from a marine source was only

very recently isolated (2003) from the sponge *Phakellia fusca*.[74] The *Phakellia* sponges have been reported to yield alkaloids, peptides and polyether acids and it was during attempts to isolate new biologically active molecules that five fluorinated natural products **56-60** were identified. All of these metabolites were derivatives of 5'-fluorouracil based alkaloids.

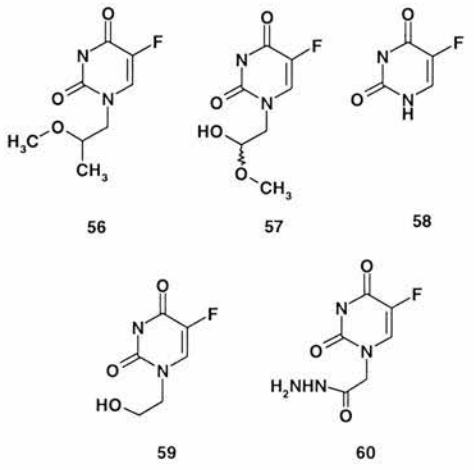


Figure 1.7 5'-Fluorouracil derivatives isolated from the sponge *Phakellia fusca* **56-60**.

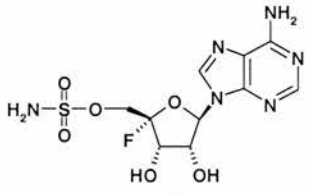
Of the five metabolites two (**58**[75] and **59**[76]) were previously known as drugs that posses anti-tumor activity.[77] It is plausible that all of these alkaloids derive from the metabolism of either **58** or **59** that may have been present as pollutants in the sea water due to their commercial production as pharmaceuticals. However from the short account of their isolation, this is not clear.

1.4.4 Fluorinated natural products from bacteria

1.4.4.1 Nucleocidin from *Streptomyces calvus*

Despite the low bioavailability of fluorine and the considerable difficulties surrounding the reactivity of the fluoride ion in an aqueous media, a few fluorometabolites have been

isolated from micro-organisms. The first of these was nucleocidin **61** a nucleoside antibiotic, which was isolated from the bacterium *Streptomyces calvus*.[78]



61

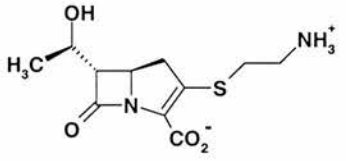
S. calvus was isolated from an Indian soil sample and was shown to produce a molecule of a similar composition to adenosine which displayed a broad range antibacterial spectrum.[79] Ironically it took twelve years to discover the presence of a fluorine atom in **61**. The unusual coupling of certain signals in the ^{1}H NMR spectrum caused by H-F couplings was initially attributed to a hindered rotation within the molecule. A revised structure 4′-fluoro-5′-*O*-sulfamoyl-adenosine **61** including the fluorine atom was proposed in 1969.[80] Subsequent work by Shuman *et al.*[81] confirmed the β-D configuration of the ribose moiety in the proposed structure as upon heating in DMF nucleocidin **61** was converted to an N3, 5'-anhydronucleoside. Finally, in 1976 some nineteen years after it was first isolated from *S. calvus* the proposed structure of nucleocidin **61** was confirmed by total synthesis.[82]

Nuclocidin **61** has a considerably different structure to the other fluorinated metabolites such as FAc **15** and the ω-fluorinated fatty acids. The position of the fluorine atom on the ribose moiety of the nucleoside offers no obvious biosynthetic hypothesis, whereas the ω-fluorinated fatty acids can clearly derive from FAc **15**. It must be assumed that the biosynthesis of nucleocidin **61** proceeds by a pathway that utilises a very distinct and novel enzymatic fluorination step. Unfortunately recent attempts to re-isolate **61** from cultures of *S. calvus* have been unsuccessful.[83] This has prevented any further work being carried out to elucidate the mechanism by which the carbon fluorine bond is generated in the

bacterium.

1.4.4.2 Biosynthesis of fluorinated natural products in *S. cattleya*

S. cattleya derives from the same genus as *S. calvus* and is currently the only other reported micro-organism capable of elaborating fluorometabolites. The bacterium attracted attention as the source of the antibiotic thienamycin **62**, which was the first example of a new sub-class of β-lactam antibiotics.[84,85] It was the first of the naturally occurring β-lactam antibiotics to be isolated that contained the carbpenem ring system and many of the carbpenems discovered subsequently are derivatives or epimers of **62**.[86]



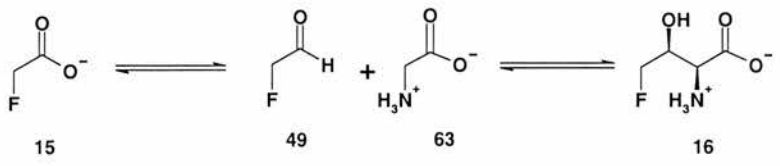
62

In 1986 whilst trying to optimise the production of the antibiotic from *S. cattleya* Sanada *et al.*[18] discovered that the addition of fluoride ion to the culture medium resulted in the production of two organo-fluorine compounds. The compounds were identified as fluoroacetate (FAc) **15** (^{19}F NMR, t, -216.9 ppm, ^{2}J 48.3 Hz) and 4-fluorothreonine (4-FT) **16** (^{19}F NMR, dt, -231.6 ppm, ^{2}J 46.9 and ^{3}J 25.0 Hz). This discovery represented the first example of a micro-organism capable of biosynthesising a fluorinated amino acid. Clearly *S. cattleya* offered a second system in which to study the biosynthesis of fluorinated natural products to develop a greater understanding of how nature generates a carbon-fluorine bond.

1.5 An overview of fluorometabolite biosynthesis of *S. cattleya*

1.5.1 A role for fluoroacetaldehyde.

Initially Sanada *et al.*, suggested that 4-FT **16** might originate from FAc **15**.[18] This would involve the generation of fluoroacetaldehyde **49** from FAc **15** followed by the condensation of **49** with glycine **63** to give 4-FT **16** (Scheme 1.6).



Scheme 1.6 Biosynthesis of 4-FT **16** from FAc **15** as proposed by Sanada *et al.*[18]

This hypothesis was supported by Tamura *et al.*[87] who showed that FAc **15** was detectable in the culture medium before 4-FT **16**. Tamura *et al.*, therefore reasoned that FAc **15** and then fluoroacetaldehyde **49** may serve as the precursors to 4-FT **16**.

In order to test this hypothesis Reid *et al.* incubated resting cell suspensions for 96 h with a range of FAc **15** concentrations.[88] They reasoned that if 4-FT **16** was derived from FAc **15** then one would expect see an increase in the concentration of 4FT **16** production if additional FAc **15** was added to cell suspensions from *S. cattleya*. In the event it emerged that incubation in the presence of 2 mM F ion gave rise to equal concentrations of the both fluorometabolites (0.9 mM), whereas incubation with 1.6 mM FAc **15** led to the production of less than 0.05 mM 4-FT **16**. The rate of interconversion of the fluorometabolites appeared to be 20 fold too slow to support a role for FAc **15** in 4-FT **16** biosynthesis. The low inter-conversion that was observed was attributed to the release of fluoride ion from the biodegradation of FAc **15**.[89,90,91] The idea that the interconversions are the result of fluoride ion release is supported by the fact that a variety of other organo-fluoro

compounds (eg. 3-fluoropyruvate, 4-fluoroglutamate, fluorofumarate and 3-fluoroalanine) could also support the biosynthesis of the fluoro-metabolites **15** and **16**. It is interesting that fluoroethanol was not metabolized by the cell suspensions suggesting that *S. cattleya* does not possess an alcohol dehydrogenase capable of oxidising this molecule. In conclusion, the results of this study implied that there was little inter-conversion between the two fluorometabolites (FAc **15** and 4-FT **16**) and therefore that it is unlikely that either compound was derived by metabolism of the other.

To further explore the biosynthetic origin of the fluorinated secondary metabolites further work was carried out by O'Hagan and coworkers.[92] in which isotopically labelled (^{2}H and ^{13}C) molecules were explored as potential metabolic precursors. A variety of labelled (^{13}C and/or ^{2}H) compounds were tested and their incorporation into the secondary metabolites was determined by both GC-MS and ^{19}F NMR analysis (Figure 1.8).

Several interesting conclusions emerged from these experiments. The overall incorporation of [1,2-$^{13}C_2$] and [2-^{13}C]glycine into C-1 and C-2 of FAc **15** and C-2, C-3 and C-4 of 4-FT **16** was high and of a similar magnitude in both cases. It was also noted that there was very low incorporation of labelled glycine into the C-1 / C-2 fragment of 4-FT **16**. This would appear to support the conclusion that FAc **15** is not derived from the condensation of fluoroacetaldehyde **49** and glycine **64**. An interesting result to emerge was the observation that the labelling patterns in FAc **15** were almost identical to that found in the C-3 and C-4 fragment of 4-FT **16**. The results from the GC-MS studies were supported by ^{19}F NMR analysis recorded after incubation with [2-^{13}C]glycine and [1,2-$^{13}C_2$]glycine (Figure 1.8). The doublet of doublets (dd) obtained for both FAc **15** and 4-FT **16** indicated that each molecule contained two ^{13}C atoms. From the results of the GC-MS and ^{19}F NMR analysis it was clear the C_2 units of both FAc **15** and 4-FT **16** had an identical origin. This

implied that there was only a single fluorination enzyme active within *S. cattleya.*

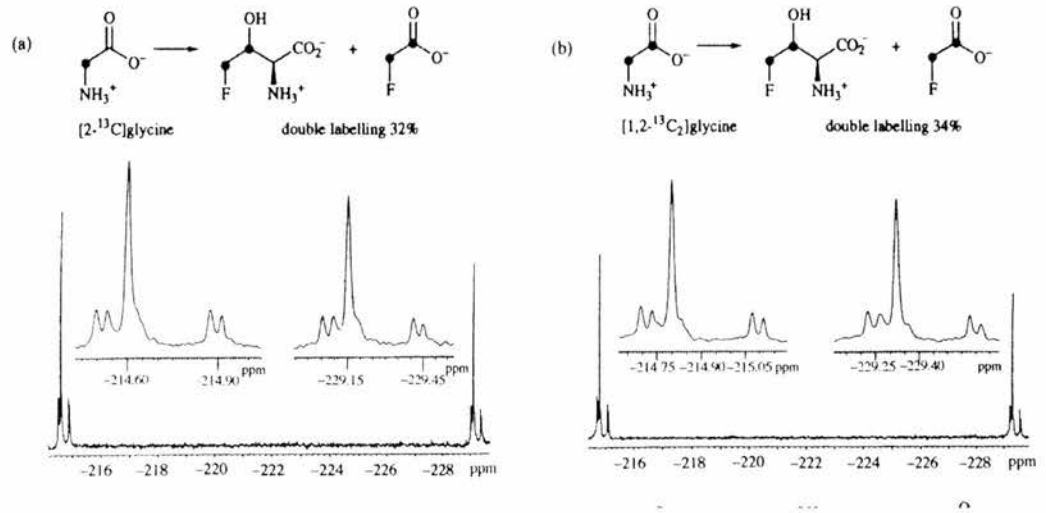


Figure 1.8 ^{19}F NMR spectra showing incorporations of ^{13}C from [2-^{13}C]glycine and [1,2-$^{13}C_2$]glycine.[92]

Despite the fact that 4-fluorothreonine was not generated by the condensation of fluoroacetaldehyde **49** with glycine **63** (Scheme 1.6) it was proposed by Moss *et al.*[93] that **49** was still an attractive precursor for the C-3/ C-4 fragment of the 4-FT **16**. To explore a potential role for fluoroacetaldehyde **49**, it was incubated with resting cell suspensions of *S. cattleya*. Analysis of the culture supernatant by ^{19}F NMR spectroscopy revealed an almost stoichiometric conversion of fluoroacetaldehyde **49** to FAc **15** after only 3 hours. Further investigations revealed that the enzymatic oxidation would only occur in the presence of (NAD^+), which indicated that an aldehyde dehydrogenase in *S. cattleya* was responsible for mediating the conversion.

It was also found that cell suspension supplemented with 2 mM fluoroacetaldehyde **49** could yield almost twice the level of 4-FT **16** compared with fluoride ion alone (2 mM) over the same incubation period (6 h). To further confirm this hypothesis, deuterium

labelled [1-$^{2}H_1$]fluoroacetaldehyde and [1,2-$^{2}H_2$]fluoroacetaldehyde were incubated with cell suspensions and the production of 4-FT **16** was monitored over time by GC-MS. The GC-MS data showed significant incorporation of a single ^{2}H atom into the (C-2+C3+C4) fragment of 4-FT **16**. The label could only reside on the C-3-C-4 fragment as it was already established that there is no significant incorporation into the C-2 position. These experiments provided strong evidence that fluoroacetaldehyde **49** was also the direct precursor to 4-FT **16** providing the C-3-C-4 fragment of the molecule. This was a defining moment in understanding fluorometabolite biosynthesis within *S. cattleya*. The identification of a substrate opened up prospects for the isolation of the first enzymes on this pathway.

1.5.2 Identification of a fluoroacetaldehyde dehydrogenase from *S. cattleya*

The first enzyme to be isolated from the fluorometabolite pathway was an NAD dependent aldehyde dehydrogenase that catalysed the conversion of fluoroacetaldehyde **49** to FAc **15**.[94] Aldehyde dehydrogenases have been isolated and studied in a variety of organisms however none of the previously isolated enzymes had been shown to utilize fluoroacetaldehyde **49** as a substrate,[95,96] whereas for this enzyme it was the optimal substrate.

Substrate	Relative activity (%)[a]	v_{max} (U/mg)	K_m (μM)
Fluoroacetaldehyde	100	0.143	80
Glycoaldehyde	150	0.206	150
Chloroacetaldehyde	80	ND	ND
Acetaldehyde	40	0.129	810
Propionaldehyde	28	ND	ND
DL-Glyceraldehyde	10	ND	ND
Benzaldehyde	65	0.099	320

[a]Activities expressed relative to the rate obtained with 0.25 mM fluoroacetaldehyde. ND = not determined.

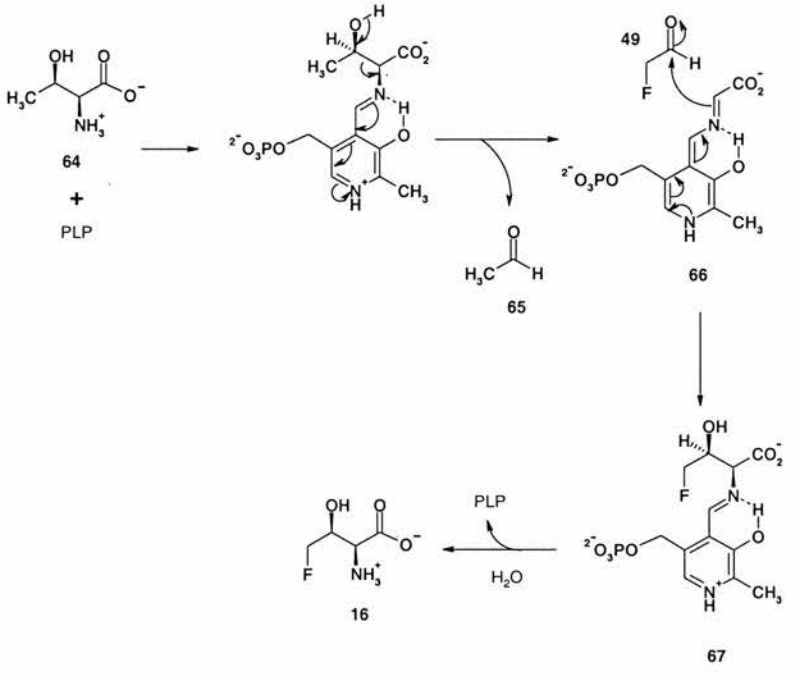
Table 1.2 Substrate specificity of fluoroacetaldehyde dehydrogenase isolated from *S. cattleya*.[94]

The aldehyde dehydrogenase was shown to process a variety of substrates (Table 1.2). The reaction rates observed for the various substrates indicate that electronic factors might be more important than steric factors for efficiency. Chloroacetaldehyde was shown to be a time dependent inhibitor of the enzyme possibly acting by a mechanism which involves alkylation of an essential active site residue.

1.5.3 Identification of a PLP-dependent threonine transaldolase from *S. cattleya*

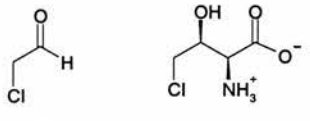
Labelling studies and incubation experiments carried out in cell suspensions of *S. cattleya* provided strong evidence to support fluoroacetaldehyde **49** as a precursor to 4-FT **16**. Cell free extracts were then incubated with fluoroacetaldehyde **49** and various amino acids and co-factors. In a successful combination it was found that the incubation of L-threonine **64**, pyridoxal 5'-phosphate (PLP) and fluoroacetaldehyde **49**, gave rise to 4-FT **16** production. The enzyme responsible for this conversion was purified and assayed with a range of amino acids including glycine **63**, but only L-threonine **64** generated 4-FT **16**.[97] The fact that the enzyme does not accept glycine **63** as a substrate sets it apart for the more classical PLP-threonine transaldolases in bacteria.[98] A mechanism for the transformation was proposed (Scheme 1.7). The first step involves the enzyme PLP complex binding a

molecule of L-threonine **64**. A retro-aldol process then releases acetaldehyde **65**. The resultant-PLP complex **66** then condenses with a molecule of fluoroacetaldehyde **49** to form the imine **67**, which subsequently releases a molecule of 4-FT **16** after hydrolysis.



Scheme 1.7 A mechanism for the enzymatic formation of 4-FT **16** by the PLP- dependent threonine transaldolase from *S. cattleya*.[97]

When incubated with chloroacetaldehyde **68** the enzyme is capable of generating 4-chlorothreonine **69**, a known secondary metabolite from a *Streptomyces sp.*[99,100] However whether or not a similar PLP-dependent threonine transadolase can account for the biosynthesis of 4-chlorothreonine **69** in other *Stremptomyces* remains to be established.



1.6 Enzymatic fluorination

1.6.1 An electrophilic, nucleophilic or radical based mechanism?

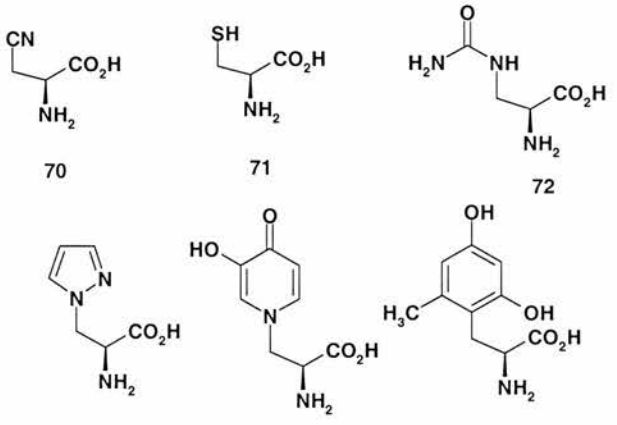
A great deal of work has been has been carried out to investigate various aspects of the biosynthesis of the fluoro-metabolites discussed in the previous sections. Prior to the work carried out during this thesis, a fluorination enzyme had not been isolated and consequently the exact nature of carbon-fluorine bond formation was still unknown.

As section 1.3 has shown, nature has evolved enzymes that can handle (in the case of chloride) halogenation reactions that occur by either electrophilc, nucleophilic or possibly radical mechanisms. Perhaps the key factor in being able to understanding the mechanism by which fluorination occurs lies in determining the exact nature of the fluorinating species. The majority of the halogenated compounds discovered in nature most probably result from electrophilic halogenation utilising enzymatically generated hypochlorous acid. However, electrophilic fluorination in nature is unattainable due to the extremely unfavourable redox potential required to generate an F^+ species. It follows that the extreme chemical characteristics exhibited by fluorine, in comparison to the other halogens, renders the possibility of a radical fluorination process also unlikely.

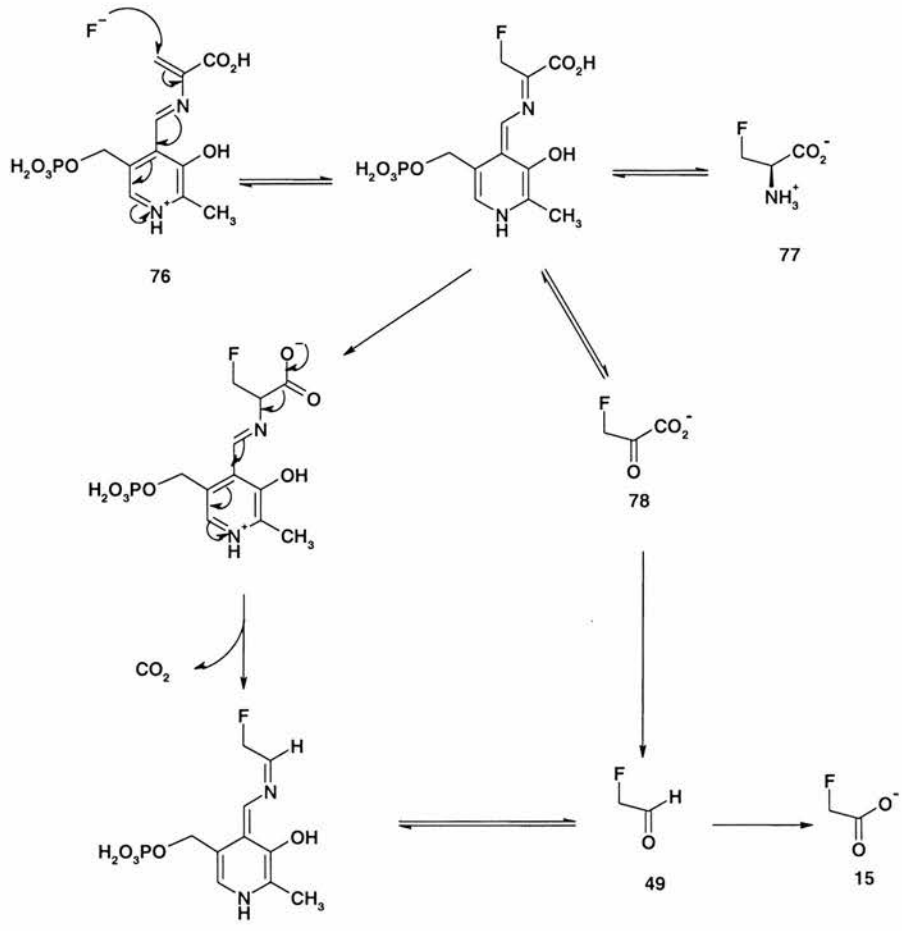
Despite the fact that there is some uncertainty that nucleophilic halogenation reactions occur in nature, this must surely be the most plausible route by which enzymatic fluorination would occur. Despite the weak nucleophilicity of fluoride ion in an aqueous environment, a "naked" fluoride is a powerful nucleophile and an enzyme that has evolved to handle C-F bond formation must have also developed an ability to generate dehydrated fluoride.

1.6.2 Enzymatic fluorination by nucleophilic attack on a double bond

The role of PLP in the metabolism of amino acids is widely understood but it can also serve to mediate the biosynthesis of several unusual β-substituted amino acids. Mead and Segal have shown that *Acacia spp.* is capable of producing seven β-substituted alanines (**70-75**) via an enzymatic reaction that is catalysed by PLP.[101]



The considerable range of amino acids produced implies that the PLP dependant enzyme responsible is relatively non-specific and that a range of nucleophiles can be processed. The hypothesis presented by Mead and Segal was that that FAc **15** may be produced by fluoride ion attack on the enamine **76** (Scheme 1.8). The enamine **76** is formed *via* the condensation of either serine or cysteine with PLP followed by the subsequent elimination of a hydroxyl (serine) or thio group (cysteine) from the resulting molecule. The resulting PLP-bound 3-fluoroalanine **77** could be metabolised in two ways. Decarboxylation would result in fluoroacetaldehyde **49** being released and subsequent oxidation by an aldehyde dehydrogenase enzyme would give FAc **15**. Conversely if the complex is hydrolysed then 3-fluoropyruvate **78** would be released and this intermediate could then be converted to FAc **15** by an oxidative decarboxylation reaction.

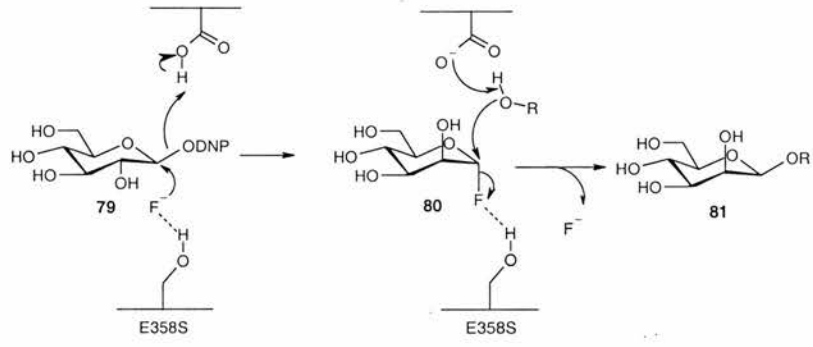


Scheme 1.8 Putative biosynthesis of FAc **15**, Mead and Segal.[102]

In order to test their hypothesis Mead and Segal investigated the influence of fluoride on protein extracts of *Acacia georginae*.[102] In the event they found no evidence for carbon fluorine bond formation as neither β-fluoroalanine **77** nor β-fluoropyruvate **78** could be detected when fluoride, the appropriate amino acids and PLP were added to the system. It was noted that the products might not have been stable under the reaction conditions as β-fluoroalanine **77** is unstable at pH 8.5 and also cell-free extracts have been shown to have the ability to generally defluorinate β-fluoropyruvate **78**.[103,104] Therefore it is possible that both fluorinated products could have degraded *in situ*.

1.6.3 The first reported "enzymatic" C-F bond formation

Withers and Zechel[105] have reported C-F bond formation by mutants of a glycosidase enzyme from an *Agrobacterium* sp. β-Glucosidase (Abg) in which the catalytic glutamate nucleophile is replaced with either an alanine or serine residue was able to form α-fluoroglycosides in the presence of fluoride (2 M). The fluoride ion acts as an alternative nucleophile restoring the enzyme's activity and results in the formation of a transient α-fluoroglycoside **80** which is then subject to a second nucleophilic displacement to generate **81** (Scheme 1.9).



Scheme 1.9 Enzymatic C-F bond formation in a mutant glycosidase enzyme.[105]

These experiments with mutant enzymes are exciting and demonstrate that a nucleophilic fluorination can be enzymatically catalysed in an aqueous environment albeit at high fluoride ion concentrations.

1.7 The discovery of fluorinase activity from *S. cattleya*

1.7.1 Adenosine-triphosphate (ATP) supports enzymatic fluorination

C. Schaffrath (St. Andrews) found that when washed cells of *S. cattleya* were re-suspended in water (0.04 g wet wt ml^{-1}), supplemented with glycerol (5 mM) and KF (2 mM) at 28 °C

for 5 days, low levels of fluorophosphates were detected in the culture medium of the bacterium, as determined by ^{19}F NMR spectroscopy (Figure 1.9).

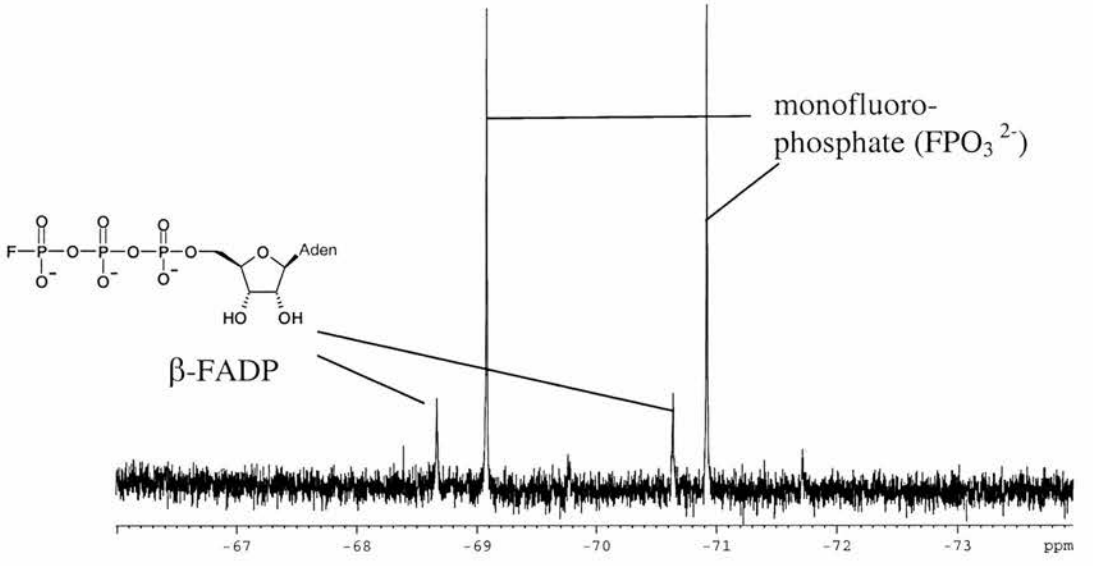


Figure 1.9 ^{19}F NMR showing fluorophosphates from *S. cattleya.*

It was considered that fluorophosphates may overcome some of the problems associated with water solvation of fluoride and provide an activated form of fluoride ion for further metabolism.[106]

C. Schaffrath investigated in a preliminary way if fluorophosphates had a role to play in the formation of the fluorometabolites of *S. cattleya.* A series of cell-free extract experiments were carried out in which adenosine-triphosphate (ATP) and several other potential phosphate sources such as UTP, GTP and CTP were added. It was found that only ATP gave rise to the production of organo-fluoro products. The resultant ^{19}F NMR spectrum recorded from this experiment is shown in Figure 1.10.[107]

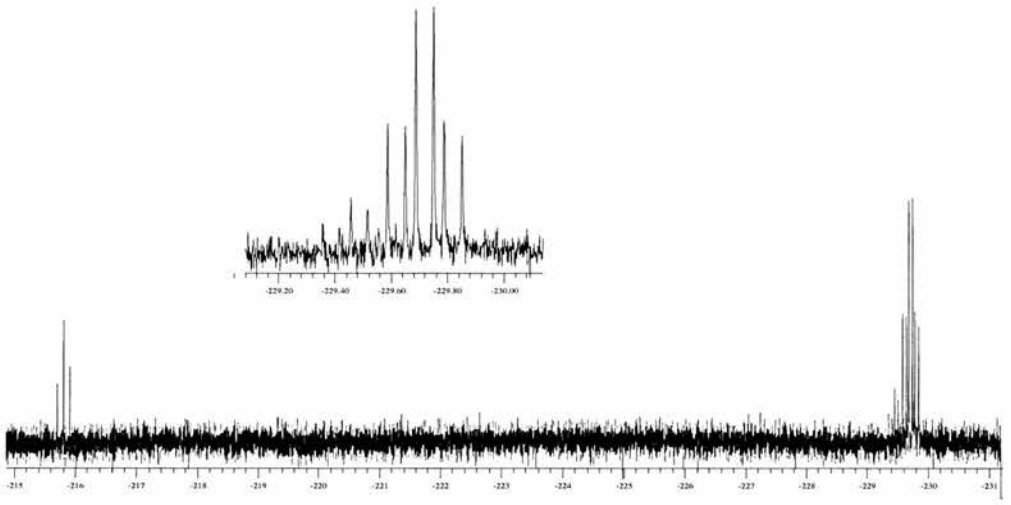


Figure 1.10 ^{19}F NMR spectrum after incubation with ATP. Three organofluorine signals can be detected of which one is assigned to FAc **15** (-215.8 ppm) and two are unknown (-229.5 ppm and -229.7 ppm).[107]

The triplet (t) at -215.8 ppm was assigned to be FAc **15** but the two remaining signals at -229.5 ppm and -229.7 ppm had never been observed in previous experiments. The production of FAc **15** under the conditions used implied that enzymatic fluorination was occurring to give new organo-fluoro compounds (expansion Figure 1.10). It was clearly possible that the first organo-fluoro compound formed is represented by one of the two unassigned fluorine signals. The two signals observed in the ^{19}F NMR spectrum were both doublets of triplets implying that the fluorine in each molecule was present as a fluoro-methyl group with an adjacent vicinal hydrogen. Furthermore, the fact that the J_{FH} coupling constants (^{2}J 47 and ^{3}J 29 Hz) were identical in each case, implied, that their structures were very similar. Previous labelling studies carried out with [2-$^{2}H_1$, 2-^{18}O]glycerol have shown that the carbon oxygen bond remains intact during the biosynthesis as the isotope is found in FAc **15** and 4-FT **16**.[108] With this background and from the ^{19}F NMR data available a minimal structure for the two potential intermediates was devised (Figure 1.11).

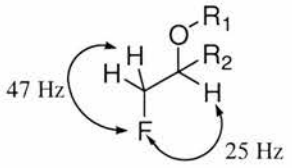
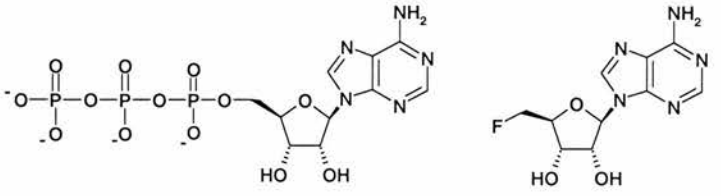


Figure 1.11 Minimal structure for new organo-fluorine compounds.

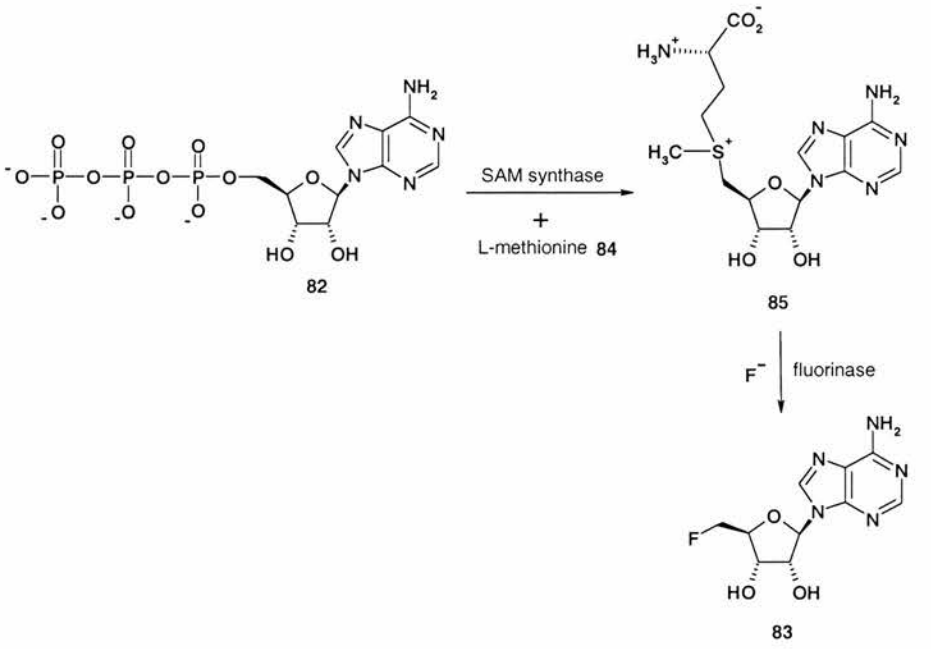
In order to generate the chemical environment shown in Figure 1.10 the only realistic place that the fluorine atom could reside in a molecule derived from ATP **82** would be C-5'. The obvious product that would arise from fluorination of ATP **82** is 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83** which has a structure in keeping with Figure 1.11.



82 83

1.7.2 *S*-Adenosyl-L-methionine (SAM) as a potential substrate for the fluorination enzyme

Cell free investigations into C-F bond formation using ATP **82** revealed that the amino acid L-methionine **84** could enhance fluorometabolite formation. This suggested that the substrate for enzymatic fluorination might actually be *S*-adenosyl-L-methionine (SAM) **85**, as SAM could clearly be generated *in situ* from ATP **82** and L-methionine **84** by the action of SAM synthase (Scheme 1.10) which should be present in a crude cell free extract from *S. cattleya*. This would account for the enhanced levels of FAc **15** production when the incubations were supplemented with L-methionine **84**. This led to the working hypothesis shown in Scheme 1.10.



Scheme 1.10 Working hypothesis for biological fluorination.

Subsequent incubation of SAM **85** with fluoride ion in a cell-free extract but in the absence of ATP **82** resulted in an identical product profile to that which was observed with ATP **82** (Figure 1.10). This result reinforced the working hypothesis that SAM **85** and not ATP **82** is the actual precursor for the fluorination enzyme.

1.7.3 Isolation of a fluorinated nucleoside from the cell free extract (CFE)

It was found that incubation of SAM **85** and KF in CFE of *S. cattleya* in the presence of iodoacetamide arrested FAc **15** formation but resulted in the build up of two other organo-fluorine compounds (Figure 1.12).

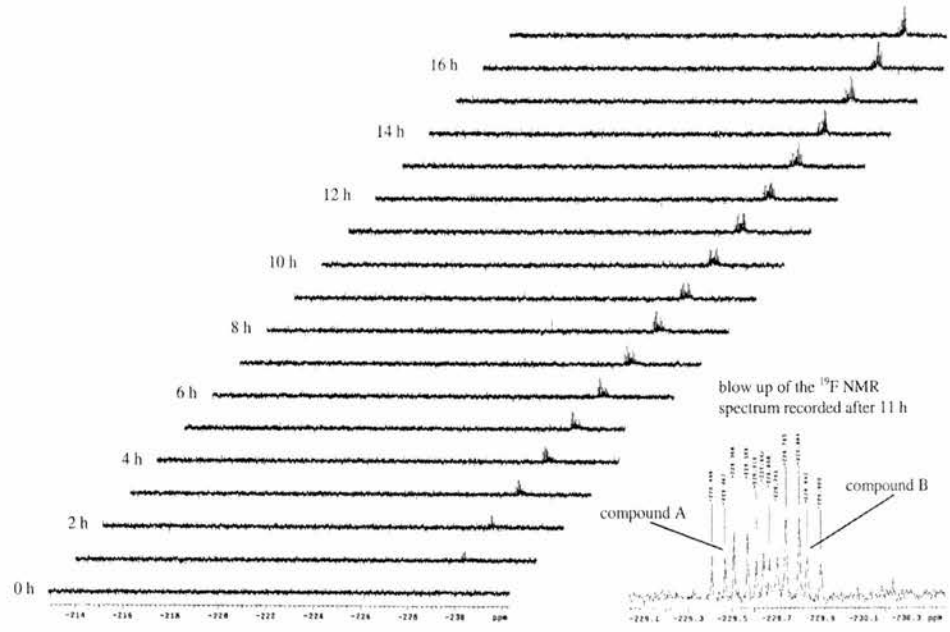
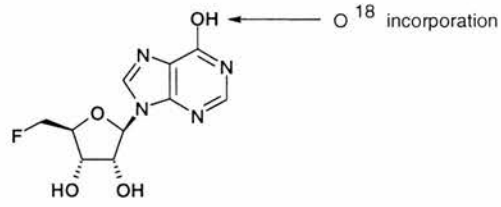


Figure 1.12 ^{19}F NMR spectra showing the build up of two fluorinated compounds.[107]

It appeared that iodoacetamide had the ability to inhibit one of the enzymes on the biosynthetic pathway and to prevent the formation of FAc **15**. Clearly the inhibition must occur after the fluorination. The identity of these compounds was of great importance to understanding the biological fluorination. It was possible to obtain a sample of one of these fluorinated compounds (compound B) by preparative HPLC using UV detection. ^{1}H NMR and ^{19}F NMR analysis were consistent with 5'-fluoro-5'-deoxyadenosine (5'-FDA) **83**. However both MALDI-TOF mass spectrometry and GC-MS analysis (after derivatisation with MSTFA) gave $[M+H]^+$ peaks that were one mass unit greater than that expected for 5'-FDA **83**. After an experiment in H_2O^{18} and based on the results from the mass spectrometry it emerged that the molecule isolated was 5'-fluoro-5'-deoxy-inosine (5'-FDI) **86**. Unfortunately, at that time a synthetic standard was not available and thus it was not possible to confirm this unambiguously. Confirmation of the identity of 5'-FDI

constitutes part of this thesis.



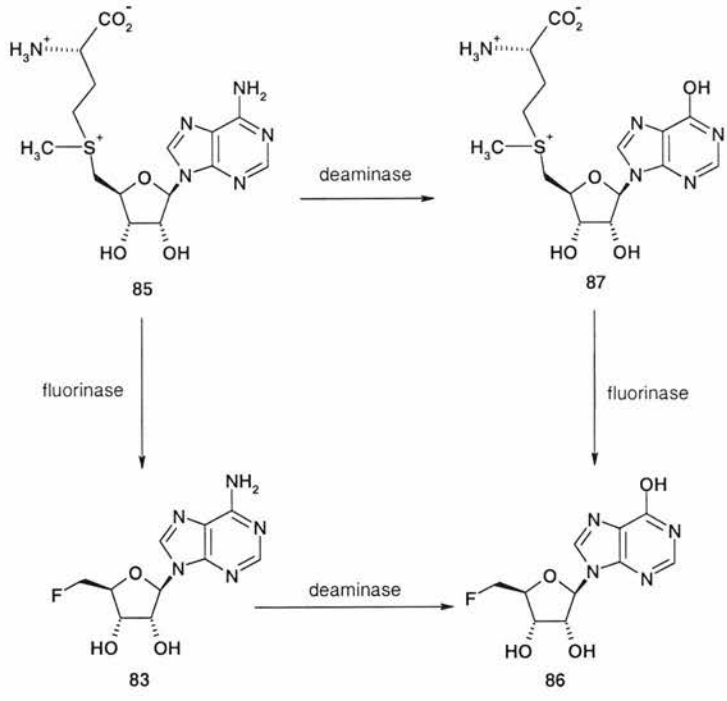
86

1.7.4 Proposed mechanism of fluorination in *S. cattleya*

The isolation of 5'-FDI **86** was not anticipated by the working hypothesis as fluorination was expected to generate 5'-FDA **83**. However, the production of 5'-FDI **86** within the cell free extract (CFE) can be accounted for by the presence of a deaminase enzyme (Scheme 1.11).

If 5'-FDI **86** and not 5'-FDA **83** was the direct product of the fluorination enzyme then SAM **85** would have to undergo deamination to yield **87**, prior to its acceptance as a substrate for the fluorination enzyme. This seems unlikely as SAM **85** is not known to be metabolised in this way. Furthermore, re-incubation of what was believed to be 5'-FDI **86** isolated from the cell free extract, showed that the molecule would not support the biosynthesis of any other fluorinated molecules.

Therefore, the more plausible explanation for the accumulation of 5'-FDI **86** is based on the original hypothesis that SAM **85** is the substrate for the fluorination which would give 5'-FDA **83**. 5'-FDA could then undergo deamination to produce the shunt metabolite 5'-FDI **86**. This pathway seems most likely, particularly as the enzymatic deamination of adenosine to generate inosine is a well documented process in metabolism.[109]



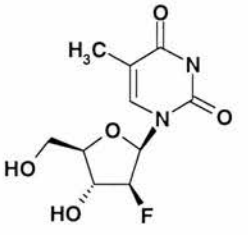
Scheme 1.11 SAM metabolism in the CFE in the presence of fluoride ion.

2 The role of 5'-FDA and 5'-FDI during fluorometabolite biosynthesis

2.1 The synthesis and general properties of fluorinated purine nucleosides

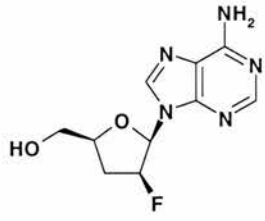
2.1.1 Purine nucleosides fluorinated at C-2', C-3' and C-4'.

In 2000 a review by Pankiewicz reported that 362 nucleoside structures had been prepared that contained a fluorine atom on the furanose moiety of the nucleoside.[110] Of these molecules 278 consisted of one or two fluorine atoms at the C-2' position (238 mono, 40 di-fluorinated). Some 39 derivatives were fluorinated at C-3' and a further 13 at both the C-2' and C-3' positions. Only two analogues were reportedly fluorinated at the C-4' carbon. Finally, 42 nucleosides were identified that carried a fluorine atom at the C-5' position. The majority of the fluorinated nucleosides reported are pyrimidines and they include important pharmaceutical products such as FMAU[111] **88** a potent antiviral agent.

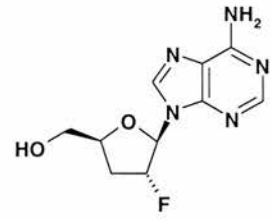


88

Since the discovery that the inclusion of a 2'(β)-fluorine atom onto the furanose ring of a nucleoside can dramatically alter its biological activity there has been considerable interest in this area. Such a fluorine substitution can increase both the chemical and metabolic stability of the molecule. The electron-withdrawing nature of the fluorine renders the 2'-fluoro-2'-deoxy-nucleosides more stable to chemical and phosphorylase catalyzed hydrolysis. This has been exploited to great effect in the development of potential anti-HIV drugs such as 9-(2,3-dideoxy-2-fluoro-β-D-*threo*-pentofuranosyl)adenosine **89** (β-FddA, Lodenosine).[112]



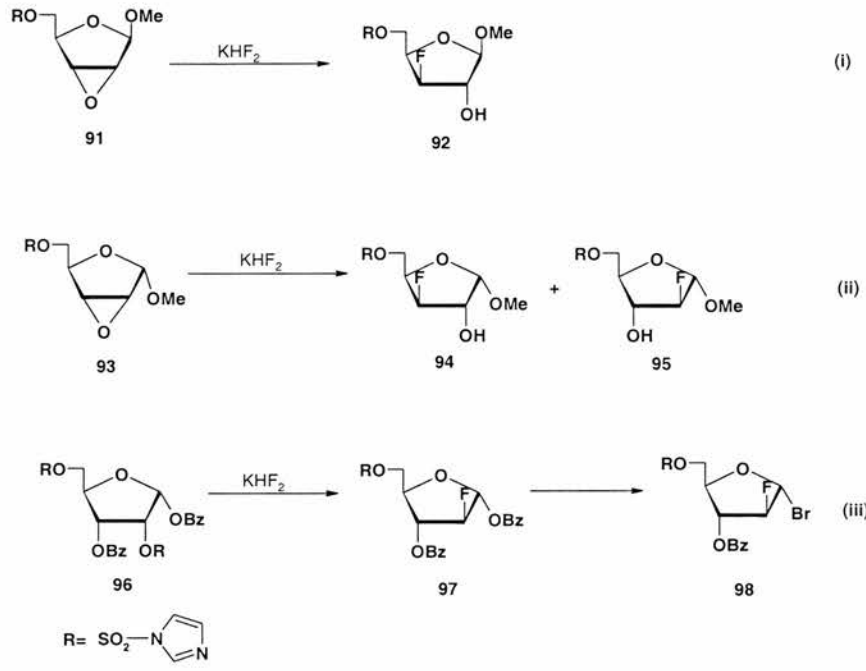
89



90

The inclusion of a fluorine atom at the C-2' (or C-3') position also imparts strong stereoelectronic effects that can alter the conformation of the sugar ring.[113,114] The synthesis of these compounds requires some consideration. The fluorine atom cannot be introduced by the direct displacement of a good leaving group at the C-2' position. This is because in the ribo configuration, the fluoride ion cannot attack the C-2' position from the β-face due to the steric effect of the base. The C-3' position does not suffer from this steric constraint and the fluorination of 2,3-anhydro-adenosine generally results in exclusive formation of 3'(β)-fluoro-3'-deoxy-adenosine.[115] It is possible to prepare the corresponding 2'(α)-fluoro-nuclosides such as α-FddA **90** by direct displacement of suitable leaving group at the C-2' position.[116,117] In this case the fluoride ion attacks from the α-face of the sugar and the nucleophile is not effected by the base.

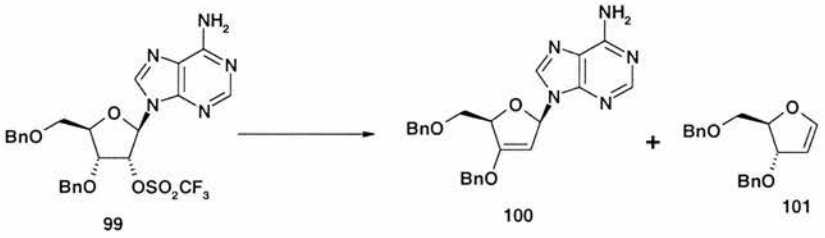
Therefore the original synthetic strategy to prepare 2'(β)-fluoro-nucleosides involved the preparation of a 2'(β)-fluoro-sugar which could then be activated at the anomeric position (C-1') and coupled to the appropriate base using a glycosylation reaction. However the problems associated with nucleophilic attack favouring the C-3' over C-2' position persisted in the preparation of 2'(β)-fluoro-sugars. An example of this is shown for the attempted fluorination of methyl 2,3-anhydro-5-O-benzyl-β-D-riboside **91** with KHF_2 which yielded only the unwanted C-3' fluoro isomer **92** (Scheme 2.1 (i)).[118]



Scheme 2.1 Effects of riboside confirmation on the regiochemistry of fluorination.[112,116,119]

It was found that fluorination of the corresponding α-riboside **93** afforded a mixture of the desired product **95** and the unwanted C-3' fluoro-sugar **94** (Scheme 2.1, (ii)).[116] This work was further improved by a team of researchers at Bristol-Myers who developed a more efficient route to the activated 2'(β)-fluoro-sugar **98**, a key intermediate that they utilised for the preparation of several fluorinated nucleosides. The key step involved the nucleophilic displacement of 2'-imidazolylsulfonate from **96** with KHF_2 to give a desired 2'(β)-fluoro-sugar. (Scheme 2.1, (iii)).[112] Interestingly, the reaction was unsuccessful when tetrabutylammonium fluoride (TBAF) or an alternative leaving group such as a 2'-triflate or 2'-mesylate was utilised. This approach offered easy access to 2'(β)-fluoro-sugars and subsequently led to an increase in research carried out to investigate the properties of 2'-fluorinated nucleosides. However, much of the research has focused on pyrimidine[110,111] rather than purine based nucleosides, because the glycosylation reaction required in the synthesis the purine nucleosides does not proceed efficiently.[116, 120,121] To address this problem Pankiewicz *et al.* developed a clever method that could be used introduce the

fluorine atom directly into a suitably functionalised adenosine.[122] They noted that the direct nucleophilic attack of a leaving group at C-2' such as a triflate, gave rise to elimination rather substitution products (Scheme 2.2).[123] This was attributed to the electro-negative triflate substituent at the C-2' forcing **102** to assume the C-3' *endo* conformation (Figure 2.1).[124] In such a conformation the triflate and the hydrogen at C-3' are in a *trans di-axial* configuration and are thus perfectly set up to facilitate an elimination reaction.



Scheme 2.2 Attempted nucleophilic fluorination of **99** results in the formation of elimination products **100** and **101**.[123]

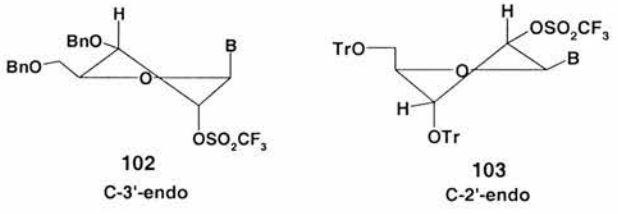
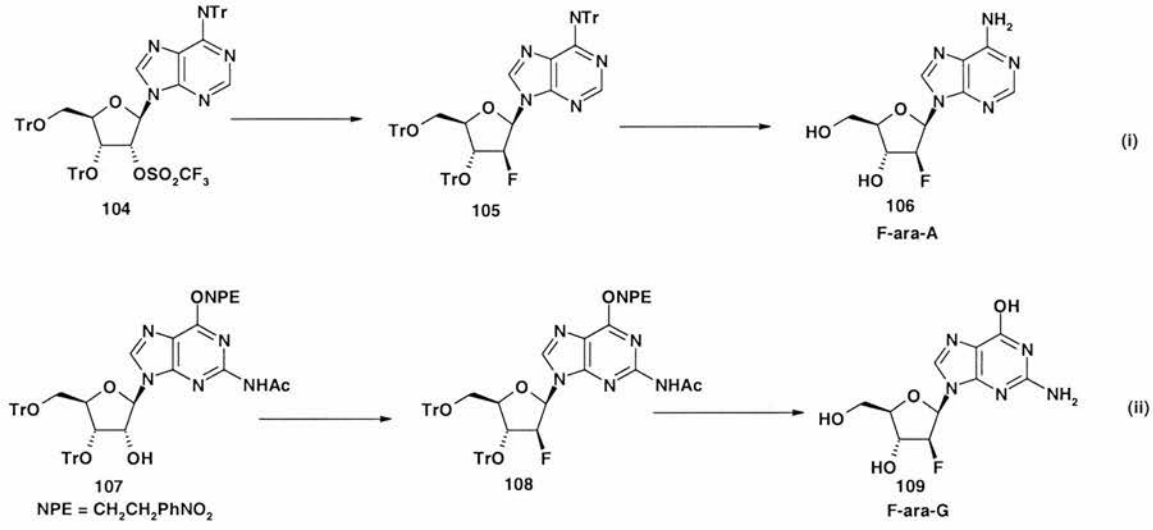


Figure 2.1 An increase in steric bulk favours a shift from a C-3'-*endo* to a C-2'-*endo* conformation.

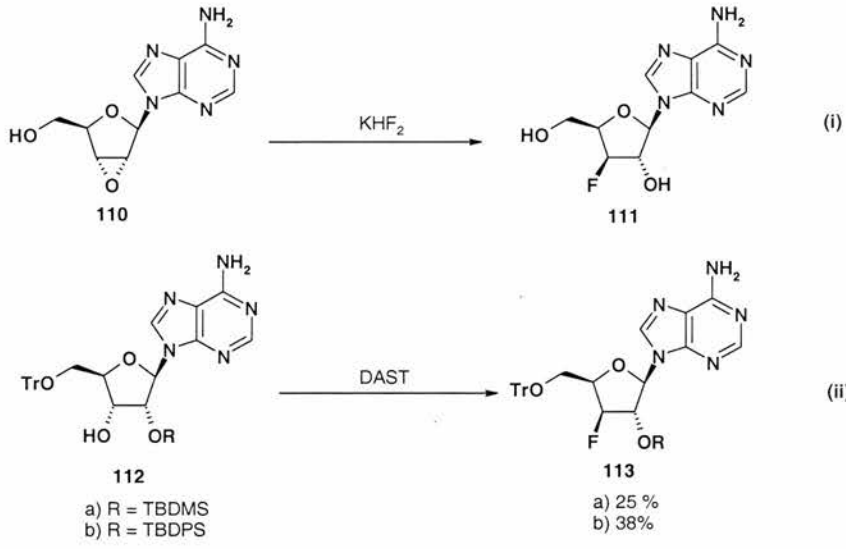
Pankiewicz *et al.* reasoned that if the conformation of the ring could be shifted to favour a C-2'-*endo* setup then nucleophilic displacement of the triflate might be possible. They envisaged that the shift in the conformation could be induced by utilising bulky protecting groups at the C-3' and C-5' positions. Their hypothesis was confirmed when the fluorination of **104** was achieved using tris(dimethylamino)sulfur (trimethylsilyl)difluoride (TASF) to afford the 2'-fluoro-2'-deoxy-nucleoside **105** albeit in a low yield (30%). De-protectection of **105** gave the nucleoside 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)adenosine (F-ara-A) **106** (Scheme 2.3 (i)). The same research group have reported higher fluorination yields with diethylaminosulfur trifluoride (DAST).

The resulting fluorinated product **108** was then used as an intermediate in the synthesis of the nucleoside 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine (F-ara-G) **109** which has been shown to exhibit selective T-cell toxicity (Scheme 2.3 (ii)).[125]



Scheme 2.3 Preparation of F-ara-A **106** (i) and F-ara-G **109** (ii) by the direct fluorination strategy developed by Pankiewicz *et al.*[122,125]

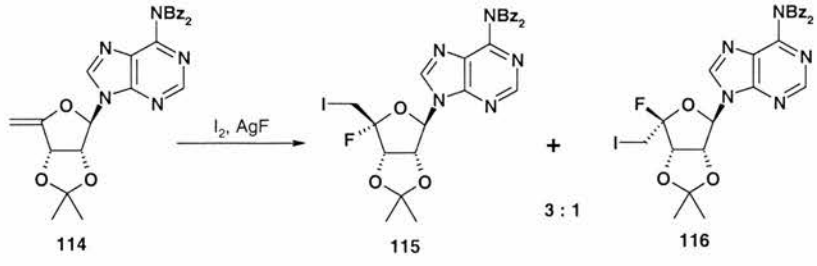
The preparation of a 3'-deoxy-3'-fluoro-β-nucleoside **111** can achieved with relative ease by ring opening of the 2',3'-anhydro-adenosine **110** (Scheme 2.4 (i)).[115] The preparation of 3'-(α)-adenosine and its derivatives is more laborious but a variety of methods to prepare these compounds have been reported.[118,126,127] Many of the early synthetic methods to prepare 3'-fluoro-nucleosides involved the coupling of a 3'-fluoro-sugar to the appropriate base.[119] Recently the direct fluorination strategy has been applied to various suitably protected adenosine molecules such as **112**. Battistini *et al.* showed that the free 3'-OH group of **112** could be converted to fluorine with DAST (Scheme 2.4 (ii))[128] however, these DAST reactions rarely exceed a reaction yield of 40%.



Scheme 2.4 Preparation of a 3'(β)-fluoro-nucleoside **111** (i) and a 3'(α)-fluoro-nucleoside **113** (ii) by direct fluorination of appropriate nucleosides.

Biological testing has revealed that the purine based nucleoside 3'-fluoro-3'-deoxy-adenosine was more active and cytotoxic as an antiviral agent than all of the other 3'-fluoro-3'-deoxy-nucleosides that have been examined to date.[110]

The only naturally occurring fluorinated nucleoside to have been reported is the 4'-fluoro-nucleoside, nucleocidin **61** isolated from the bacterium *S. calvus*.[78] This is particularly noteworthy because the synthesis of 4'-fluoro-nucleosides is relatively rare. During the total synthesis of **61,** the fluorine atom was incorporated into the C-4' position by treatment of **114** with iodine and silver fluoride as illustrated in Scheme 2.5.



Scheme 2.5 Introduction of the 4'-fluorine atom C-4' during nucleocidin synthesis.[83]

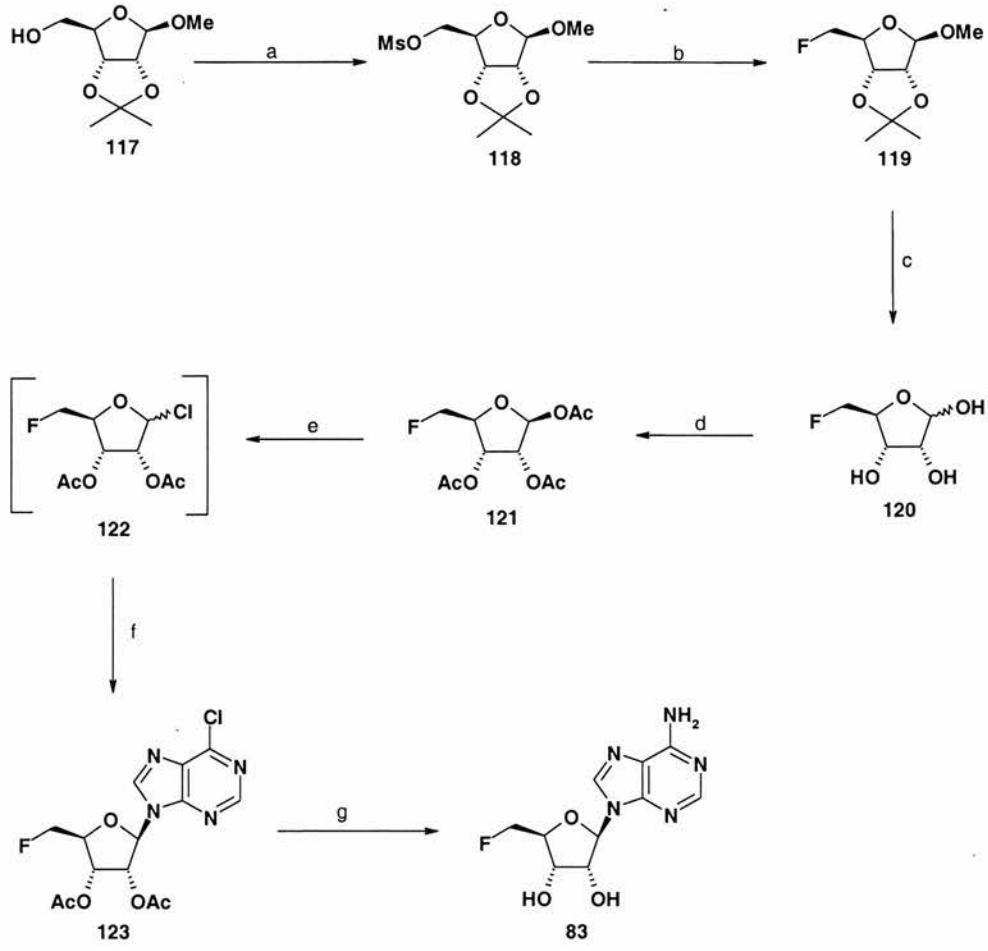
Fortuitously, the reaction favoured the formation of the desired product **116** in a 3:1 ratio.[83] The synthesis of 4'-fluorinated nucleosides is complicated by the lability of these

compounds due to acid-catalysed glycosidic bond cleavage.[129] This is presumably the main reason that few 4'-fluorinated molecules have been explored as pharmaceutical agents.[130,131] Interestingly this lability was exploited for 5'-deoxy-4'-5-difluorouridine, which is the pro-drug to the anti-tumor agent 5'-fluorouracil.[132]

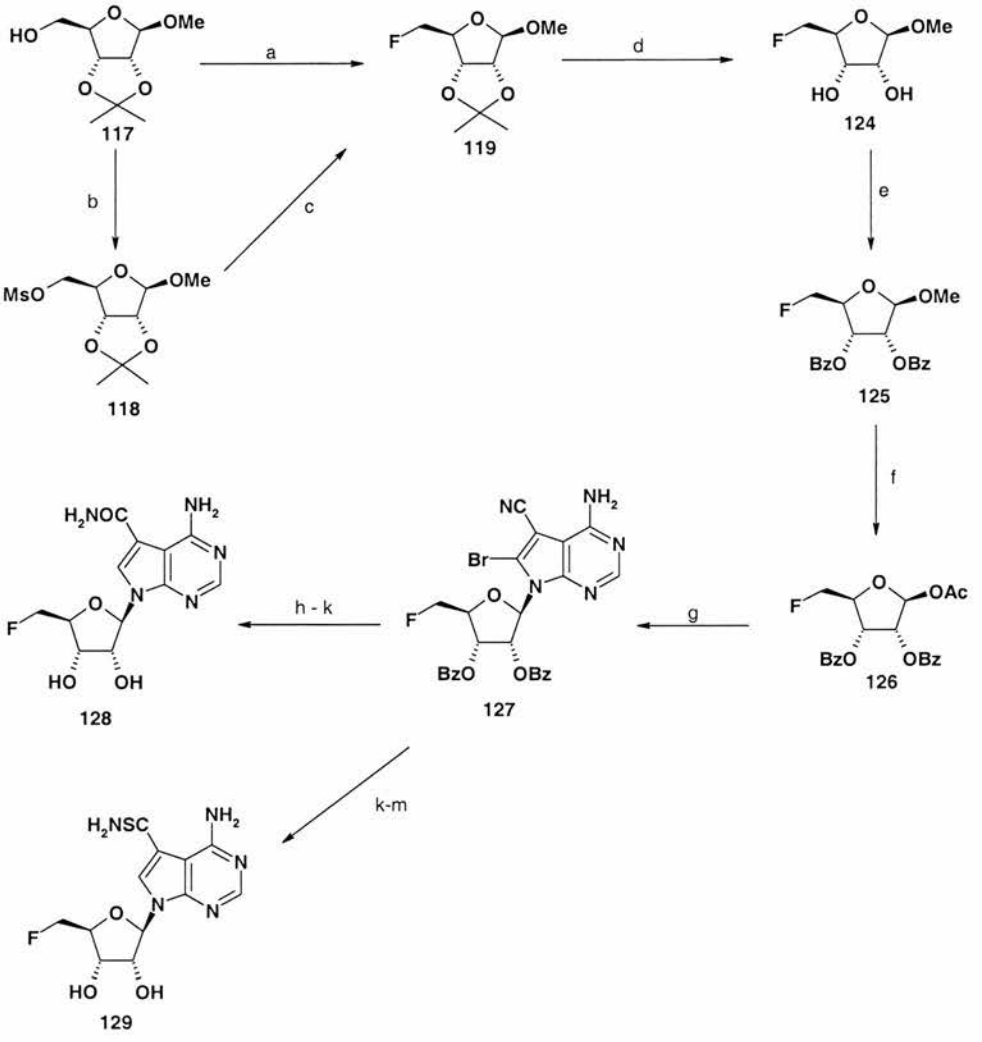
2.1.2 C-5' fluorinated purine nucleosides

A search of the literature revealed only a handful of nucleosides that were mono-fluorinated at the C-5' position and there was only one reported synthesis of our desired synthetic target 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83**.[133] The overall yield for the synthetic sequence was 4% and this does not include the additional step that is required to prepare the starting material **117**. The only other 5'-fluorinated nucleosides that have been reported are derivatives of toyomycin **128** and the antibiotic sangivamycin **129** that were synthesised in an attempt to produce molecules that were resistant to phosphorylation.[134] All of these 5'-fluoro-nucleosides **83**, **128** and **129** have been prepared *via* synthetic routes that incorporate the fluorinated sugar, 5'-fluoro-5'deoxy-2,3-*O*-isopropylidene-β-D-ribofuranoside **119** as the key intermediate (Schemes 2.6 and 2.7).

In the synthesis reported by Kissman and Weiss (Scheme 2.6) the fluorine atom was introduced into the C-5' position by nucleophilic displacement of a mesylate group using anhydrous KF.[135] The yield for the reaction is moderate (68%) but the reaction conditions required to carry out the transformation are fairly harsh. Sharma *et al.* showed that the same transformation could be achieved in a much better yield (90%) and under milder conditions when TBAF[135] was employed as the fluorinating reagent. Sharma *et al.* also introduced the fluorine atom directly onto the C-5' position by using DAST[135] as a fluorinating agent (Scheme 2.7, step a). The reaction yield was lower than that achieved with TBAF even when the required mesylation step is taken into consideration.



Scheme 2.6 The synthesis of 5'-FDA **83** reported by Kissman and Weiss.[133] Reagents and conditions: a) Pyridine / MsCl, 3 °C, 71% b) KF / MeOH, 160 °C, 68% c) 0.02 M H_2SO_4, 100 °C, 100% d) Pyridine / acetic anhydride, 21% e and f) Acetyl chloride. HCl /ether, chloro-mercuric-6-chloropurine, 54% g) MeOH / NH_3, 100 °C, 64%.



Scheme 2.7 The synthesis of 5'-fluoro-nucleosides **128** and **129** reported by Sharma *et al.*[134] Reagents and conditions: a) DAST, benzene, 80 °C, 3 h, 35% b) MsCl, pyridine, 0 °C, 24 h, 78% c) TBAF MeCN, reflux, 24 h, 90% d) Formic acid (60%) (crude) e) Pyridine, benzoyl chloride, 0 °C, 95% f) Acetic acid / acetic anhydride, 78% g) 4-amino-6-bromo-5-cyanopyrrolo[2,3-d]pyrimidine, hexamethyldisilazane, TMSTF, 77% h) NEt_3, Pd charcoal / H_2, 90% j) NH_3/ MeOH, 91% k) NH_4OH, H_2O_2, 75% l) Hydrogen sulfide / pyridine m) NH_3/ MeOH, 27% (for two steps).

The synthetic strategy followed by both Kissman (Scheme 2.6) and Sharma (Scheme 2.7) involved the incorporation of a fluorine atom into a suitably protected sugar molecule. The 5'-fluoro-sugar is then subsequently activated at the anomeric carbon and coupled to the desired base using a glycosylation reaction. This synthetic strategy allows a reasonable degree of freedom to generate a variety of 5'-fluoro-nucleosides as the glycosidic coupling

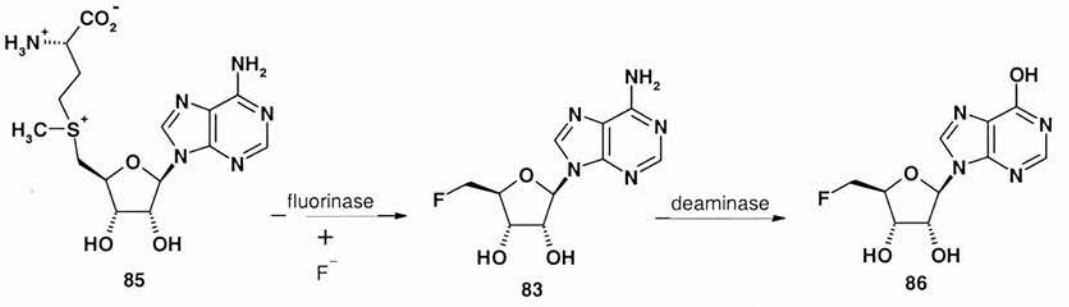
can be carried out between the 5'-fluoro-sugar and a range of bases. However, the disadvantages of these procedures are that both require a minimum of seven synthetic steps and the coupling reaction used to generate the nucleoside is often problematic. The condensation reaction between the sugar and purine moieties, although well documented, is often low yielding and can lead to the generation of unwanted α-nucleosides. Despite these problems Sharma *et al.* reported that the generation of **127** occurred to furnish only the β-nucleoside in a good yield. It is worth noting, however, that the condensation reaction en-route to the synthesis of 5'-FDA (Scheme 2.7) requires the use of a different base. The method used to achieve this condensation as outlined by Kissman (Scheme 2.6, steps e and f) proceeds in a moderate yield (54%) despite the use of toxic mercury based reagents. It should be possible, and is desirable, to remove the need for such toxic reagents by utilising a more modern synthetic glycosylation procedure.[136]

2.2 Synthesis of 5'-FDA and 5'-FDI

2.2.1 Introduction

At the start of my work, *S*-adenosyl-L-methionine (SAM) **85** was implicated as the substrate for the fluorination enzyme (Chapter 1). It was proposed that the fluorinase mediated a reaction between inorganic fluoride and SAM **85** to yield 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83**. Unfortunately, attempts to isolate 5'-FDA **83** from the crude cell-free extract (CFE) were unsuccessful. It was possible however to isolate 5'-fluoro-5'-deoxy-inosine (5'-FDI) **86** (Chapter 1) the inosine analogue of 5'-FDA **83**. As discussed previously, the isolation of 5'-FDI **86** provided substantial evidence in support of the proposed mechanism of enzymatic fluorination. This was based on the assumption that the most likely pathway to 5'-FDI **86** is by enzymatic deamination of 5'-FDA **83**.

In order to confirm the nature and product of the enzymatic fluorination reaction and to investigate the proposed conversion of 5'-FDA **83** to 5'-FDI **86** (Scheme 2.8) synthetic samples of both 5'-FDA and 5'-FDI were required. In addition, to allow the correct identification all of the fluorinated metabolites detected by ^{19}F NMR analysis, synthetic samples of the known fluorinated metabolites 4-FT **16** and fluoroacetaldehyde **49** were also required. The preparation of FAc **15** was not required as it is commercially available. If 5'-FDA **83** could be confirmed as the product of the enzymatic fluorination reaction, then an assay for enzyme purification could be developed.



Scheme 2.8 Relationship between SAM **85**, 5'-FDA **83** and 5'-FDI **86** in *S. cattleya* cell free extracts.

2.2.2 Synthesis of 5'-fluoro-5'-deoxy-adenosine (5'-FDA)

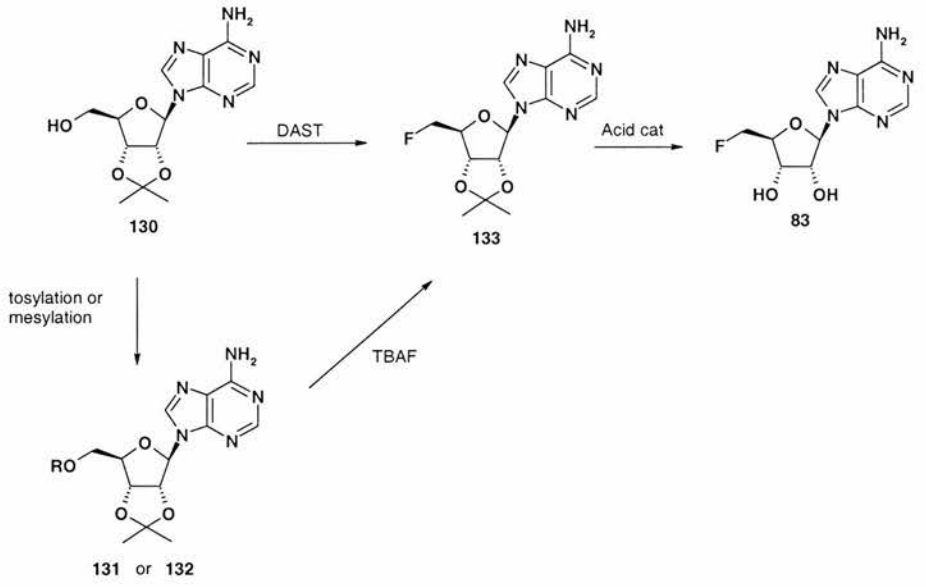
Having considered the literature it is clear that a method incorporating a combination of the procedure detailed in Schemes 2.6 and 2.7 could be used to prepare 5'-FDA **83**. However, a more attractive route would involve introduction of the fluorine atom into C-5' of a suitably protected adenosine molecule in which the glycoside bond is already formed. This would circumvent any problems that may arise from the sugar - base coupling reaction.

Modification of the C-5' of adenosine is an extensively covered topic in the nucleoside literature and the reaction is usually carried out with the 2'-OH and 3'-OH groups protected. This limits unwanted side reactions that can occur at these secondary hydroxyl groups and leads to the adenosine being more soluble in organic solvents. There are

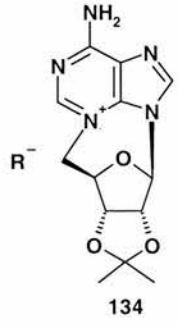
numerous ways in which the *trans* 2',3' diol in adenosine can be protected[137] but it was felt that the most convenient approach would be to utilise the isopropylidene group.

2',3'-Isopropylidene-adenosine **130** can be prepared from adenosine using a standard protection protocol (ie. acetone, 2,3-dimethoxypropane with an acid catalyst)[138] but its wide spread application in the preparation of modified nucleosides has resulted in **130** being commercially available. From **130** there are two potential routes which could be used to introduce the C-5' fluorine atom. The first strategy involves the direct fluorination of the free 5'-OH using DAST to generate 2',3'-isopropylidene-5'-fluoro-5'-deoxy-adenosine (5'-FIA) **133** an unknown compound. If the fluorination of the free 5'-OH group using DAST is not achievable then a second route can be investigated. This method would initially require the activation of the 5'-OH group in **130**, followed by fluoride ion displacement e.g. with TBAF to give **133**. Acidic deprotection[139] of **133** should then afford the target 5'-FDA **83** (Scheme 2.9).

It is somewhat surprising that the latter method utilising TBAF had not previously been explored given that nucleophilic displacement of an activated C-5' hydroxyl or halide has been used to introduce a variety of functionalities.[140,141] The reason for this could be due to the reported problem of intramolecular displacement in which N-3 of adenine attacks C-5' ejecting the leaving group.[142] This internal displacement reaction leads to the formation of unwanted 3',5'-cyclo-nucleosides **134**.[143]



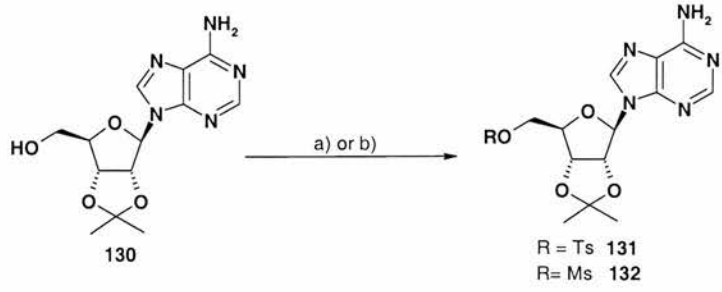
Scheme 2.9 Synthetic routes to 5'-FDA **83** avoiding the problematic glycosylation reaction.



However, the conditions required to generate these compounds are normally relatively forceful requiring a prolonged reaction time at reflux in a high boiling solvent such as DMF.[144] Therefore, it was anticipated that fluorination under relatively mild conditions would limit or possibly exclude the formation of 3',5'-cyclo-nucleosides. Also, it is has been reported that introduction of a benzoyl (Bz) protecting group on to the free amine at C-6 drastically reduces the nucleophlicity of the N-3 atom and thus suppresses the formation of these 3',5'-cyclo-nucleosides.[145] This strategy could clearly be employed if required.

A first attempt to prepare **133** involved the direct fluorination of the free 5'-OH of the commercially available **130** using DAST. The reaction was monitored by ^{19}F NMR spectroscopy but even after a prolonged reaction time at 25 ^{o}C (+ 48 h) no fluorinated

products of any kind were observed. No attempt was made to run the reaction at a higher temperature because based on the work carried out by Sharma *et al.* (Scheme 2.7) it was felt that a fluorination strategy that utilised TBAF might be more rewarding. Such an approach would clearly require the activation of the 5'-OH. Both 2',3'-*O*-isopropylidene-5'-*O*-tosyl-adenosine[146] **131** and 2',3'-*O*-isopropylidene-5'-*O*-mesyl-adenosine[147] **132** were therefore prepared according to known protocols (Scheme 2.10).



Scheme 2.10 Reagents and conditions: a) TsCl, pyridine, 0 °C, 5 h, 66% b) MsCl, pyridine, 0 °C, 4 h, 73%.

Both the toyslation and mesylation reactions occurred to furnish the desired products in good yields and the analytical and spectroscopic data for the compounds were in full agreement with the literature. It is worth noting that in both cases the free amine at the C-6 position of the adenine base in **130** did not participate in, or complicate, the reactions and only the 5'-*O*-tosyl **131** and 5'-*O*-mesyl **132** compounds were isolated.

Tetrabutylammonium fluoride (TBAF) is available as its trihydrate (TBAF. $3H_2O$). Although there are procedures detailed to prepare anhydrous TBAF[148] there are problems associated with the stability of anhydrous TBAF and there is evidence that the use of anhydrous TBAF increases the formation of elimination products due to the increased basicity of dry fluoride ion.[149] Therefore, a trial fluorination using TBAF.$3H_2O$ was conducted. The fluorination of both **131** and **132** was carried out using 2.5 equivalents of TBAF.$3H_2O$ in refluxing acetonitrile (MeCN). After 16 h thin layer chromatography (TLC) analysis showed that in both cases all of the starting material had been consumed and that several new products had appeared. The solvent was removed to afford the crude reaction

mixture as a brown oil which was analysed by ^{19}F NMR spectroscopy prior to work up. The ^{19}F NMR spectra recorded in each case were identical -228.9 ppm (dt, ^{2}J 46.7 and ^{3}J 23.6 Hz) (Figure 2.2).

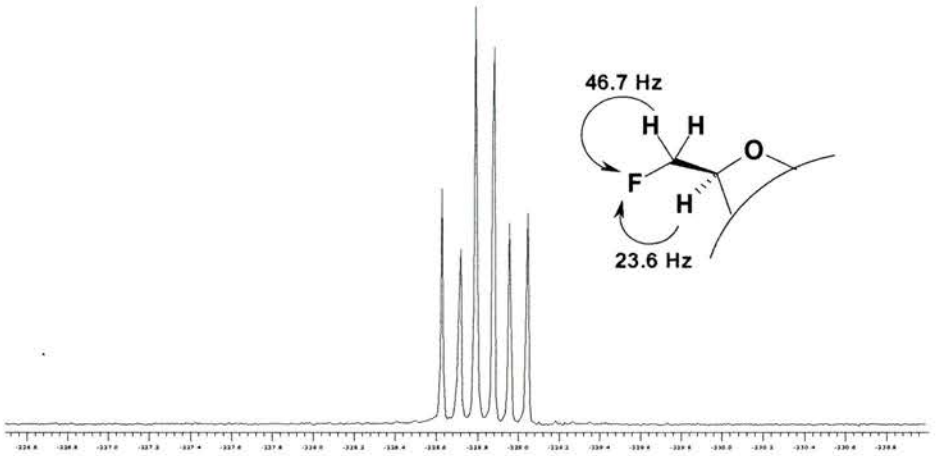
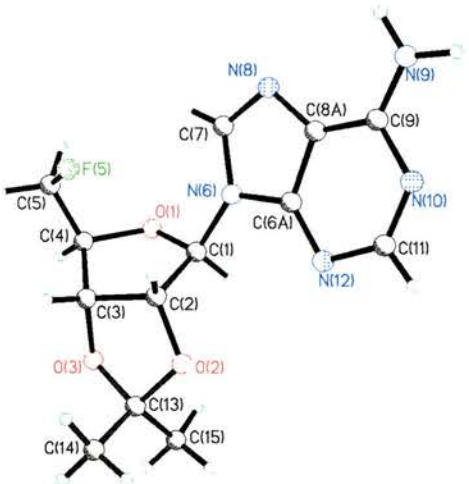
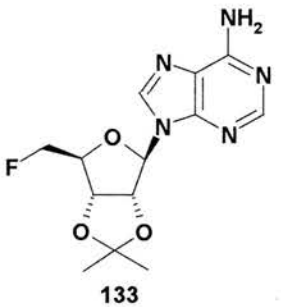


Figure 2.2 ^{19}F NMR (282 MHz; $CDCl_3$) spectrum of the crude reaction mixture from the fluorination of **131**.

Purification by column chromatography gave analytically pure material and the X-ray crystal structure determined for **133** is shown in Figure 2.3.



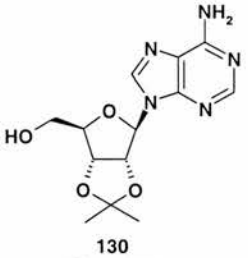


133

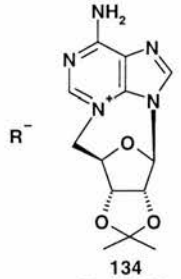
Figure 2.3 X-ray structure of **133**

Further investigation into the reaction conditions revealed that TBAF.$3H_2O$ could be reduced to 2 molar equivalents without a significant change in the reaction yield. This is beneficial to the process overall as the removal of by-products that arise from the

degradation of TBAF.$3H_2O$ can sometimes be problematical as they often separate into the organic phase. Extending the reaction beyond 6 h had little effect on the yield. During one attempted fluorination of the toyslate **131** a considerable amount of white precipitate was formed after the reaction had been heated to reflux overnight. The precipitate was not soluble in $CDCl_3$, but NMR analysis in D_2O indicated that two compounds were present. ES-MS of the sample showed two signals with masses of 290 and 308 consistent with compounds **130** and **134** respectively. The formation of these two compounds could arise from hydrolysis of the 5'-*O*-toysl group and displacement of the tosyl by adenine in an intramolecular reaction. It is interesting to note that the counter ion R^- for **134** is not TsO^- as no aromatic signals were apparent by ^1H NMR analysis. ^{19}F NMR analysis revealed that fluoride ion was present (s, -120.1 ppm) and it is therefore possible that it is acting as the counter ion in **134**.



130
Mw = 307



134
Mw = 290

The formation of these impurities may clearly be encouraged by the reaction being carried out at reflux. However when **131** was treated with TBAF.$3H_2O$ (2.5 eq) at ambient temperature and the reaction was left to run for 24 h the reaction yield remained moderate (46%). Thus lowering the reaction temperature did not have an obvious beneficial effect.

Cleavage of the iso-propylidene protecting group from **133** was accomplished by heating in dilute aqueous sulfuric acid (0.02 M). The reaction was followed by ^{19}F NMR spectroscopy and was stopped when the majority (>90%) of the starting material (-228.9

ppm, dt) had been converted to 5'-FDA **83** (-230.0 ppm, dt). The reaction was typically complete after heating for 4-5 hours at 90 °C.

Upon cooling the reaction was lyophilised and recrystallised from ethanol / water to afford 5'-FDA **83** in an excellent yield (85%). HPLC analysis showed that the nucleoside had a purity of greater than 95% (Figure 2.4).

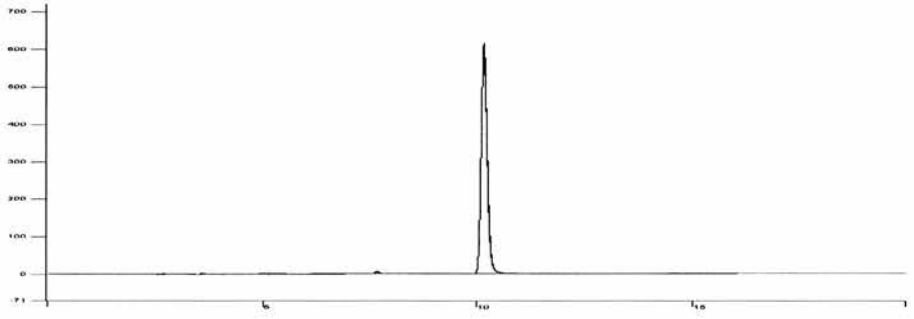
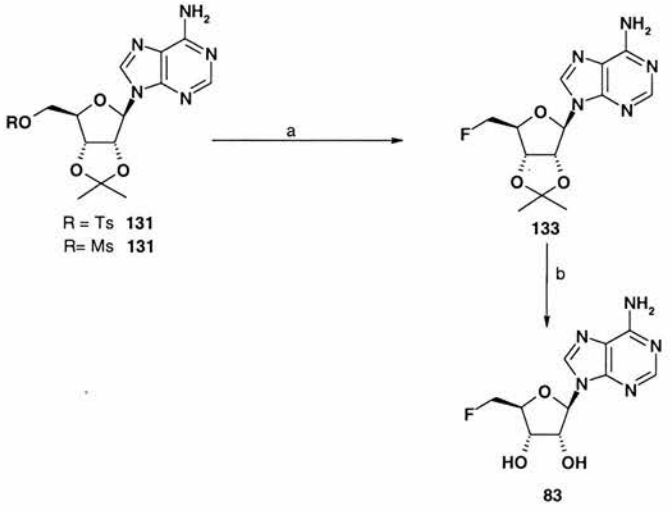


Figure 2.4 HPLC (C-18, UV detection, 0-20 min) analysis of synthetic 5'-FDA **83**.

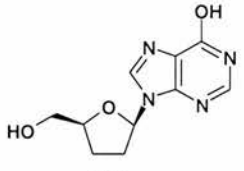
In summary the preparation of 5'-FDA **83** was achieved by a new protocol which involved the fluorination of suitably activated adenosines **131** and **132**. Removal of the protecting group from **133** afforded 5'-FDA **83** in three steps and in a total overall yield of 35%.



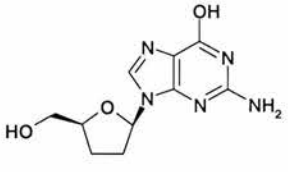
Scheme 2.11 Reagents and conditions: for R = Ms; a) TBAF.3H_2O, MeCN, reflux, 16 h, 46% and for R = Ts a) TBAF.3H_2O, MeCN, reflux, 16 h, 57% b) 0.02 M H_2SO_4, 100 °C, 85%.

2.2.3 Synthesis of 5'-fluoro-5'-deoxy-inosine (5'-FDI)

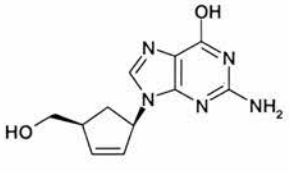
Nucleoside analogues have been shown to posses antitumor,[150] anti-inflammatory[151] and anti-viral[152] activities. These nucleosides have great therapeutic value in chemotherapy or as potential drug molecules for the treatment of viral infections such as AIDS.[153] Analogues of the various nucleoside classes for example adenosine, guanosine and inosine, all figure prominently as broad spectrum anti-viral agents.[154-156] The practical synthetic routes to some fluorinated adenosine analogues have already been discussed (Section 2.1). The synthesis of inosine and guanosine analogues can be achieved by hydrolysis of the corresponding adenosine (6-amino) or 6-chloro-adensoine derivatives under basic conditions. Unfortunately, these approaches are limited due to the inherent instability of many pharmaceutically important nucleoside analogues such as 2',3'-dideoxyinosine **135** (ddI).[157] This problem has been solved in part by employing a chemo-enzymatic strategy to the preparation of these sensitive molecules. To this end, the enzyme adenosine deaminase (ADA; EC 3.5.4.4) has gained popularity as an efficient catalyst to convert purine ribosides to their corresponding 6-oxopurine ribosides. The enzyme ADA has been successfully utilised in the synthesis of the anti-HIV compounds, 2',3'-dideoxy-guanosine **136** (ddG)[158] and Carbovir[159] **137**.



135



136



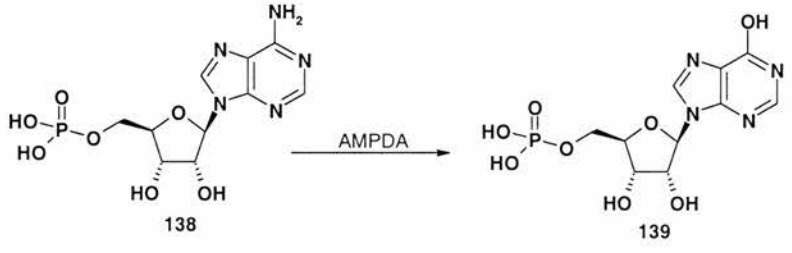
137

It was felt that a similar enzymatic approach towards the synthesis of 5'-FDI **86** presented an attractive route for two main reasons. Firstly, 5'-FDI **86** has not previously been synthesised and the stability of the molecule was unknown, thus an enzymatic synthesis

offered a mild approach. Secondly, the substrate required for the enzymatic transformation is 5'-FDA **83** a compound which has already been prepared and thus no further synthetic chemistry is required.

Accordingly the nucleosides 5'-FDA **83** (100 μl, 10 mM) and adenosine (100 μl, 10 mM) were both treated with ADA (Sigma, calf intestinal mucosa, 1 mg in 1ml ultra-pure water) at 25 °C. Adenosine was shown to be fully converted to inosine within 2 h by HPLC analysis but 5'-FDA remained completely unchanged even after 48 h. This result supports the work of Ciuffreda[160] who reported that although some substrate specificity is displayed by these enzymes at the C-2' and C-3' position, a free 5'-OH group appeared to be essential for catalytic activity. The absence of any deamination of 5'-fluoro-5'-deoxy-adenosine with ADA is consistent with these observations and highlights a limitation of this approach.

In order to overcome the problems associated with this limited substrate range Margolin *et al.*[161] developed the use of 5'-adenylic acid deaminase from *Aspergillus sp.* (AMPDA; EC. 3.5.4.6) as a practical alternative to ADA. AMPDA mediates the deamination of 5'-AMP **138** to 5'-IMP **139** (Scheme 2.12) and the enzyme is inexpensive and commercially available because of its application in the food industry.



Scheme 2.12 Enzymatic deamination of 5'-AMP **138** to 5'-IMP **139** mediated by AMPDA (EC. 3.5.4.6).

AMPDA was shown to have a much broader substrate tolerance than ADA and did not appear to require a free 5'-OH for catalytic activity a factor of particular importance in terms of the preparation of 5'-FDI **86**. Accordingly, 5'-FDA **83** (50 mg) with AMPDA (10

mg) in ultra-pure water (10 ml) at 25 °C showed complete conversion to a new compound within 6 h. Upon completion of the reaction the enzyme was simply denatured by heating (100 °C / 3 min) and the protein removed by centrifugation. Lyophilisation of the sample afforded the product **86** as an analytically pure white powder in almost quantitative yield (98%) and with a high degree of purity (>95%) as judged by HPLC (Figure 2.5 (ii)). It was found that it was also possible to carry out the enzymatic reaction on a larger scale (up to 1g of substrate) without any detrimental effects on either yield or product purity.

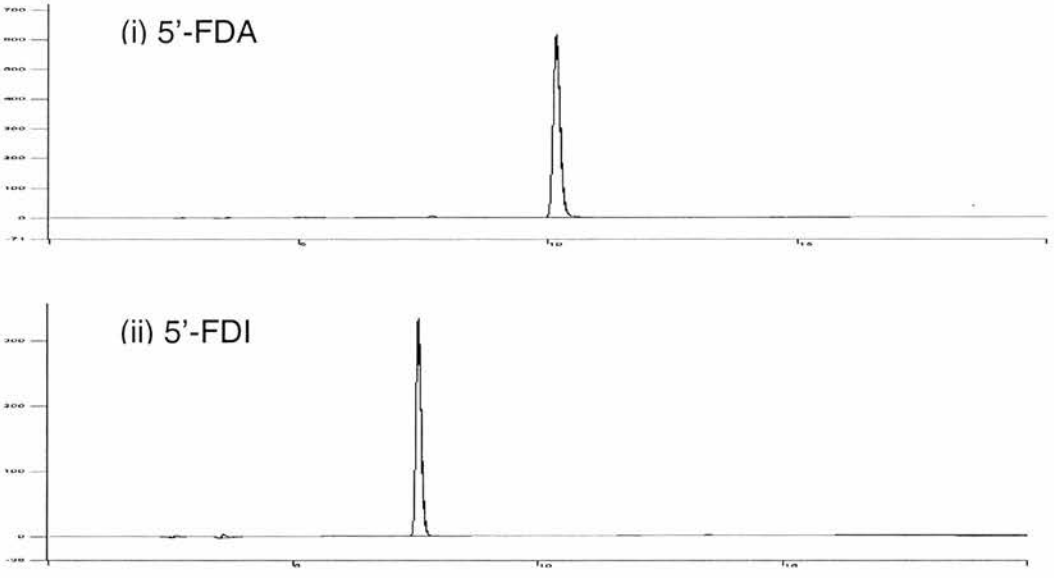


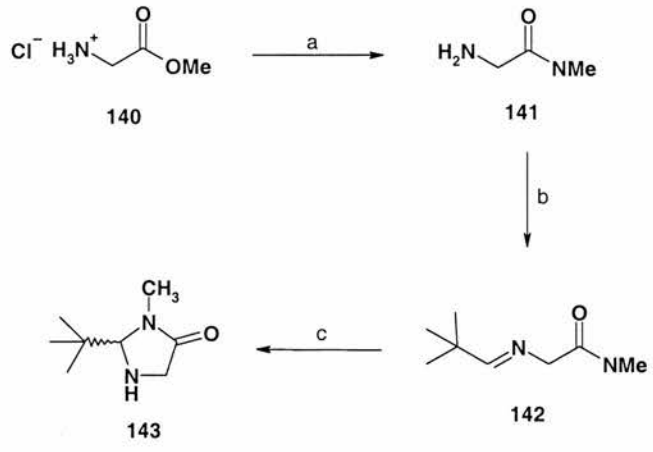
Figure 2.5 HPLC (C_{18}-UV detection, time shown is 0-20 min) analysis showing the conversion of 5'-FDA **83** (i) to 5'-FDI **86** (ii) mediated by AMPDA.

2.3 Synthesis of 4-fluorothreonine (4-FT) and fluorocetaldehyde

2.3.1 Synthesis of 4-fluorothreonine (4-FT)

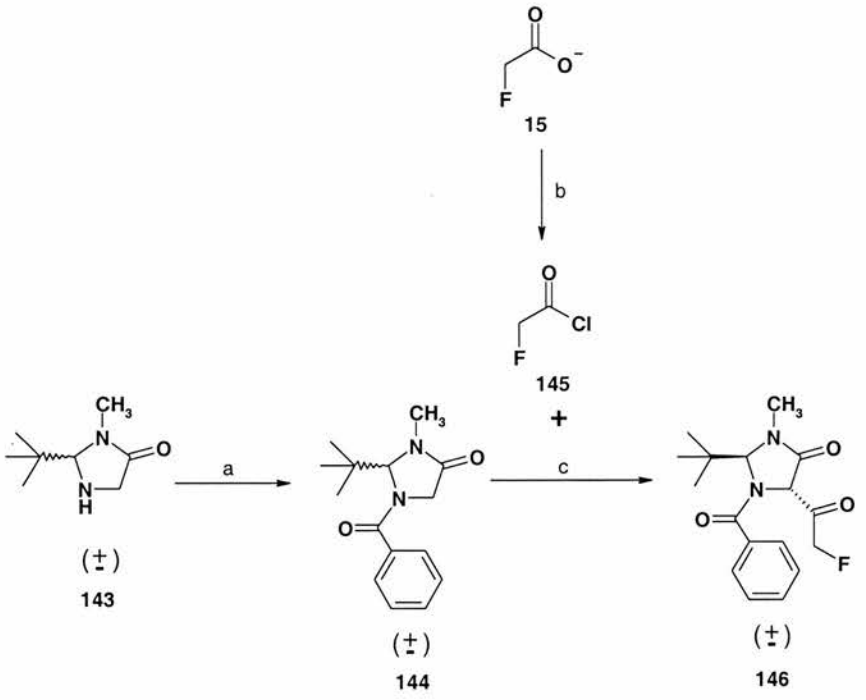
The synthesis of (*rac*)-4-fluorothreonine (4-FT) was carried out using the synthetic protocol developed previously by O'Hagan and Amin.[162] The method utilised a modification of Seebach's imidazolidinone amino acid methodology. The (*rac*)-imidazolidinone **143** was prepared in two steps from glycine methyl ester hydrochloride **140** *via* the un-isolated *N*-methylamide **141**.[163] The Schiff base that was produced upon the

reaction of **140** with pivaldehyde was cyclised using methanolic HCl to produce imidazolidinone **143**.[164]



Scheme 2.13 Reagents and conditions: a) 8M EtOH / $MeNH_2$, R.T, 15 h b) Pivaldehyde, NEt_3, DCM, reflux, 10 h, 78 % yield for two steps c) HCl_g sat.MeOH, 0 °C to R.T, 4 h, 76%.

Subsequent treatment of **143** with benzoyl chloride generated 1-benzoyl-2-(tert-butyl)-3-methyl-imidizolidinone **144**. Condensation of **144** with fluoroacetaldehyde **49** using the standard Seebach approach was not possible due to the difficulty of preparing dry fluoroacetaldehyde **49**. However, the modified strategy involved condensation with fluoroacetyl chloride **145** instead of fluoroacetaldehyde **49**. Accordingly fluoroacetyl chloride was prepared from sodium fluoroacetate and phthaloyl chloride and was reacted with **144** in the presence of a base to afford **146** in a moderate yield after purification.



Scheme 2.14 Reagents and conditions: a) Benzoyl chloride, 1M NaOH, 83% b) Phthaloyl chloride (excess), 74% c) BuLi (2M hexane), HN*i*P$_2$, -78 °C, 54%.

The X-ray crystal structure of the (±) β-ketone **146** was in keeping with the results originally observed by Amin and O'Hagan. An *anti* relationship between the newly formed carbon bond at C-5 and the existing t-butyl group at C-2 was observed. This confirmed that only two out of the four possible isomers had been formed. Also a combination of the fact that the α-carbon is tetrahedral and the presence of a t-butyl group prevents epimerisation at the otherwise labile α-carbon of the β-ketone **146**.

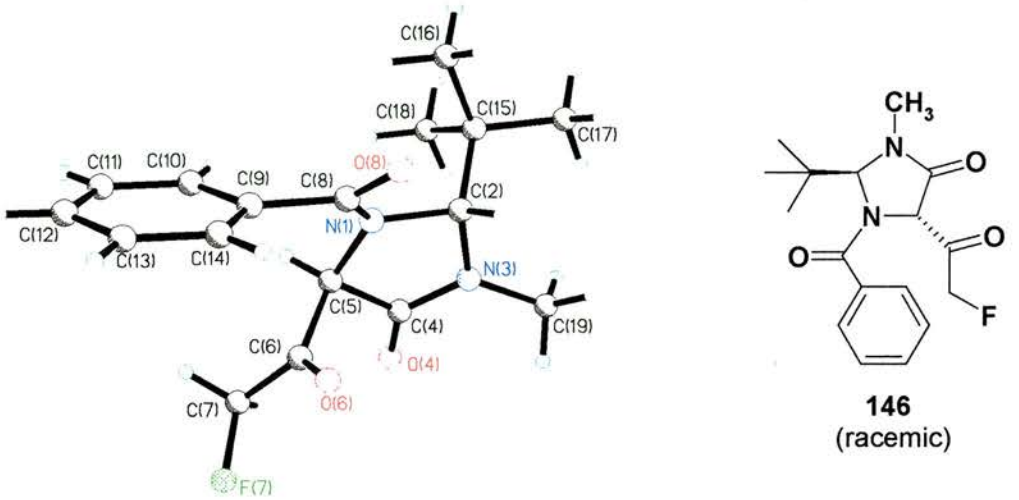
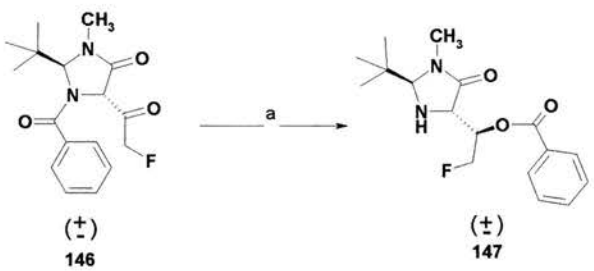


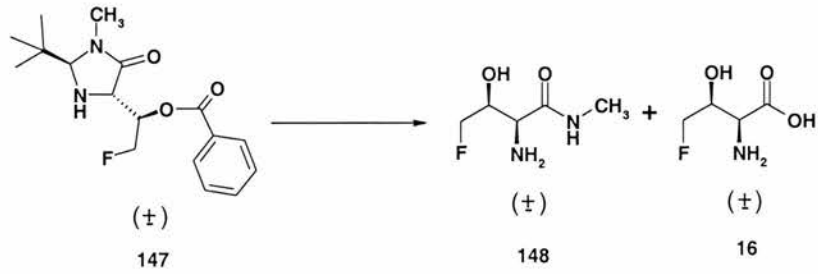
Figure 2.6 X-ray structure of (±)-β-ketone **146**

Reduction of the β-ketone **146** with an excess of $NaBH_4$ resulted in an intramolecular transacylation and the formation of ester **147**. This facile transacylation has previously been observed by Seebach for his systems.[164]



Scheme 2.15 Reagents and conditions: $NaBH_4$, MeOH, R.T, 5 mins (65%).

4-FT **16** was then released after hydrolysis of **147** in 10 M HCl at 100 °C for 72 h. ^{19}F NMR analysis of the crude product mixture indicated the presence of a single fluorinated compound corresponding to the expected shift and splitting pattern for (*rac*)-4-FT **16**. However ^{1}H NMR analysis also confirmed the presence of the partially hydrolysed product, 4-fluoro-methylamide **148**. The ratio of **148** to 4-FT **16** was determined to be approximately 3:1 based on the integration of the 2'-H protons of each product.

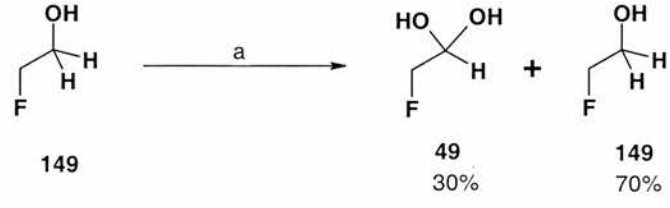


Scheme 2.16 Reagents and conditions: 10 M HCl / 100 °C / 72 h.

In order to afford complete cleavage of the amide stronger reaction conditions were employed. The crude product was redissolved in 10 M HCl and heated in a steel bomb at 100 °C for two separate periods of 24 h. After such time ^{1}H NMR analysis indicated complete conversion to 4-FT **16**.

2.3.2 Synthesis of fluoroacetaldehyde

The synthesis of fluoroacetaldehyde **49** was carried out using the previously described procedure as outlined in Scheme 2.17.[107] Fluoroethanol **149** was oxidised using PDC in DCM to generate fluoroacetaldehyde **49** which was collected as its hydrate by distillation. The method unfortunately does not oxidise all of the starting material and ^{19}F NMR analysis of the resultant fluoroacetaldehyde (-231.0 ppm, dt, $^{2}J_{F,H}$ 46.7 and $^{3}J_{F,H}$ 10.0 Hz) typically shows 70% residual fluoroethanol **149** (-224.5 ppm, tt, $^{2}J_{F,H}$ 47.6 and $^{3}J_{F,H}$ 32.1 Hz). A quantitative estimate of fluoroacetaldehyde can be determined by ^{19}F NMR analysis with a reference sample of fluoroacetate (FAc) **15** of known concentration.



Scheme 2.17 Reagents and conditions: a) PDC, DCM, reflux, 16 h, 30-40%.

2.4 Cell free incubation experiments

2.4.1 Preparation of the cell free extract from *S. cattleya*

Cells from *S. cattleya* were grown using the protocol detailed in section 5.2. The cells were then used to prepare a cell free extract (CFE) using the method described in section 5.2. The CFE (500 µl of 0.4 mg /ml, tris-HCl buffer, 50 mM, pH 7.8) was tested for fluorinase activity by incubating *S*-adenosyl-L-methionine SAM (4 mM) with KF (5 mM) for 16 h at 37 °C. After incubation the protein was removed by centrifugation after heating (100 °C / 3 min). The production of FAc **15** in the supernatant was confirmed by ^{19}F NMR (-215.7 ppm, t, $^{2}J_{F,H}$ 29.5 Hz) against a reference. The production of FAc **15** from SAM **85** and KF indicates that the fluorinase and all of the proceeding enzymes on the biosynthetic pathway are active within the CFE. It is also worth noting that it was found that the production of 4-FT **16** was not normally observed under these conditions. This is because the transaldolase enzyme (Chapter 1) required to generate 4-FT **16** requires both L-threonine and PLP, and if the concentration of these molecules within the CFE is low then the overall production of 4-FT is low. However the production of 4-FT is observed if the CFE is supplemented with the appropriate cofactors. Likewise the addition of NAD^{+} leads to enhanced production of FAc **15**. As both of the fluorinated secondary metabolites, are biosynthesised from fluoroacetaldehyde **49** the presence of either metabolite is sufficient to indicate active cells. The major fluorinated compound present within the CFE was shown by co-injection with a reference sample to be 5'-FDI **86**. It has already been discussed that 5'-FDI **86** is believed to arise from the deamination of 5'-FDA **83**.

2.4.2 Incubation of 5'-FDA with the cell free extract

In order to establish whether or not 5'-FDA **83** is an intermediate on the biosynthetic pathway and significantly the product of the fluorination enzyme, incubation studies were

carried out using synthetic 5'-FDA. Accordingly 5'-FDA **83** (3.7 mM, final conc.) was incubated with the CFE preparation from *S. cattleya*. The reaction was conducted at 37 °C for 16 h and the products of the reaction were analysed using ^{19}F NMR. The resultant ^{19}F NMR spectrum (Figure 2.7) looked almost identical to that recorded after treatment of the CFE with SAM and KF.

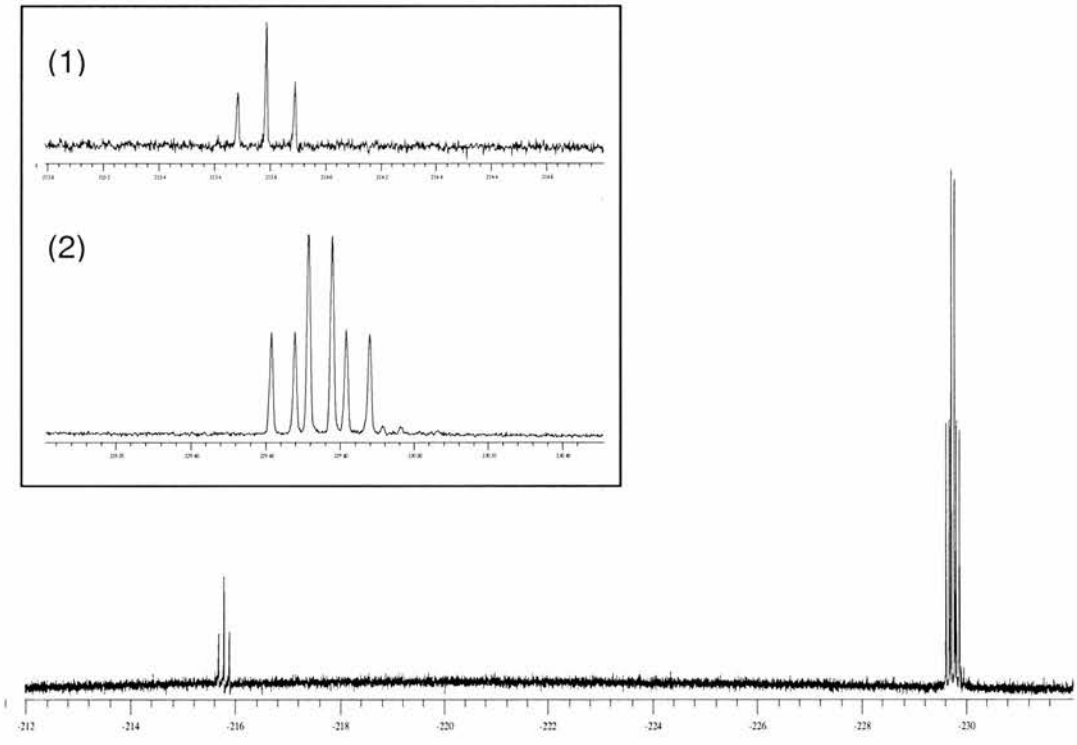


Figure 2.7 ^{19}F NMR (10% D_2O) analysis of crude CFE sample after 16 h incubation at 37 °C with 5'-FDA **83** (3.7 mM).

The signal at -215.8 ppm (Figure 2.7; expansion 1) represents FAc **15** showing that 5'-FDA **83** has the ability to support the entire biosynthesis within *S. cattleya* in the absence of fluoride ion. The result suggests that 5'-FDA **83** represents the product of the first committed enzymatic step on the biosynthetic pathway.

In the CFE studies carried out using SAM and KF, the major product was 5'-FDI **86**. 5'-FDI **86** was also the major compound produced in this experiment (Figure 2.7; expansion 2). This was confirmed by correlation of ^{19}F NMR and HPLC against synthetic 5'-FDI **86**. The fact that 5'-FDI **86** dominates the products of this CFE study is consistent with it being

a shunt metabolite. The exact role, if any, that 5'-FDI **86** may have in relation to fluorometabolite biosynthesis within *S. cattleya* is discussed in the next section (2.4.4).

2.4.3 The origin and role of 5'-FDI within *S. cattleya*

The synthetic reference was important in confirming the identity of 5'-FDI **86** which was originally isolated from the CFE by Dr. C. Schaffrath.[107] The levels of 5'-FDI **86** observed when either SAM **85** or 5'-FDA **83** (Figure 2.7) are incubated with the CFE suggests that it might not be an intermediate on the biosynthetic pathway. One would not expect an intermediate on a biosynthetic pathway to accumulate in such a manner unless one of the enzymes required on the pathway was inhibited or missing. Incubation of a synthetic sample of 5'-FDI **86** with the CFE confirmed this hypothesis. No matter how long the incubation with 5'-FDI was left to run the nucleoside remain metabolically inert within the CFE. It is interesting to note that the results indicated that the bioconversion of 5'-FDA **83** to 5'-FDI **86** is an irreversible process. Clearly there is a deaminase in the *S. cattleya* extract mediating this transformation.

5'-FDA **83** was shown to be inactive when treated with a commercial adenosine deaminase (from calf intestines) but the fact that the conversion of 5'-FDA **83** to 5'-FDI **86** occurs so readily in the CFE implies that the transformation is probably mediated by another class of deaminase with a broader substrate specificity.

It may be that *S. cattleya* posses a specific deaminase that has evolved to accept 5'-FDA **83**. If this were the case then 5'-FDI **86** may have a specific role in fluorometabolite biosynthesis. In this context the most obvious role is that it is a secondary metabolite. However it is notable that it never accumulates in whole cells and has only been identified in cell free extracts. It may be a storage molecule to prevent excessive levels of the toxic, FAc **15** accumulating within the cells. However if 5'-FDI was acting in this capacity then it

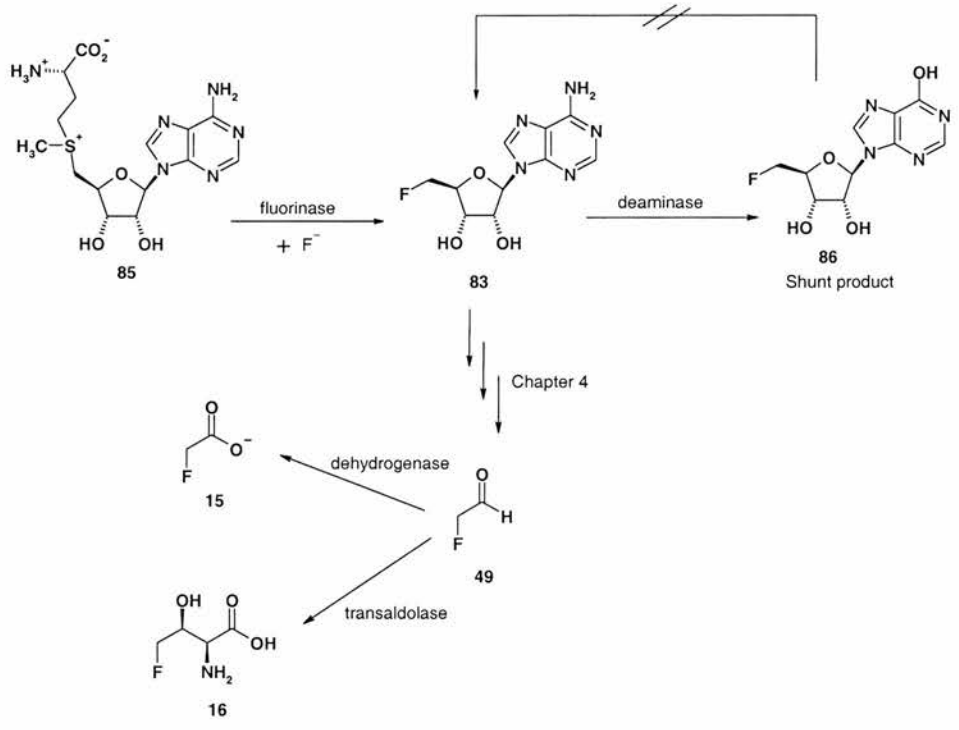
should be possible to regenerate 5'-FDA **83** from 5'-FDI **86** when fluoride ion is not available, but incubation studies show that this is not case. Also if 5'-FDI **86** does not feed back into the pathway then it may act to reduce the overall fluorometabolite concentrations by being released from the cells. Again this does not appear to be the case as 5'-FDI has never been isolated from experiments carried out using whole cells. Therefore the most likely explanation at present is that the deaminase has an alternative role in the cell and it is usually partitioned from 5'-FDA **83**, however in the CFE "soup" this is not the case and it acts to convert 5'-FDA **83** to 5'-FDI **87**.

2.5 Conclusions

The synthesis of 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83** has been accomplished using a new and shorter synthetic protocol. The reaction sequence involved the activation of the 5'-OH of 2',3'-isopropylidene-adenosine **130** by either mesylation or toyslation followed by nucleophilic fluorination using TBAF. Finally acid catalysed deprotection of the isopropylidene group in **133** afforded the target molecule 5'-FDA **83**. The synthesis of 5'-FDI **86** was achieved in one enzymatic step from 5'-FDA **83** by using the commercially available AMPDA enzyme. The reaction gave an almost quantitative yield and could be employed to prepare up to 1g of material. The synthesis of reference compounds (*rac*)-4-FT **16** and fluoroacetaldehyde **49** were carried out using previously established procedures. These compounds were required to underpin the biotransformation studies.

Incubation of the cell free extract (CFE) of *S. cattleya* with synthetic 5'-FDA **83** resulted in the formation of FAc **15** (detected by ^{19}F NMR analysis, Figure 2.7). This confirmed that 5'-FDA **83** was an intermediate on the pathway and supported the hypothesis present in Scheme 2.8 which was devised on the basis of earlier studies carried out with SAM **85** and KF. In addition to FAc **15,** 5'-FDA **83** was also a precursor to 5'-FDI **86** the presence of

which was confirmed by correlation to a synthetic reference. Synthetic 5'-FDI **86** was shown to be metabolically inert when incubated in the CFE. There was no evidence for the reversible conversion of 5'-FDI **86** to 5'-FDA **83**. All of these observations suggest that 5'-FDI **86** is not involved in fluorometabolite biosynthesis in *S. cattleya*. It most probably arises from the presence of an adventitious deaminase within the CFE acting on 5'-FDA **83**. In conclusion the results from both the 5'-FDA and 5'-FDI with the CFE feeding allowed an overview of the steps involved in fluorometabolite biosynthesis to be constructed and is shown in Scheme 2.18. The diagram also takes into account the previously identified aldehyde dehydrogenase and transaldolase (Chapter 1). The enzymes and intermediates responsible for the bioconversion of 5'-FDA **86** to fluoroacetaldehyde **49** will be discussed in detail in Chapter 4.



Scheme 2.18 An overview of fluorometabolite biosynthesis in *S. cattleya*.

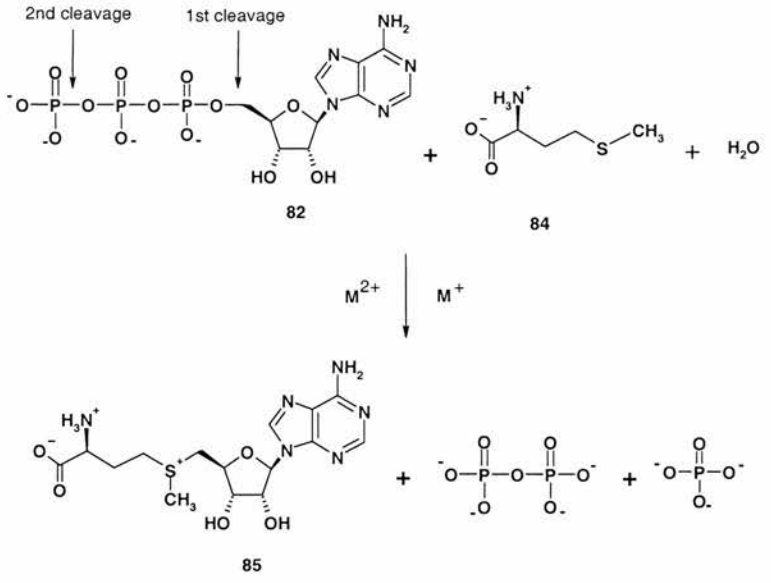
3 The fluorinase

3.1 *S*-Adenosyl-L-methionine (SAM) and its role in nature

For a long time the ability of SAM **85** to function as a methyl donor was assumed to represent the molecule's sole biological role.[165] However enzymological studies principally carried out over the past 15 years have identified several other SAM dependent enzymes.[166] The discovery of these enzymes, including the identification of SAM as the substrate for the fluorinase from *S. cattleya* (Chapter 2), have revealed a much broader role for SAM in biochemistry.

3.1.1 The biosynthesis of *S*-adenosyl-L-methionine (SAM)

SAM **85** was first discovered in 1953 by Cantoni.[167] Since then SAM **85** has become one of the most ubiquitous enzyme substrates second only to adenosine tri-phosphate **82** (ATP).[166] SAM **85** is generated by the enzymatic coupling of ATP **82** and L-methionine **84** catalysed by *S*-adenosyl-L-methionine synthase (MAT; EC 2.5.1.6).[168] This transformation proceeds by a two step reaction mechanism in which the complete triphosphate chain is released from ATP **82** as SAM **85** is formed by nucleophilic attack of L-methionine **84**. The triphosphate is then hydrolyzed by the same enzyme to give PP_i and P_i leading to the overall reaction detailed in Scheme 3.1.[169] Kinetic and isotopic studies have shown that the reaction of ATP **82** and L-methionine **84** occurs by an S_N2 reaction mechanism involving the direct nucleophilic attack of the sulphur atom in **84** on C-5' of ATP **82**.[170,171]

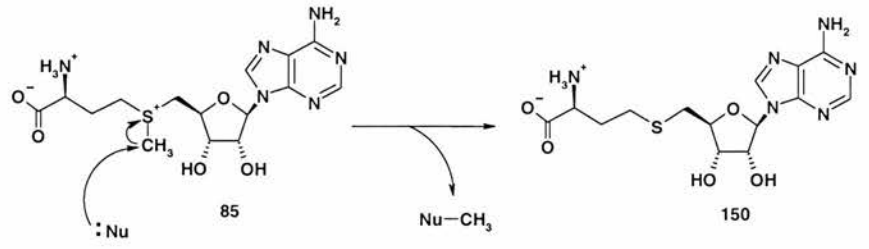


Scheme 3.1 The action of *S*-adenosyl-L-methionine synthetase.[169]

3.1.2 SAM dependent enzymes

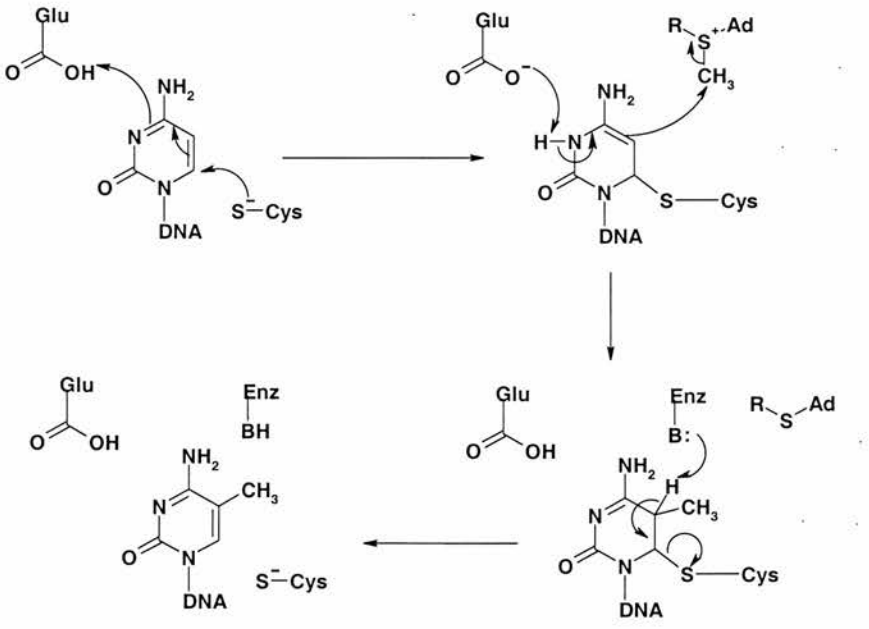
3.1.2.1 SAM as biological methylating agent

In biological systems numerous reactions occur that involve the enzymatically catalysed transfer of a methyl group from a donor molecule to an acceptor.[165] Several different methyl group donors exist in nature but undoubtedly the most widely utilised is SAM **85**. The preference that nature exhibits for SAM **85** over other methyl group donors such as methylcobalamin or 5-methyltetrahydrofolic acid has been attributed to the highly favourable thermodynamics associated with SAM-dependent methyl-transferase reactions.[168] Methylation substrates can vary enormously in size ranging from arsenite to DNA[172] and proteins. In addition methyl transfer to these substrates can occur at carbon, nitrogen, oxygen, sulfur or even halides.[165,173,174] The general mechanism by which SAM-dependent methyl transferase (MTase) enzymes operate is shown in Scheme 3.2.



Scheme 3.2 General mechanism of a SAM-dependent methyl transferase.

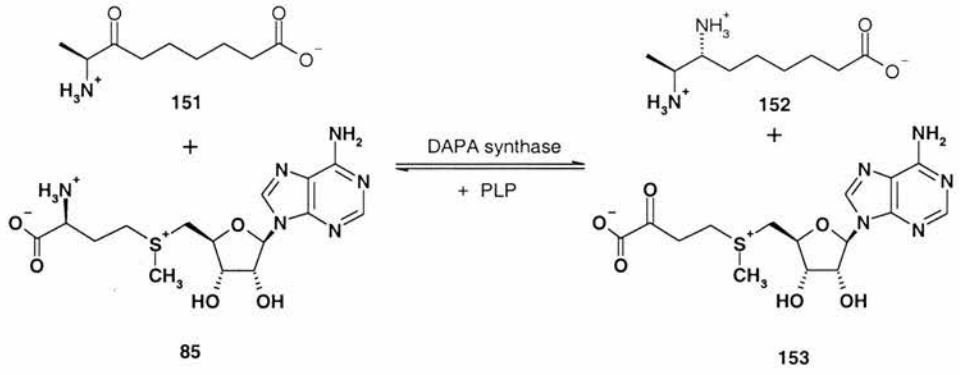
The strong electrophilic character of the methyl group in SAM **85** encourages nucleophilic attack leading to methylation of the substrate and the generation of *S*-adenosylhomocysteine (SAH) **150**. All MTase enzymes are thought to proceed by this direct transfer of the methyl group to the substrate. In general the reaction proceeds with an inversion of stereochemistry at the methylation site, implying an S_N2 type mechanism.[175,176] An example of enzymatic methylation of DNA is shown in Scheme 3.3. In this case the methylation of the C-5 position of the electron-deficient base cytosine proceeds by a Michael addition pathway instead of a direct nucleophilic attack (Scheme 3.3).[177-180]



Scheme 3.3 Mechanism for the methylation of cytosine in DNA.[172]

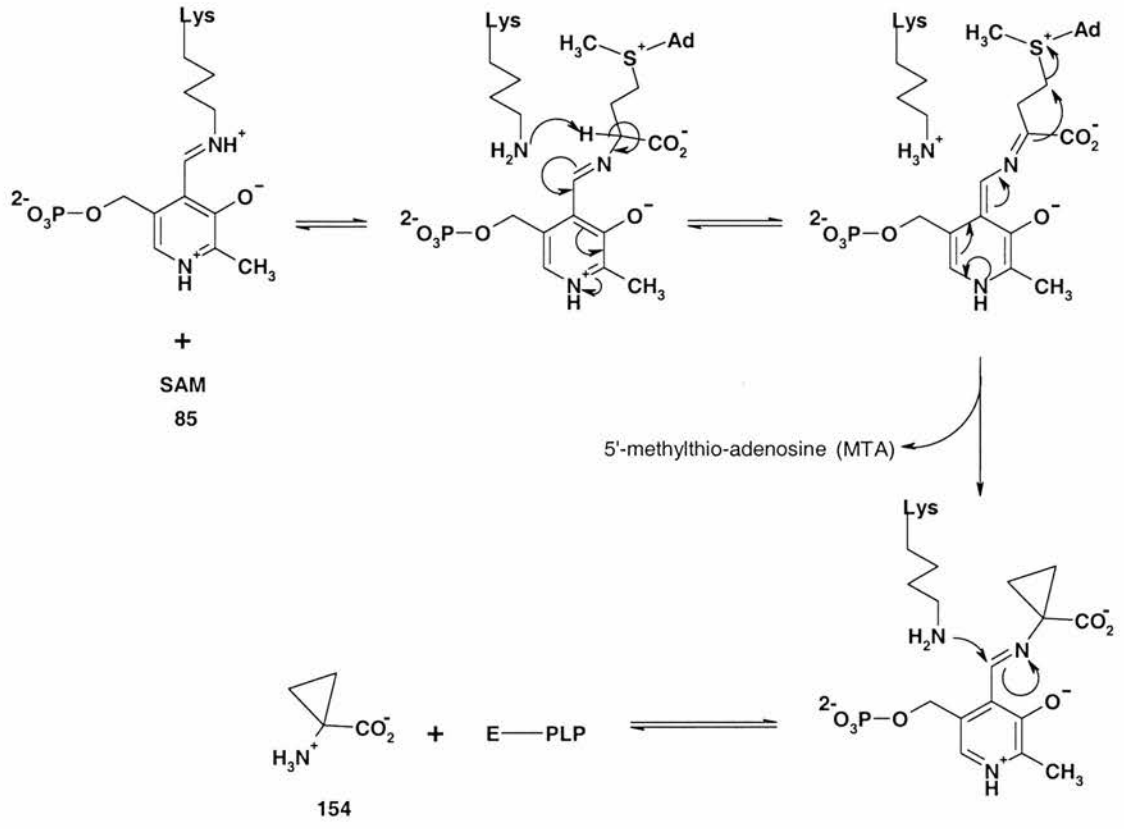
3.1.2.2 SAM as an aminotransferase substrate

DAPA synthase is a PLP dependent aminotransferase that catalyses the formation of 7,8-diamino pelargonic acid (DAPA) **152**, an intermediate on the biotin biosynthetic pathway, from 7-keto-8-amino pelargonic acid (KAPA) **151** (Scheme 3.4).[181,182] The amino group required for the transformation is provided by SAM **85** which is converted to **153**.[183] The mechanism is believed to be similar to that which has already been elucidated for several PLP-dependent aminotransferases.[184]



Scheme 3.4 The action of DAPA synthase.

Amino-cyclopropane-1-carboxylic acid (ACC) **154** is a biosynthetic precursor to the plant hormone ethylene. ACC itself is derived from SAM **85** in a reaction that is catalysed by the PLP-dependent enzyme ACC synthase (EC 4.4.1.14).[185] In this enzymatic process SAM **85** acts as an aminoalkyl donor and the proposed catalytic mechanism by which ACC synthase acts is detailed in Scheme 3.5.

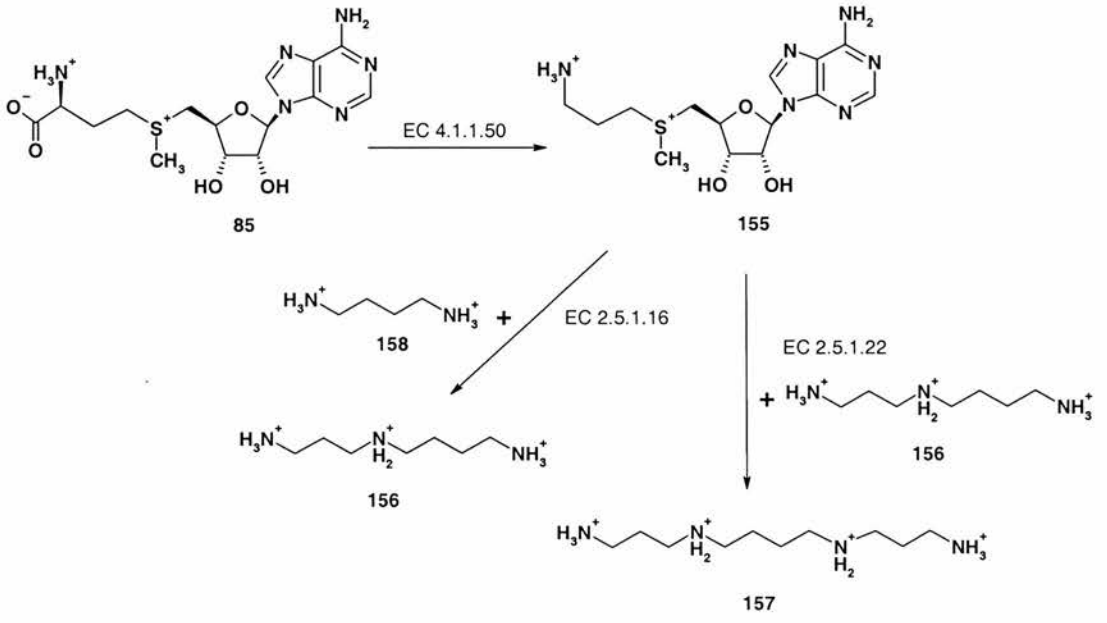


Scheme 3.5 Proposed catalytic cycle accounting for the formation of ACC **154** from SAM **85** promoted by the PLP-dependent ACC synthase enzyme.[185]

SAM **85** also acts indirectly as an aminoalkyl group donor in the preparation of diamines required for polyamine biosynthesis. SAM **85** is converted to *S*-adenosyl-methioninamine **155** (decSAM)[186] by the enzyme *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50).[187,188] DecSAM **155** is then further metabolised by either spermidine synthase (EC 2.5.1.16) or spermine synthase (EC 2.5.1.22) to give spermidine **156** or spermine **157** respectively (Scheme 3.6).[189] In both cases it is decSAM **155** derived from SAM **85** that acts as the aminopropyl donor.

The decarboxylation of SAM **85** by *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) is not a reversible process. This coupled with the fact that decSAM **155** does not act as a methyl donor means that once **155** has been generated the original molecule of SAM **85** is

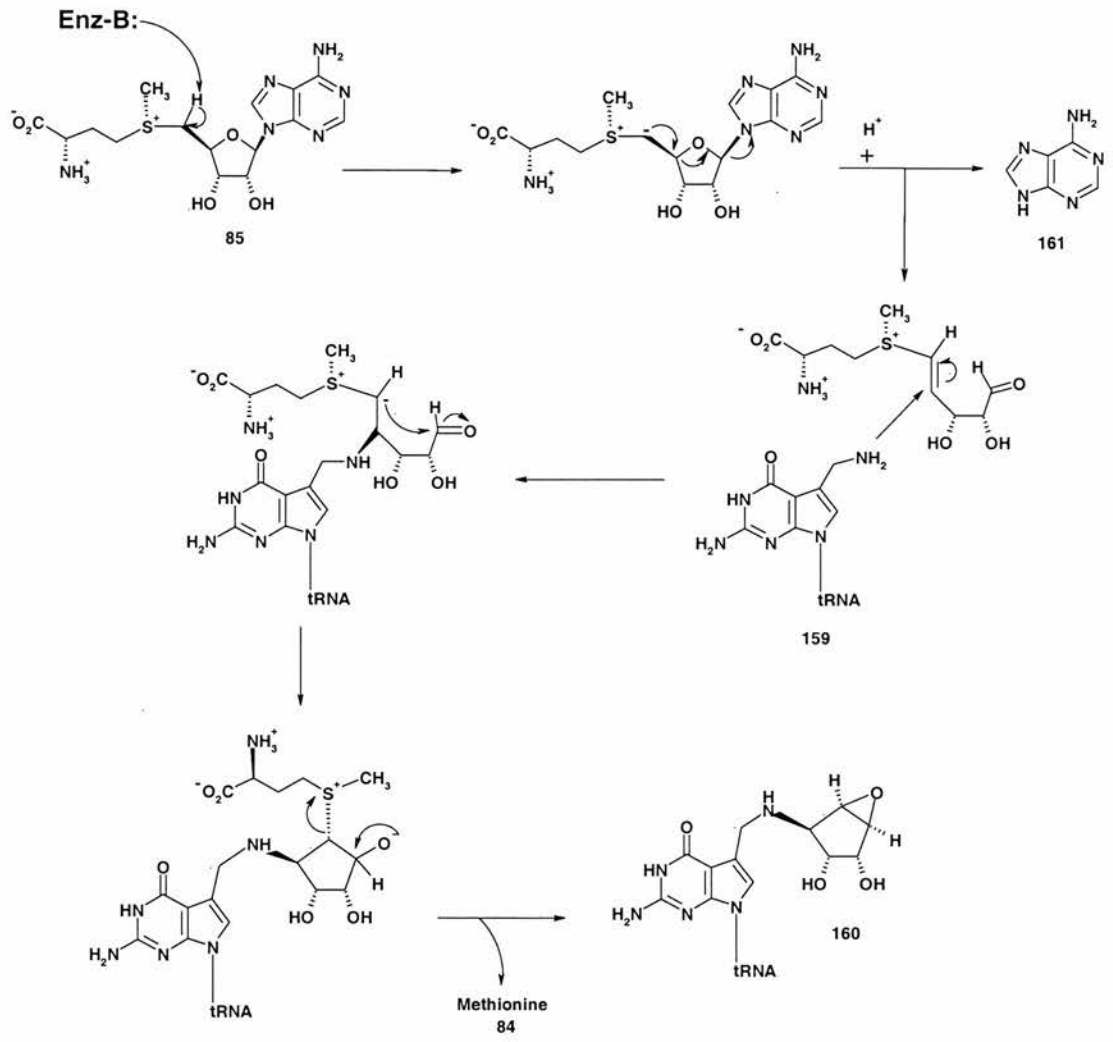
committed to polyamine biosynthesis. This has promoted speculation that the enzyme might have a role to play in regulating the production of ethylene in some plants.[190]



Scheme 3.6 Polyamine biosynthesis from SAM.

3.1.2.3 SAM as a ribosyl donor

SAM has several important roles in post-transcriptional modifications serving as a methyl donor in the methylation of bases[172] or providing the ribose moiety for a variety of nucleosides.[191] A role has also been found for SAM as a ribosyl group donor in the biosynthetic pathway to epoxyqueuosine **160** an unusually modified tRNA nucleoside (Scheme 3.7).[2]



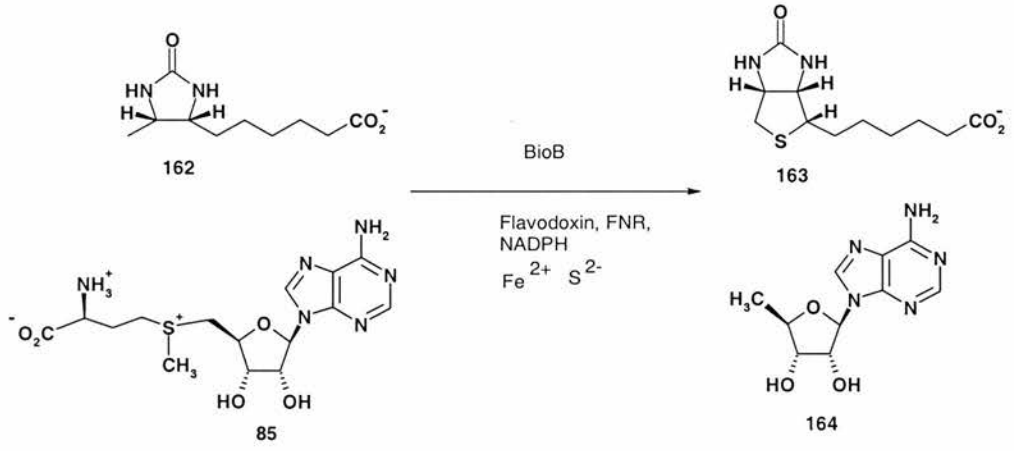
Scheme 3.7 The biosynthesis of the modified tRNA nucleoside queuosine **160**.[166]

The enzyme SAM-tRNA ribosyl transferase-isomerase (QueA) is involved in the catalytic transfer of a ribose group to a 7-amino-methyl-7-deaza-guanosine **159** in the modified tRNA.[192] Subsequent rearrangement with the loss of methionine leads to the formation of the epoxy-carbocycle **160**.[193,194] The mechanism presented in Scheme 3.7 has largely been proposed on the basis of isotopic labelling and kinetic studies. The use of SAM labelled at C-1' of the ribose unit has shown that this carbon is incorporated into the C-3 of epoxy-carbocycle **160**.[195] This result implies that C-4' of the ribose is therefore the likely site of attack by the modified tRNA to generate the initial C-N bond. Kinetic studies using purified enzyme in conjunction with inhibitors also support this sequence of events. The

results show that tRNA binds first to the enzyme followed by SAM and that the products are released in the order of adenine **161**, L-methionine **84** and then finally the product epoxyqueuosine-tRNA **160**.

3.1.2.4 SAM as a source of 5'-dexoyadenosyl radicals

The last step on the biotin biosynthetic pathway is mediated by biotin synthase (BioB) an enzyme which the catalyses the transformation of dethiobiotin **162** to biotin itself **163** (Scheme 3.8).[196]



Scheme 3.8 Final step of biotin biosynthesis.

BioB is a member of the "radical SAM" superfamily of enzymes which are characterised by their ability to use SAM **85** to generate radicals in biological systems.[197-199] The radical generated by these particular enzymes is the 5'-deoxyadenosyl radical which occurs as a consequence of homolytic cleavage of the carbon (C-5') - sulfur bond in SAM **85**. The generation of this radical occurs by the transfer of one electron to the carbon – sulfur bond and in *E. coli* this is provided by reduced flavodoxin which transfers the electron to SAM **85** *via* a Fe_4S_4 cluster.[200] The reaction catalysed by BioB involves some remarkable organic radical chemistry resulting in the insertion of a sulfur atom between the non-activated carbons C-6 and C-9 of dethiobiotin **162**. The exact nature and mechanism of this

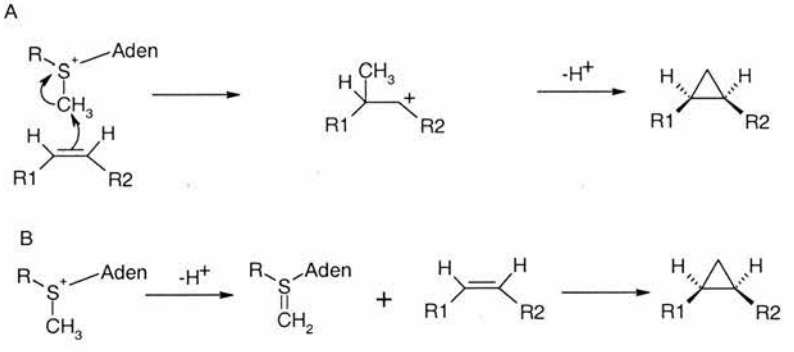
reaction has been the subject of considerable debate in recent years. One hypothesis is that the sulfur atom is provided by a Fe_2S_2 cluster which is consistent with ^{34}S labelling studies[201] and that the destruction of a Fe_2S_2 complex has been shown to accompany the turn over of the enzyme.[202] The role of the 5-deoxyadenosyl radical generated from SAM **85** is believed to be related to the hydrogen abstraction from both the C-9 and C-6 positions of dethiobiotin **163** to allow the complete ring closure of the biotin thiophane ring. The distance from the C-5' of the 5'-deoxyadenosyl radical to both of these hydrogens has recently been established by crystal structure analysis and they appear to be close enough to support this idea (~3.9 Å and ~ 4.1 Å respectively).[196] The double hydrogen abstraction in the proposed mechanism would require the enzyme to generate two 5'-deoxyadenosyl radicals but the use of two molecules of SAM **85** in the overall enzymatic process is still a matter of debate.[203]

3.1.2.5 SAM as a source of methylene groups

Nature has developed several ways to generate natural products that contain the unusual cyclopropane functional group.[204,205] One of the mechanisms by which this is achieved is the direct methylation of a double bond catalysed by cyclopropane fatty acid synthase (CFAS). *E.coli* CFAS is one of the best studied representatives of this class of enzyme.[206] Experiments carried out using [methyl-^{14}C]SAM have confirmed that CFAS utilises the methyl group of SAM as a precursor for the methylene group.[207]

The unsaturated double bond of a fatty acid is believed to attack the electrophilic methyl group of SAM **85** which leads to the formation of SAH **150** and a carbocation intermediate. Deprotonation and ring closure then leads to the generation of a *cis* cyclopropane ring in which the stereochemistry of the original double bond has been retained. Even though this carbocation mechanism (Scheme 3.9, A) is widely accepted[208]

and gains some support from recent crystallographic data[209] in some fatty acid derivatives the position of the reactive double bond infers that an alternative mechanism may be in operation. For fatty acids in which there is a thio-ester group attached to the double bond a "ylide" mechanism (Scheme 3.9, B) has been proposed.[210] This mechanism proposes the formation of a sulfur ylide derived from SAM **85** that can add to the polarised double bond. Ring closure of the resulting enolate results in the formation of the cyclopropane ring. The instability of purified CFAS enzyme has presented considerable problems for detailed mechanistic studies. At present there is no overwhelming evidence to support one mechanism over the other. Recently there has been renewed interest in cyclopropane synthases because the cylcopropanation of mycolic acids in *M. tuberculosis* has been associated with the virulence and persistence of the pathogen.[211] Consequently this class of enzyme might prove to be a good target for new antituberculosis drugs.



Scheme 3.9 The reaction catalysed by CFAS, and the two possible reaction mechanisms (A) carbocation mechanism (B) ylide mechanism.[206]

3.2 5'-Fluoro-5'-deoxy-adenosine synthase (fluorinase) a novel SAM dependent enzyme

3.2.1 Purification of 5'-fluoro-5'-deoxy-adenosine synthase (fluorinase) from *S. cattleya*

With 5'-fluoro-5'deoxy-adenosine (5'-FDA) **83** confirmed as the direct product of the fluorination enzyme (Chapter 2) an HPLC assay was established to allow purification of the fluorinase. The purification of the fluorinase (EC 2.5.1.63) was carried out by Dr. C. Schaffrath and Dr. H. Deng and full details have been published.[212] The enzyme was shown to be a hexamer with a native mass of approximately 180 KDa. The kinetic parameters determined for the enzyme showed that the Km value for fluoride (8.56 mM) ion was 20-fold higher than the Km calculated for SAM **85** (0.42 mM). It was reasoned that the higher Km value for fluoride may relate to the high energy of solvation that must be overcome in order for fluoride to bind to the enzyme. As for many SAM dependent enzymes the fluorinase is inhibited in a competitive manner by SAH **150**.

3.2.2 X-ray crystal structure of the fluorinase

On the basis of the amino acid sequence from native protein and trypsin digests, the fluorinase gene was cloned by Dr. J. Spencer and Dr. F. Huang at the University of Cambridge. This allowed the FDAS gene to be placed in a suitable vector and to be over-expressed in *E. coli*. The availability of mg quantities of the enzyme allowed a fuller characterisation of the enzyme including X-ray structure analysis by Prof. J. Naismith and Dr. C. Dong (University of St. Andrews).[213] Crystallised fluorinase was shown to contain a bound molecule of SAM **85** suggesting that SAM does not readily dissociate from the enzyme during purification. SAM **85** was found to be bound at the interface between two monomer units and is completely buried within the structure. It was proposed that the extensive contacts made between SAM **85** and the two monomer units may drive the closure of the domains to form the enveloped binding site. The structure showed that SAM **85** bound in a high energy conformation in which the C'2-O'2 and C'3-O'3 bonds of the ribose are eclipsed. It was postulated that this high energy conformation was adopted in

order to weaken the C5'-S bond of the substrate (SAM) and thus encourage the fluorination reaction. Data for crystals of the fluorinase, that had been incubated with KF (50 mM) and SAM (10 mM) for 4 h prior to crystallisation, were also collected. In this case the X-ray structure of the protein showed both a product molecule, 5'-FDA **83** and L-methionine **84** were present in the active site (Figure 3.1). SAM had been converted to 5'-FDA on the surface of the enzyme.

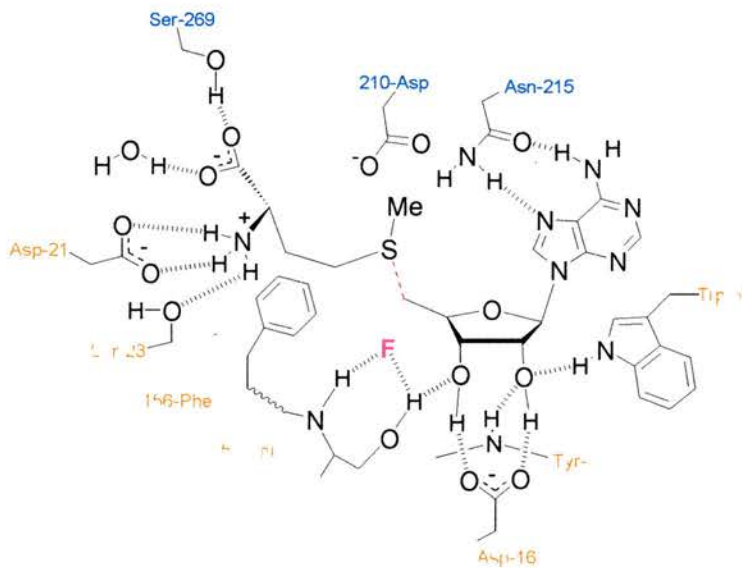


Figure 3.1 Representation of the 5'-FDA-fluorinase complex.

This crystal structure (Figure 3.1) revealed that the fluorine atom at C-5'of the product (5'-FDA **83**) resided in a binding pocket that was unoccupied in the substrate (SAM **85**) complex. This binding pocket is hydrophobic in nature and appears to permit a hydrogen bond between the backbone amide NH of Ser158 and the F atom (N-F, 3.1Å). This was an interesting observation as it is well documented that organic bound fluorine is generally a poor hydrogen bond donor.[214] No water molecules were detected in the vicinity of the fluorine atom, an observation that suggests that the fluoride ion is completely desolvated prior to the formation of the C-F bond. For the enzyme to fully desolvate fluoride ion it would require to input approximately 400 KJ mol^{-1} of energy. This high activation energy

can perhaps be overcome in part by the fluoride ion making polar contacts with the enzyme. These contacts may prevent the enzyme having to pay the full free energy cost required to completely desolvate the fluoride ion.

A comparison of the product and substrate complexes show that C-F bond of the product (5'-FDA **83**) is aligned *anti* (164 °) at a distance of 1.9 Å from the C5'-S bond of the substrate (SAM **85**). This provides structural evidence in support of an S_N2 type mechanism. This observation is consistent with previous whole cell stereochemical studies[215] and also more recent work with the purified enzyme (Chapter 3; Part B).[216]

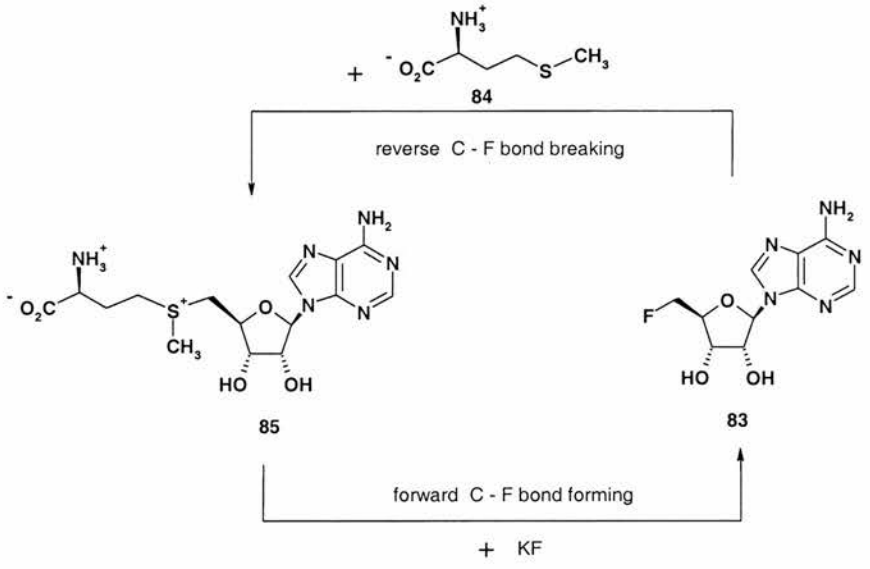
3.3 Novel properties of the fluorinase

The remainder of this chapter summarises investigations into the novel properties of the fluorinase enzyme. All of the experiments were carried out using overexpressed enzyme that had been purified by Dr. H. Deng (University of St. Andrews). Dr H. Deng was also responsible for evaluating the kinetic data discussed in this section and additional ES data. GC-MS analyses of the samples from the chlorination studies were carried out in collaboration with Dr. J. T. G Hamilton and Dr. Colin McRoberts at the Department of Agriculture at the Queens University in Belfast.

3.3.1 The enzymatic preparation of SAM catalysed by the fluorinase

3.3.1.1 The reverse reaction

The fluorinase from *S. cattleya* catalyses the formation of a carbon - fluoride bond between SAM **85** and inorganic fluoride ion to give 5'-FDA **83** (Scheme 3.10, forward reaction). Cleavage of the C5'-S bond results in the release of L-methionine **84**. It was decided to explore the reverse reaction and investigate if 5'-FDA **83** and L-methionine **84** could be used to generate SAM **85** (Scheme 3.10, reverse reaction). This would be interesting as currently the only reported enzyme capable of generating SAM **85** is SAM-synthase (MAT) (Scheme 3.1).



Scheme 3.10 The forward and reverse reaction of the fluorinase.

In order to investigate this possibility a series of incubation experiments were carried according to the protocol shown in Table 3.1.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (µl)		
5'-FDA **83** (7.4 mM)	100	100	100
L-methionine (100 mM)	40	40	-
Fluorinase (10 mg / ml)	100	-	100
Phosphate Buffer pH 6.8 (20 mM)	200	240	300

All incubations were carried out at 37 °C for 16 h.

Table 3.1 Experimental protocol to explore the reverse reaction.

Upon completion the products from Exp 1 - 3 (Table 3.1) were analysed by HPLC (UV detection). A new compound was found to have been produced in Exp 1 (Table 3.1) that eluted approximately 5 minutes after 5'-FDA **83** (Figure 3.2, (i)). This compound was confirmed by co-injection to be SAM **85**. It was also possible to confirm the presence of SAM **85** by ES-MS (399, M^+) (Figure 3.2, (ii)).

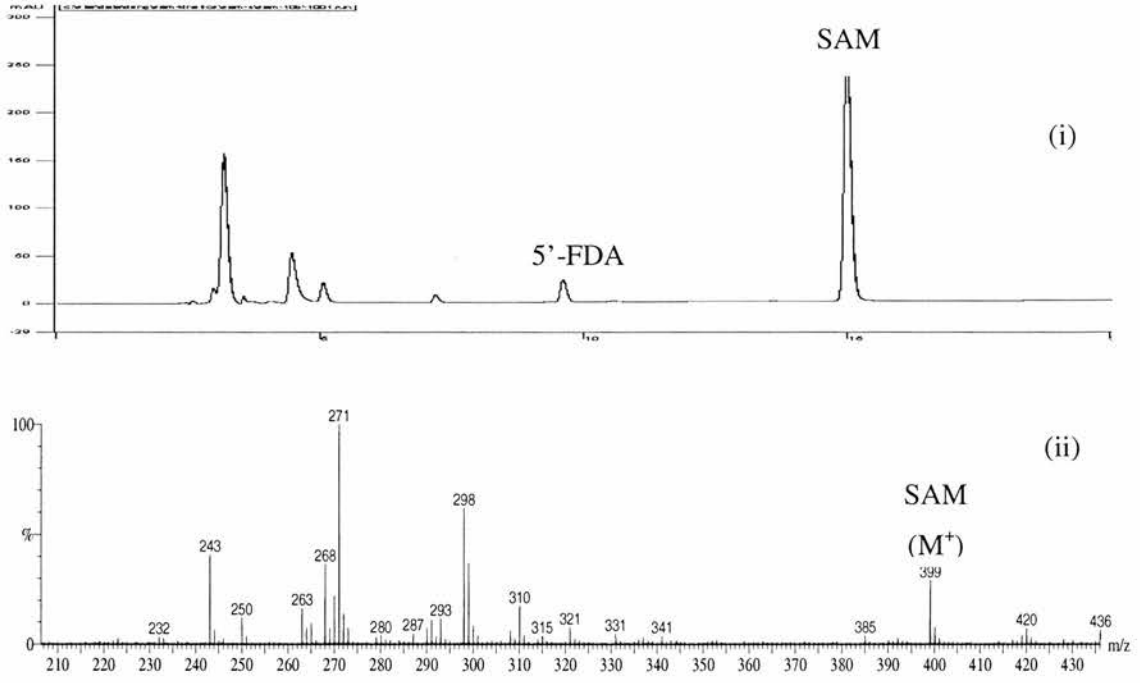


Figure 3.2 HPLC (i) and ES-MS (ii) analysis of Exp 1- confirming SAM production.

HPLC analysis of Exp 2 (Table 3.1) did not reveal SAM **85** production. Thus SAM **85** was not generated in the absence of the fluorinase. There was also no evidence of SAM production in Exp 3 (Table 3.1). Thus L-methionine **84** appeared to be essential for the production of SAM **85**. Clearly the production of SAM from 5'-FDA and L-methionine (Exp 1, Table 3.1) indicated that the fluorinase is capable of catalysing the reverse reaction. The formation of SAM **85** requires the cleavage of the C5'-F bond of 5'-FDA **83**, the strongest bond in organic chemistry, with a bond dissociation energy of 460 $KJmol^{-1}$ (Table 3.2).

X	Bond Dissociation energy ($KJ\ mol^{-1}$) CH_3-X	Bond length (nm) C-X
F	460	0.139
Cl	356	0.178
Br	297	0.193
I	238	0.214
H	414	0.109

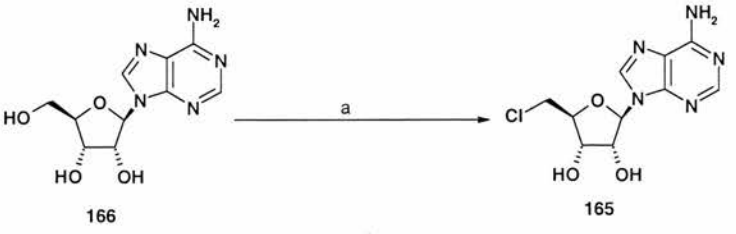
Table 3.2 Physicochemical parameters of the halogens[217]

However despite this several enzymes have been identified that can catalyse the defluorination of organo-fluorine compounds such as FAc **15**.[218,219] In addition the release of fluoride ion from aliphatic compounds is readily achievable by geminal P_{450} hydroxylation. In the case of the fluorinase it is possible that the same hydrogen bonding interactions that help deliver a desolvated fluoride ion for the substitution reaction may also aid in the liberation of fluoride after C-F bond scission, stabilising the fluoride ion as it is released.

3.3.1.2 Synthesis of 5'-chloro-5'-deoxy-adenosine (5'-ClDA)

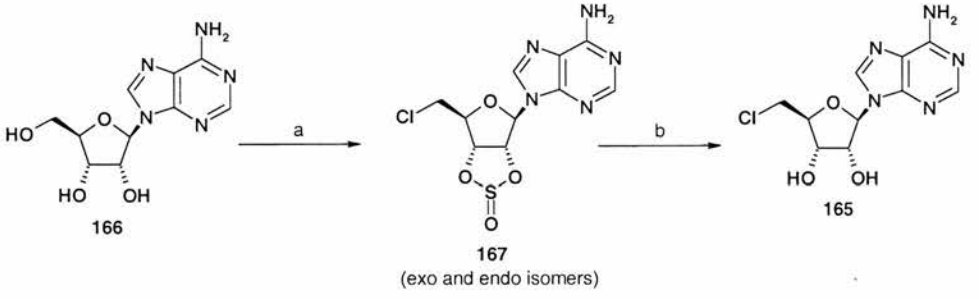
Having demonstrated that the fluorinase operates in reverse it was of interest to explore if 5'-chloro-5'-deoxy-adenosine (5'-ClDA) **165** could be used in place of 5'-FDA **83** as a substrate to support the synthesis of SAM **85**. Clearly from the bond strengths (Table 3.2) the cleavage of the C-Cl bond in 5'-ClDA **165** should occur more readily than the cleavage of the C-F bond in 5'-FDA **83**. However, the X-ray structures that have been determined for the fluorinase indicate that the binding pocket that accommodates the fluorine atom of the product (5'-FDA) has a radius estimated to be around 1.4-1.6 Å. Hence it is not obvious that the larger chlorine atom will fit this binding pocket. This in turn may prevent 5'-ClDA **165** from assuming the correct orientation to allow the reaction to proceed.

In order to investigate this further, a synthetic sample of 5'-ClDA **165** was required. It was possible to prepare **165** in a moderate yield by the selective chlorination of adenosine **166** using the procedure outlined by Francesconi and Stick.[220] The reaction was shown to be completely selective for the primary (5'-OH) hydroxyl group as only unreacted starting material **166** and 5'-ClDA **165** were isolated after column chromatography. The yield was moderate and all attempts to improve the yield of the reaction by modification of the conditions (eg. prolonged reaction time) were unsuccessful.



Scheme 3.11 Reagents and conditions: a) $P(Ph)_3$, CCl_4, pyridine, R.T, 16 h, 55%.

It was found that a more convenient synthetic protocol to prepare 5'-CLDA **165** on a larger scale (> 500 mg) involved the two step process reported by Robins *et al.*[221] Reaction of **166** with thionyl chloride afforded 5'-chloro-5'-deoxy-2',3'-O-sulfinyl-adenosine **167** in an excellent yield. Cleavage of the sulfinyl moiety was then achieved using NH_3 / MeOH methanol and afforded 5'-ClDA **165** in an excellent overall yield.



Scheme 3.12 Reagents and conditions: a) $SOCl_2$, MeCN, pyridine, R.T, 24 h, 76 % b) NH_3/ H_2O, methanol, R.T, 16 h, 91%.

3.3.1.3 The enzymatic preparation of SAM from 5'-ClDA

With a synthetic sample of 5'-ClDA **165** in hand attention turned to investigating it as a substrate for the fluorinase. Accordingly a series of incubation experiments (1-3, Table 3.3) were carried out following the protocol shown in Table 3.3.

	Exp1	Exp2	Exp3
	Volume added (μl)		
5'-ClDA **165** (10.5 mM)	50	50	50
L-methionine **84** (100 mM)	40	-	40
fluorinase (10 mg / ml)	100	100	-
Phosphate buffer pH 7.8 (20 mM)	200	240	300

All incubations were carried out at 37 °C for 16 h.

Table 3.3 Experimental protocol to investigate if 5'-ClDA **165** as a substrate.

The ability of the fluorinase to catalyse the formation of SAM **85** from 5'-ClDA **165** and L-methionine **84** (Exp 1, Table 3.3) was confirmed by HPLC analysis (Figure 3.3 and Scheme 3.13). HPLC analysis of the control experiments (Exp 2 and 3, Table 3.3) did not indicate any reaction in the absence of the fluorinase or of L-methionine **84**.

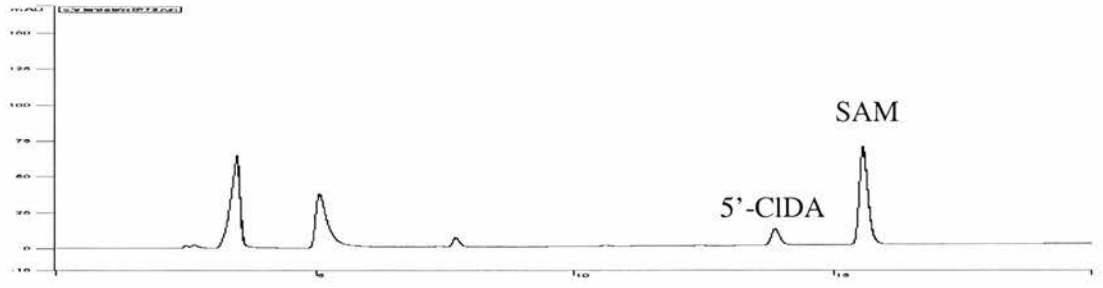
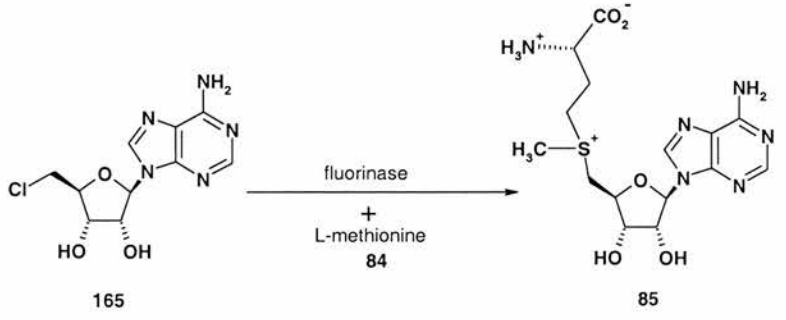


Figure 3.3 HPLC (UV detection, 0-20 min) analysis of the products form Exp 1 (Table 3.3)



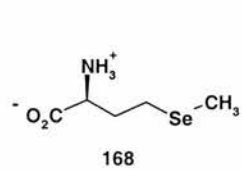
Scheme 3.13 Enzymatic preparation of SAM **85** from 5'-ClDA **165** and **84** catalysed by the fluorinase (Exp 1, Table 3.3).

The results indicated that 5'-ClDA **165** must be able to bind in the active site of FDAS. Assuming that the mechanism of the reaction is the same as for 5'-FDA **83** then it is likely that both 5'-FDA **83** and 5'-ClDA **165** bind to the protein in a similar conformation.

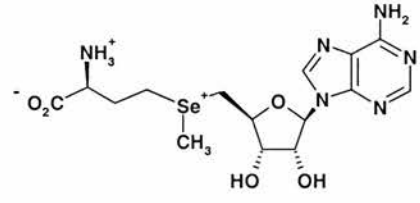
V_{max} values were determined (by Dr. H. Deng) for both 5'-FDA **83** (0.7 nmol / mg protein / min) and 5'-ClDA **165** (4.0 nmol / mg protein / min) showing that the production of SAM **85** proceeds faster when 5'-ClDA **165** is used as a substrate. This increase in rate is most likely due to the difference in bond strengths (Table 3.2) and the better leaving group ability of chloride over fluoride.

3.3.1.4 L-Methionine analogues as substrates

L-Selenomethionine **168** is a L-methionine **84** analogue in which the sulfur atom has been replaced by selenium (Se). L-Selenomethionine **168** is known to act as a substrate for *S*-adenosyl-L-methionine synthase (Section 3.1.1) and kinetic studies have shown that will react faster with ATP than L-methionine. The rate enhancement is presumably due to the increased nucelophilicity of selenium over sulfur. It was attractive to investigate if seleno-SAM **169** could be prepared by the fluorinase.



168



169

Accordingly 5'-ClDA **165** (7 mM, 10 μl) was incubated with **168** (100 mM, 2 μl) in the presence of the fluorinase (10 mg / ml, 100 μl) at 37 °C for 16 h. The protein was then denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm. / 15 min). The production of a new compound that had a later retention time than SAM **85** was detected by HPLC analysis. The incubation procedure was repeated on a larger scale and

ES-MS was used to confirm that seleno-SAM **169** had been generated (Figure 3.4). The ES-MS showed a mass peak for the seleno-SAM degradation product 5'-methylseleno-adenosine **170** (M^+ +H) that displayed the characteristic isotope fingerprint for selenium (Se^{82} 18%, Se^{80} 100%, Se^{78} 50 %, Se^{77} 15% and Se^{76} 19%). Control experiments confirmed that the reaction did not occur in the absence of the fluorinase. The production of seleno-SAM **169** was also achieved if 5'-FDA **83** was used in place of 5'-ClDA **165** as a substrate (Scheme 3.14).

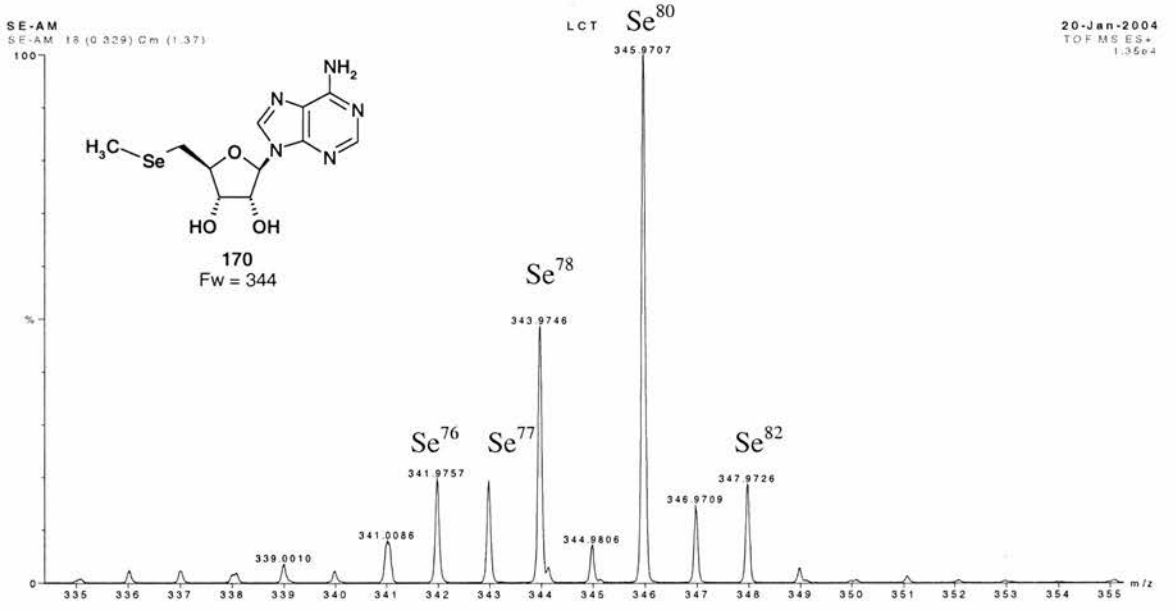
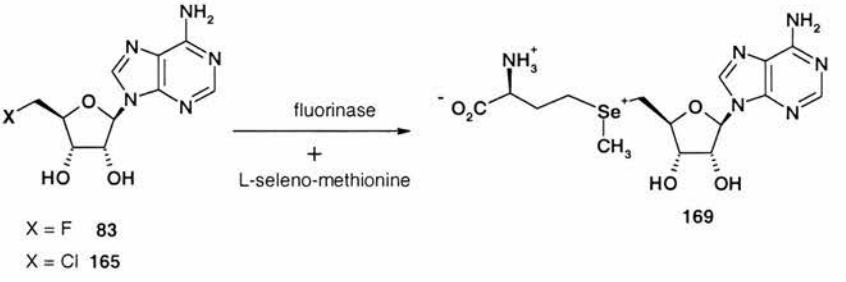
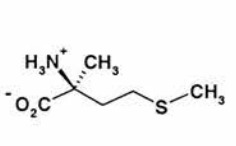


Figure 3.4 ES-MS analysis confirming the production of seleno-SAM **169** from 5'-ClDA **165**.

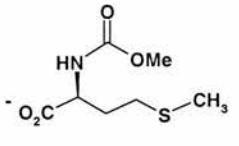


Scheme 3.14 Enzymatic preparation of seleno-SAM **169** catalysed by the fluorinase.

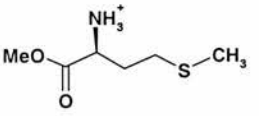
In order to further investigate the substrate specificity of the enzyme in the reverse direction; several commercially available L-methionine analogues **171-173** were also investigated as potential substrates.



171



172



173

Each analogue **171–173** was incubated with 5'-ClDA **165** in the presence of the fluorinase at 37 °C for 16 h, according to the protocol described Table 3.4. In addition to the experiments shown in Table 4 control experiments without active enzyme were also carried out. These experiments confirmed that no chemical coupling between 5'-ClDA **165** and any of the L-methionine analogues **171–173** occurred under the reaction conditions used.

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
	Volume added (µl)			
5'-ClDA **165** (3.5 mM)	25	25	25	25
L-Methionine **84** (100 mM)	5	-	-	-
α-Methyl-D/L-methionine **171** (100 mM)	-	5	-	-
N-Acetyl-L-methionine **172** (100 mM)	-	-	5	-
L-methionine methyl ester **173** (100 mM)	-	-	-	5
Fluorinase (2.6 mg / ml)	100	100	100	100
Tris-HCl buffer, pH 7.8 (50 mM)	50	50	50	50

Table 3.4 Experimental protocol used to test the L-methionine analogues **171–173** as substrates for the reverse reaction catalysed by the flurorinase

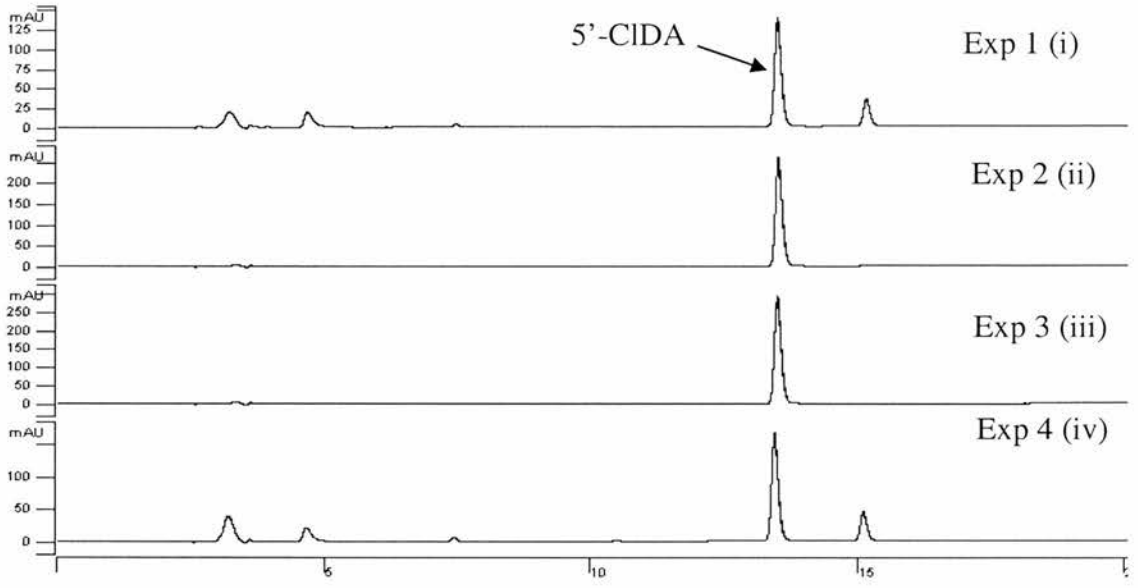
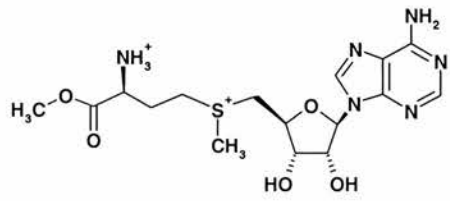


Figure 3.5 HPLC (UV detection, 0-20 minutes) analysis of Exp 1-4 (Table 3.4).

HPLC analysis of the control reaction (Exp 1, Table 3.4) confirmed that 5'-ClDA **165** had been converted to SAM under the reaction conditions used (Figure 3.5 (i)). HPLC analysis of Exp 2 and Exp 3 showed that only 5'-ClDA **165** was present confirming that the L-methionine analogues **171** and **172** were not substrates for the reaction (Figure 3.5 (ii) and (iii)). The X-ray crystal structure of the fluorinase shows that L-methionine **84** binds in the active site in a conformation that places the nucleophilic sulfur atom in close proximity to C-5' of the product (5'-FDA **83**). This positioning is due to the strong binding interactions between the amino acid and the enzyme. In particular the hydrogen bonding that occurs between the amino group of L-methionine **84** and Asp-21 and Ser-23 appears to be important in orientating the molecule into the correct position for nucleophilic attack. As both **171** and **172** are modified at this position it is reasonable to assume that they cannot participate in this binding interaction and consequently they are not accepted as substrates.

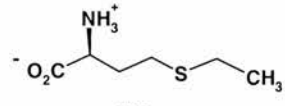
HPLC analysis of Exp 4 (Table 3.4) showed that SAM **85** (confirmed by co-injection with a standard) had been generated (Figure 3.5 (iv)). This was surprising as the product predicted in Exp 4 should be the methyl ester of SAM **174**.



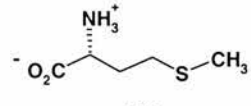
174

To explore the result of Exp 4 further a series of control experiments were carried out to determine if non-enzymatic hydrolysis of the L-methionine methyl ester **173** to L-methionine **84** could account for the result. The results indicated that significant hydrolysis of the methyl ester **173** had occurred. This clearly indicated that the fluorinase could utilise to L-methionine **84** that had been formed from the *in situ* chemical or enzymatic hydrolysis of the methyl ester **173** to prepare SAM **85**.

It was also possible to obtained samples of two other L-methionine analogues, L-ethionine **175**, which has an extended alkyl chain attached to the sulfur atom and the stereo-isomer D-methionine **176**.



175



176

Both **175** and **176** were incubated with 5'-ClDA **165** and fluorinase at 37 °C for 16 h according to the protocol shown Table 3.5. Control experiments were also carried out that confirmed that in the absence of enzyme no chemical coupling between 5'-ClDA **165** and L-ethionine **175** or D-methionine **176** had occurred.

	Exp 1	Exp 2	Exp 3
	Volume added (μl)		
5'-ClDA **165** (7.0 mM)	100	100	100
L-methionine **84** (100 mM)	40	-	-
L-ethionine **175** (100 mM)	-	40	-
D-methionine **176** (100 mM)	-	-	40
fluorinase (10 mg / ml)	100	100	100
Tris-HCl buffer, pH 7.8 (50 mM)	300	300	300

Table 3.5 Experimental protocol used to test the L-methionine analogues **175** and **176** as substrates for the reverse reaction.

Control Exp 1 (Table 3.5) confirmed that the reaction conditions used were suitable to support the biosynthesis of SAM **85** (Figure 3.6). HPLC analysis of Exp 2 (Table 3.5) showed that 5'-ClDA **165** had remained unreacted implying that L-ethionine **175** cannot act as a substrate (Figure 3.6). However HPLC analysis of Exp 3 (Table 3.5) confirmed that 5'-ClDA **165** had been converted to a second compound (Figure 3.6). This compound was shown by co-injection to co-elute with *S*-adenosyl-L-methionine (SAM) **84**. Despite the lack of access to a sample of *S*-adenosyl-D-methionine it would clearly be expected that both D and L-diastereoisomers of SAM would elute on HPLC very similarly. Hence the results tentatively imply that D-methionine **176** can be utilised as a substrate by the fluorinase. However without further investigations particularly to confirm the stereochemistry of the product SAM, it is impossible conclude beyond doubt that the fluorinase is promiscuous enough to accept both enatiomers of methionine.

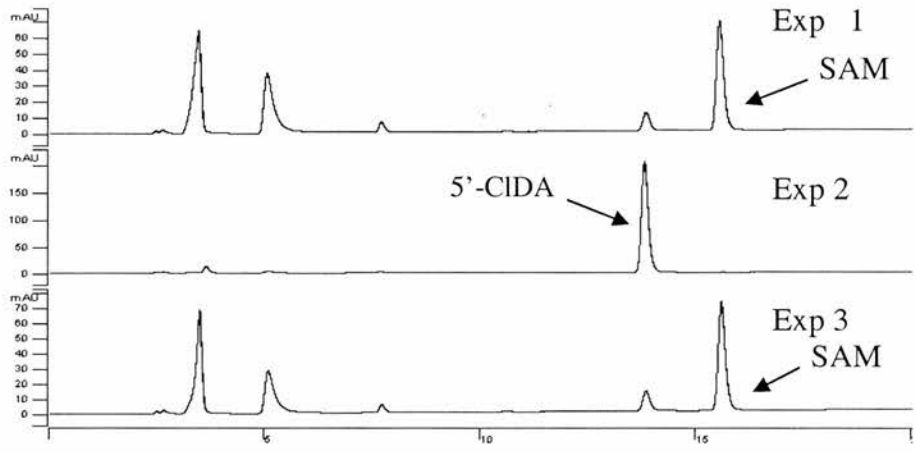
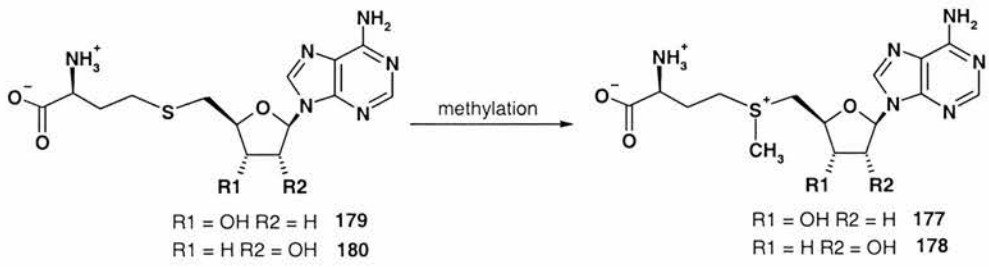


Figure 3.6 HPLC (UV detection, 0-20 minutes) analysis of Exp 1-3 (Table 3.5).

3.3.2 Preparation of deoxy-SAM analogues

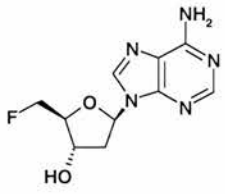
To further investigate the substrate specificity of the fluorinase analogues of SAM were required. Such compounds are not readily available and thus a method of obtaining modified SAM structures was required. The chemical synthesis of SAM analogues such as 2'-deoxy-SAM **177** and 3'-deoxy-SAM **178** could clearly be achieved by methylation[222] of the corresponding known SAH derivatives **179** and **180** (Scheme 3.15). The synthetic routes to these compounds are reported in the literature but they are complex.[223,224]



Scheme 3.15 Potential synthetic strategy to 2'-deoxy-SAM **177** and 3'-deoxy-SAM **178**.

A more attractive method of preparing SAM analogues emerges by utilising the fluorinase. For example **177** and **178** could be generated by a 5'-halo-nucleoside / L-methionine coupling reaction catalysed by the fluorinase. In order to successfully exploit this

methodology it was necessary therefore to prepare the modified adenosine nucleoside analogues **181-184**.



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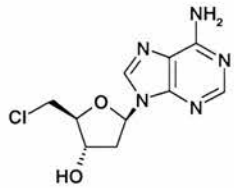
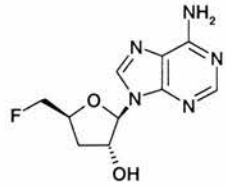
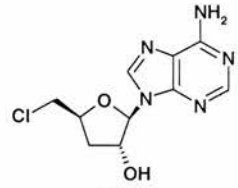


182



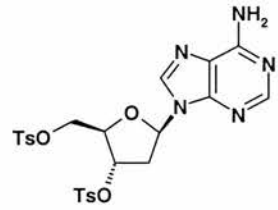
183



184

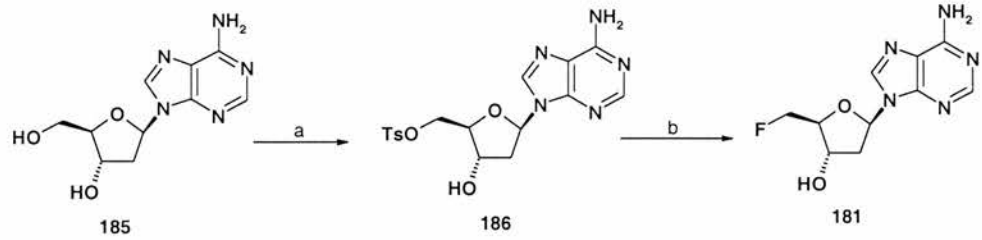
3.3.2.1 Synthesis of modified 5'-halo-nucleosides

The preparation of 5'-fluoro-2',5'-dideoxy-adenosine **181** was approached using a synthetic strategy identical to that applied to the preparation of 5'-FDA **83** (Chapter 2). An initial attempt to prepare 5'-tosyl-2'-deoxy-adenosine **186** from 2'-deoxy-adenosine **185** was carried out using 1.5 equivalents of TsCl in pyridine. The reaction was conducted at 0 ^{o}C for 12 h. ^{1}H and ^{13}C NMR analysis of the only isolated product revealed that tosylation had occurred at both the 5'-OH and 3'-OH positions giving 3',5'-ditosyl-2'-deoxy-adenosine **187**. No monotosylated product was identified.



187

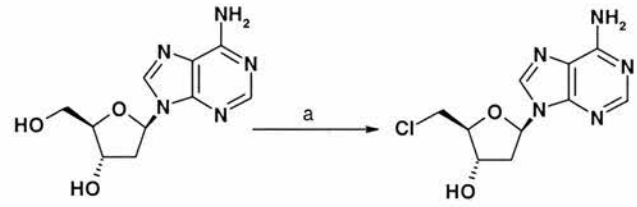
In order to try to eliminate the formation of the unwanted di-tosylate **187** the reaction temperature was lowered to -20 ^{o}C. Reaction of **185** with TsCl (3 equivalents) at -20 ^{o}C for 48 h afforded a mixture of **186** (58%) and **187** (26%) that were easily separable by chromatography. Fluorination of **186** was carried out with TBAF.3H_2O to give 5'-fluoro-2',5'-dideoxy-adenosine **181** in a moderate yield after purification (Scheme 3.16).



185 186 181

Scheme 3.16 Reagents and conditions: a) TsCl, pyridine, -20°C, 48 h, 58% b) TBAF.$3H_2O$, MeCN, reflux, 16 h, 44%.

It was possible to prepare 5'-chloro-2',5'-dideoxy-adenosine **182** in a moderate yield from **185** using the same reaction conditions utilised in the chlorination of adenosine **166** to give 5'-ClDA **165**. As found previously a considerable amount of unreacted starting material **185** was recovered during the purification.

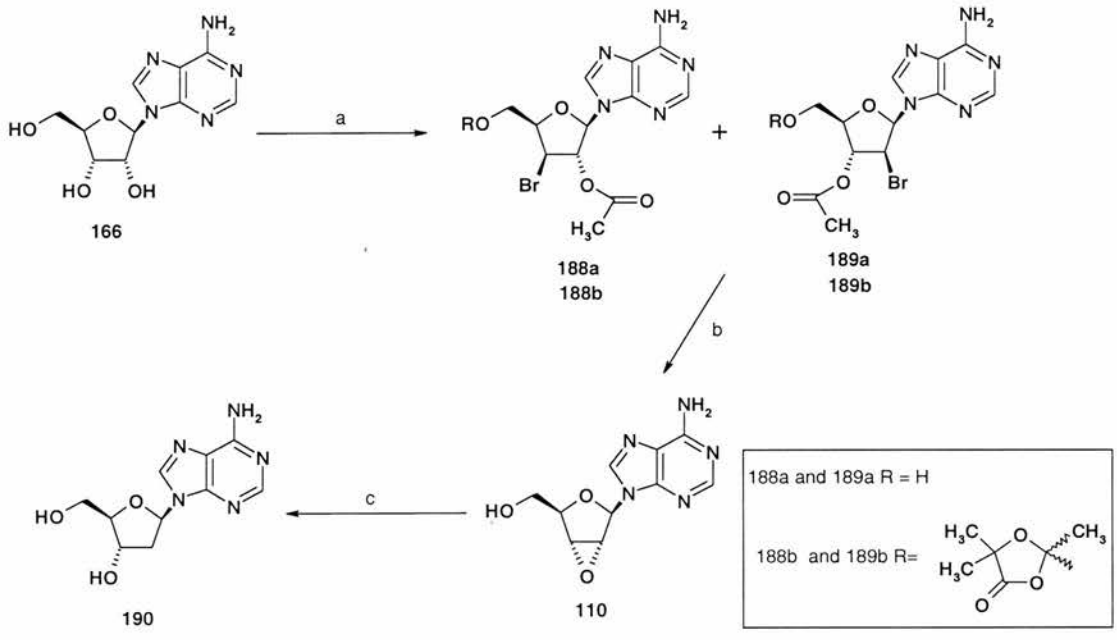


185 182

Scheme 3.17 Reagents and conditions: a) $P(Ph)_3$, CCl_4, pyridine, R.T, 11 h, 49 %.

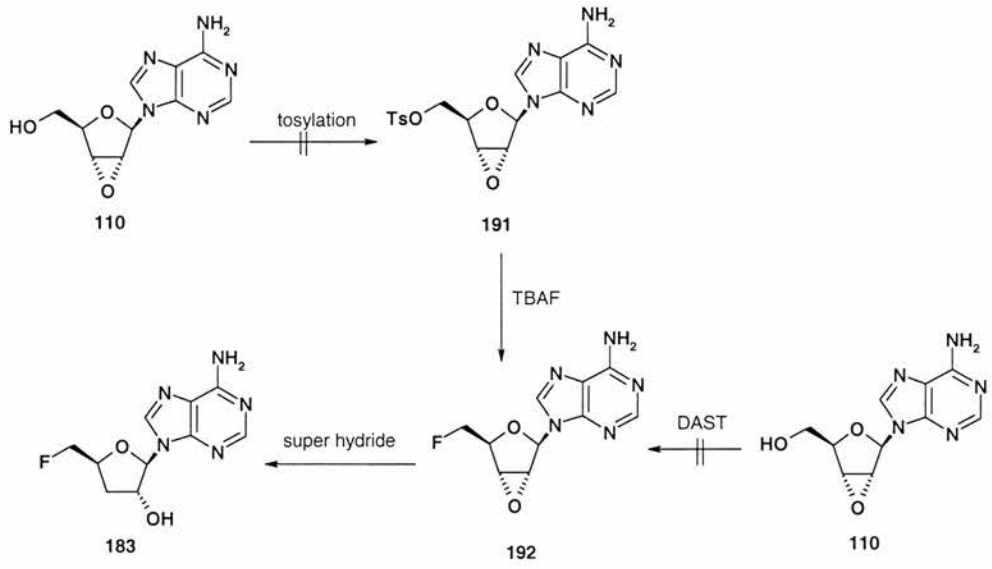
The synthesis of both 5'-fluoro-3',5'-dideoxy-adenosine **183** and 5'-chloro-3',5'-dideoxy-adenosine **184** required 3'-deoxy-adenosine **190** as a precursor. The preparation of **190** was achieved from adenosine **166** using a modification of the procedure developed by Robins *et al.* (Scheme 3.18).[225] The reaction of **166** with 2-acetoxy-2-methylpropanoyl bromide (α-acetoxyisobutryl bromide, α-AIBBr) afforded a mixture of **188a**, **188b**, **189b** and **189b**. Treatment of this reaction mixture with Dowex 1 X 2 (OH^-) resin in dry methanol followed by work up gave 2',3-anhydro-adenosine **110** in an excellent yield. Regioselective ring opening of the epoxide **110** was achieved using super hydride ($LiEt_3BH$/ THF) in 1,4-dioxane affording **190** in an excellent yield. The use of 1,4-dioxane as the solvent was preferred to DMSO as used by Robbins *et al.*[226] This was because any

residual DMSO left from the work up procedure was found to cause problems during the purification of **190**.



Scheme 3.18 Reagents and conditions: a) α-AIBBr, 1eq. water, MeCN, R.T, 1 h, (products not isolated) b) Dowex 1 X 2 (OH^-), MeOH, R.T, 1 h, 90% c) $LiEt_3BH$/ THF (1M), 1,4-dioxane, 16 h, 89%.

The preparation of 5'-fluoro-3',5'-dideoxy-adenosine (3'-deoxy-5'-FDA) **183** required the synthesis of the activated nucleoside 5'-tosyl-3'-deoxy-adenosine. However all attempts to convert 3'-deoxy-adenosine **190** into 5'-tosyl-3'-deoxy-adenosine were unsuccessful. This led to the investigation of alternative synthetic strategies to 3'-deoxy-5'-FDA **183** *via* the epoxide **110** (Scheme 3.19). Attempts to open the epoxide **110** with both HF/pyridine and $TBAF.3H_2O$ proved unsuccessful with only unreacted starting material being recovered in each case. This presented an opportunity to fluorinate **110** directly and leave the epoxide ring intact. If this was achieved then the product **192** could be converted to **183** using super hydride.

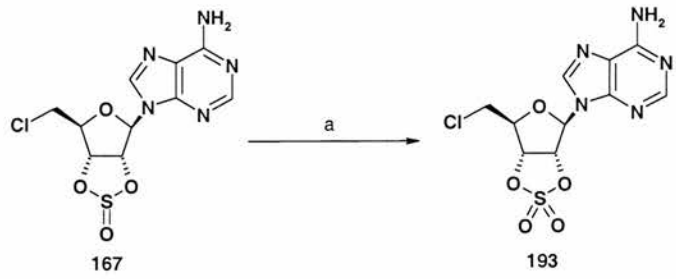


110 191 192 183 110

Scheme 3.19 Attempted synthetic route to 3'-deoxy-5'-FDA **183** from 2',3-anhydro-adenosine **110**.

In the event attempts to tosylate **110** using TsCl, pyridine were unsuccessful and it was not possible to prepare **192** by direct fluorination of **110** using DAST.

In light of these results attention was turned to the preparation of 5'-chloro-2',5'-dideoxy-adenosine **184**. Treatment of 3'-deoxy-adenosine **190** with triphenyl phosphine, CCl_4 and pyridine only afforded a complex mixture of products. The availability of 5'-chloro-5'-deoxy-2',3'-O-sulfinyl-adenosine **167** (Scheme 3.12) allowed an alternative strategy to 5'-chloro-3',5'-dideoxy-adenosine **184** to be explored. Oxidation of **167** gave 5'-chloro-5'-deoxy-2',3'-O-sulfonyl-adenosine **193** in an excellent yield (Scheme 3.20).[221,226]



167 193

Scheme 3.20 Reagents and conditions: a) $NaIO_4$, $RuCl_3$ (cat), MeCN, 1 h, 85%.

Nucleophilic ring opening of 2',3'-cyclic sulfates such as **193** have been shown to proceed with almost complete regioselectively to yield 3'-deoxy substituted products.[227] Therefore it was reasoned that it might be possible to convert **193** to 3'-deoxy-5'-ClDA **184** by hydride opening of the cyclic sulfate moiety. Sodium borohyride ($NaBH_4$) was selected as the hydride reagent as stronger hydride sources such as Super Hydride has been shown to displace a 5'-chloro atom (see preparation of 5'-deoxy-adenosine **164** Chapter 4). Treatment of **193** with $NaBH_4$ (excess), R.T for 48 h, afforded only unreacted starting material (TLC analysis). If the reaction was left to proceed for longer than 48 h, considerable degradation of the starting material was observed. In light if these results the synthesis of 3'-deoxy analogues was discontinued.

The difference in reactivity between 5'-OH groups in 2'-deoxy-adenosine **185** and 3'-deoxy-adenosine **190** can possibly be linked to the different conformational preferences that the two molecules exhibit. In solution 2'dA **185** like adenosine **166** resides predominately in a South type conformation (Figure 3.7).[228,229] In this conformation the base is positioned such that it does not cause significant steric hindrance to the 5'-OH group. Conversely 3'dA **190** prefers a North type conformation (Figure 3.7). In the North type conformation the base is positioned far closer to the 5'-OH group. It is possible that this results in the 5'-OH experiencing some steric hindrance that prevents reagents from interacting with the hydroxyl group effectively.

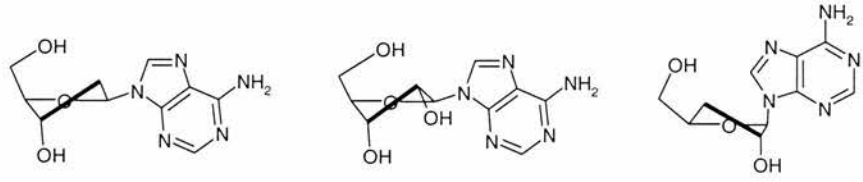
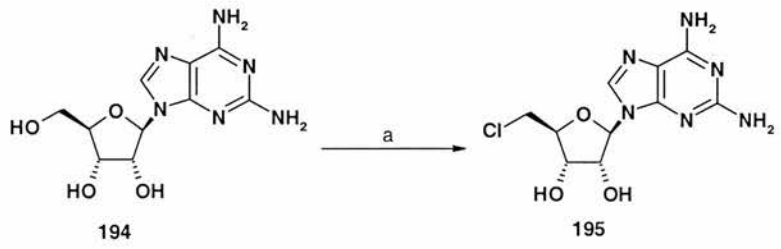


Figure 3.7 Conformational preference exhibited by 2'dA **185**, adenosine **166** and 3'dA **190**.

The fact that it is possible to chlorinate (using same reaction conditions) the 5'-OH of both **166** and **185** but not **190** suggests that the South type conformation might present a more reactive 5'-OH group than the North type conformation.

The availability of 2-amino-adenosine **194** permitted the preparation of the C-2 purine modified nucleoside 5'-chloro-5'-deoxy-2-amino-adenosine **195**. This was achieved by direct chlorination and afforded **195** albeit in a low yield, as shown in Scheme 3.21.



194 195

Scheme 3.21 Reagents and conditions: a) $P(Ph)_3$, pyridine, CCl_4, 16 h, 42%.

3.3.2.2 Enzymatic preparation of SAM analogues catalysed by the fluorinase

5'-FDI **86** the 6-oxo-purine derivative of 5'-FDA **83** was explored as a potential substrate for the fluorinase in the reverse reaction. Accordingly 5'-FDI **86** (10 mM, 50 µl) was incubated with L-methionine **84** (100 mM, 25 µl), fluorinase (10 mg / ml, 100 µl) and Tris-HCl buffer (50 mM, pH 7.8, 300 µl) for 16 h at 37 °C. HPLC analysis of the sample did not show any new products to indicate that 5'-FDI **87** was a substrate for the fluorinase. The result implies that the 6-amino group of the purine base is required for binding and hence enzyme activity (see Figure 3.1).

In order to investigate if it was possible to use the fluorinase to prepare 2'-deoxy-SAM **177**, 5'-chloro-2',5'-dideoxy-adenosine **182** was tested as a substrate. Accordingly **182** (10 mM, 100 µl) was incubated with L-methionine **84** (100 mM, 50 µl), fluorinase (10 mg / ml, 100 µl) and Tris-HCl buffer (50 mM, pH 7.8, 400 µl) for 16 h at 37 °C. HPLC (UV

detection) analysis showed the production of a new compound that eluted after 2'-deoxy-5'-ClDA **184**. ES-MS confirmed that this was 2'-deoxy-SAM **177** (Figure 3.8)

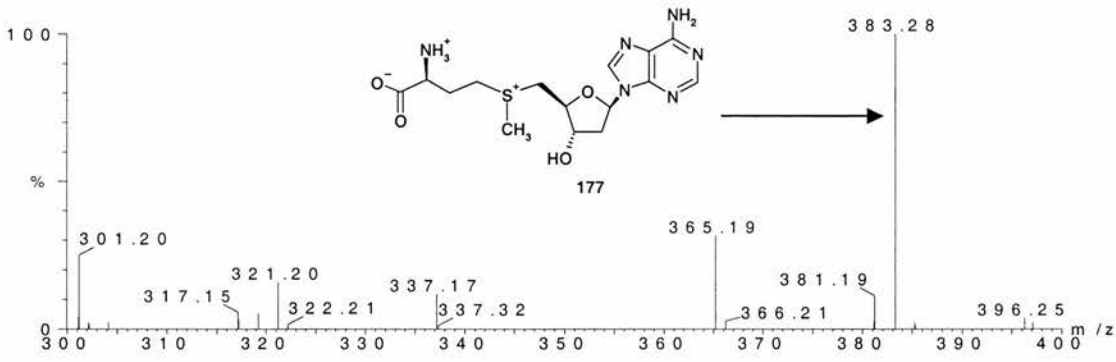
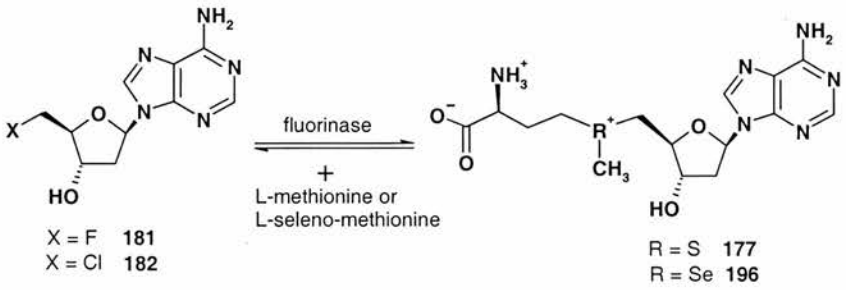


Figure 3.8 ES-MS analyses showing the enzymatic production of 2'-deoxy-SAM **178** from **184** catalysed by the fluorinase.

Control experiments showed that 2'-deoxy-SAM **177** could only be generated from 2'-deoxy-5'-ClDA **182** and L-methionine **84** in the presence of the fluorinase enzyme. Further investigations revealed that 5'-fluoro-2',5'-dideoxy-adenosine **181** could also support the preparation of 2'-deoxy-SAM **177** (see section 5.2). It was also possible to use L-seleno-methionine **168** in place of L-methionine **84** to prepare 2'-deoxy-seleno-SAM **196** using either 2'-deoxy-5'-FDA **181** or 2'-deoxy-5'-ClDA **182** as a substrate (Scheme 3.22). The production of 2'-deoxy-seleno-SAM **196** was confirmed by ES-MS (Figure 3.9). It showed a mass peak for the 2'-deoxy-seleno-SAM degradation product 2'-deoxy-5'-methylseleno-adenosine **197** (M^+ +H) that displayed the characteristic isotope fingerprint for selenium (Se^{82} 18%, Se^{80} 100%, Se^{78} 50 %, Se^{77} 15% and Se^{76} 19%).



Scheme 3.22 Enzymatic preparation of **177** and **196** catalysed by the fluorinase.

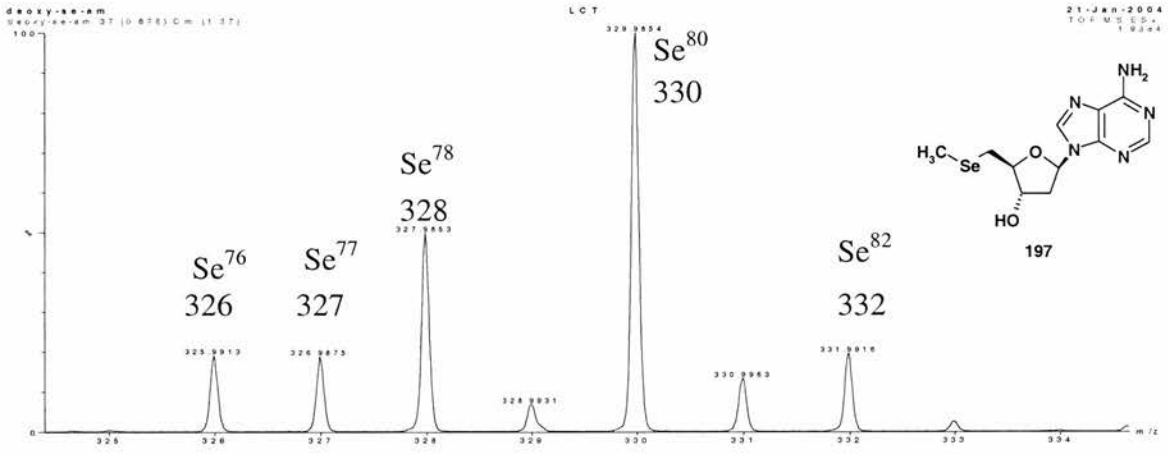


Figure 3.9 ES-MS analysis confirming the production of 2'-deoxy-seleno-SAM **196** from 2'-deoxy-5'-ClDA **182**.

Despite ongoing advances in synthetic techniques the preparation of SAM **85** for biological studies is still carried out by the enzymatic coupling of L-methionine **84** and ATP **82** catalysed by *S*-adenosyl-L-methionine synthase. However it is interesting to note that the majority of *S*-adenosyl-L-methionine synthases that have been isolated to date will not accept 2'-deoxy-ATP as a substrate. Therefore the use of the fluorinase offers a practical route to prepare SAM **85** and 2'-deoxy-SAM **177** for biological investigations.

The fluorinase was also able to catalyse the coupling of **195** and L-methionine **84** to give 2-amino-SAM **198** (Figure 3.10 and Scheme 3.23). The production of **198** was confirmed by ES-MS ***m/z*** 414 (M^+, 100%) and 313 (M^+ +H, -$C_4H_8NO_2$).

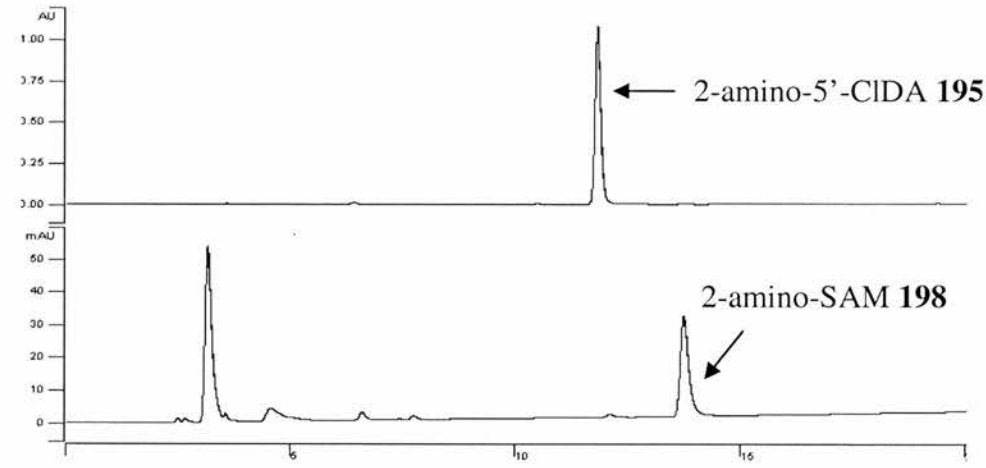
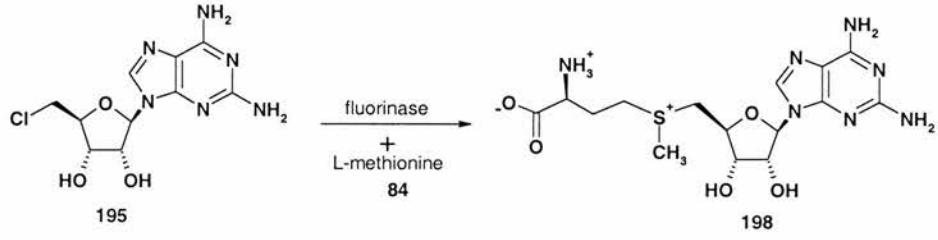
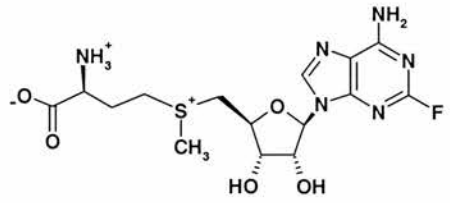


Figure 3.10 HPLC analysis (UV, 0-20 min) showing the production of 2-amino-SAM **198**.

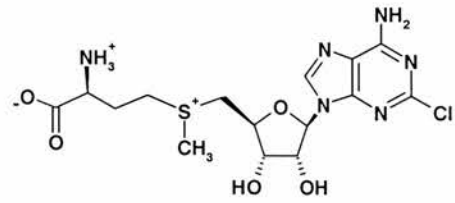


Scheme 3.23 Enzymatic preparation of 2'-amino-SAM **198** catalysed by the fluorinase.

The ability of the fluorinase to tolerate an amino group at the C-2 position identifies a site that might tolerate further modifications possibly leading to the preparation of novel SAM analogues. For examples the inclusion of a fluorine or chlorine atom (**199** and **200**) at this position might be of interest. This is because the inclusion of these particular atoms at the C-2 position has been shown to alter the chemical properties of the nucleoside. Also the inclusion of a fluorine atom at C-2 would make ^{19}F NMR active SAM. In theory this could then allow the use of ^{19}F NMR techniques to be applied to the study of SAM dependent enzymes or the metabolism of SAM in biological systems.



199



200

3.3.3 A novel transhalogenation reaction mediated by the fluorinase.

Having established that the fluorinase can be used to prepare modified SAM's in the reverse direction, it was interesting to asses if these SAM's could be used as substrates for the fluorination reaction, in the forward direction. As chlorinated substrates can be used for the preparation of the SAM derivatives the possibility that dechlorination followed by fluorination could be carried out in a one pot reaction was investigated. The overall process would involve a transhalogenation reaction. Accordingly 5'-ClDA **165** was incubated with L-methionine **84** or L-seleno-methionine **168**, KF and the fluorinase according to the experimental protocol detailed in Table 3.6.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (μl)		
5'-ClDA **165** (7.0 mM)	100	100	100
KF (0. 5 M)	20	20	20
L-seleno-methionine **168** (100 mM)	-	40	-
L-methionine **84** (100 mM)	40	-	-
fluorinase (10 mg / ml)	100	100	100
Tris-HCl pH 7.8 (50 mM)	300	300	340

Table 3.6 Experimental protocol for enzymatic transhalogenation with 5'-ClDA **165**.

HPLC and ^{19}F NMR analyses confirmed the production of 5'-FDA **83** in Exp 1 and Exp 2 (Figure 3.11). The fact that 5'-FDA **83** was not observed in Exp 3 indicated that 5'-ClDA **165** could not undergo direct enzymatic fluorination. Therefore the production of 5'-FDA **83** in Exp 1 and Exp 2 must proceed *via* enzymatic fluorination of either SAM **85** (Exp 1) or seleno-SAM **169** (Exp 2). Hence this novel transhalogenation reaction must proceed according to the sequence of events outlined in Figure 3.11 where 5'-ClDA **165** is first

converted to either SAM **85** or seleno-SAM **169** depending on whether L-methionine **84** or L-seleno-methionine **168** is used as a substrate. The presence of inorganic fluoride (KF) in the reaction mixture then permits the enzymatic fluorination of **85** or **170** to give 5'-FDA **83**. The fluorinase is first used in the reverse direction to create SAM or seleno-SAM and is utilised in the forward direction to mediate the fluorination of these compounds.

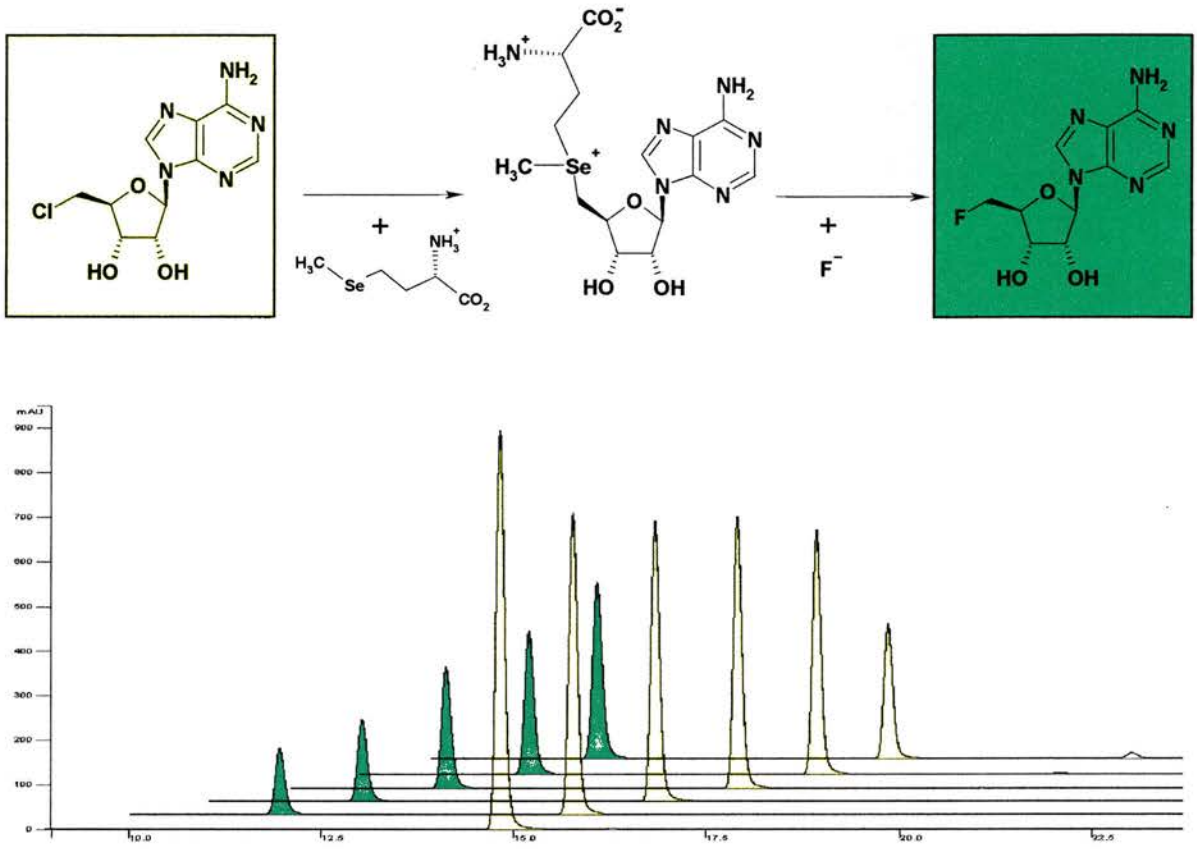
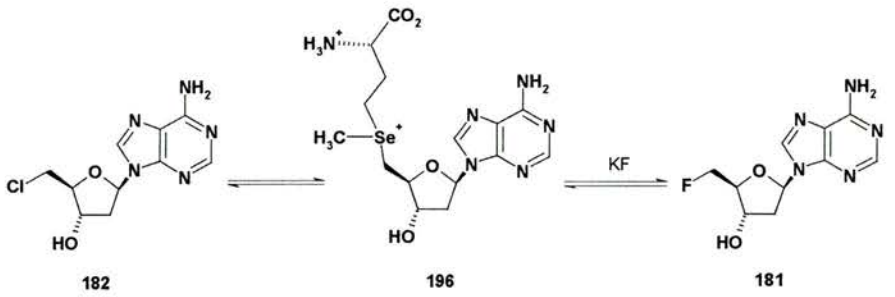


Figure 3.11 Transhalogenation reaction catalysed by the fluorinase (Exp 2, Table 3.6).

The 2'-deoxy substrate, 5'-chloro-2',5'-dideoxy-adenosine **182** was then tested as a substrate for the fluorinase catalysed transhalogenation reaction. HPLC analysis showed that in the presence of L-seleno-methionine **168**, KF and the fluorinase, 2'-deoxy-5'-ClDA **182** was converted to 5'-fluoro-2',5'-dideoxy-adenosine (2'-deoxy-5'-FDA) **181** (Scheme 3.24). The production of **181** was confirmed by co-injection with a reference sample.

Control experiments confirmed that the reaction did not occur in the absence of either the fluorinase or L-seleno-methionine **168**.



Scheme 3.24 Enzymatic preparation of 2'-deoxy-5'-FDA **181** by fluorinase mediated transhalogenation.

This experiment confirms that the 2'-OH group is not essential for fluorinase activity. Thus even without the hydrogen bonding contacts that the 2'-OH group makes with the enzyme, the substrate must still be correctly bound to facilitate fluoride ion attack. The X-ray structure of a 2'-deoxy-5'-FDA-fluorinase complex was recently solved in collaboration with Prof. J. Naismith and Dr. D. Robertson (University of St. Andrews) and supports a correct binding mode (Figure 3.12).

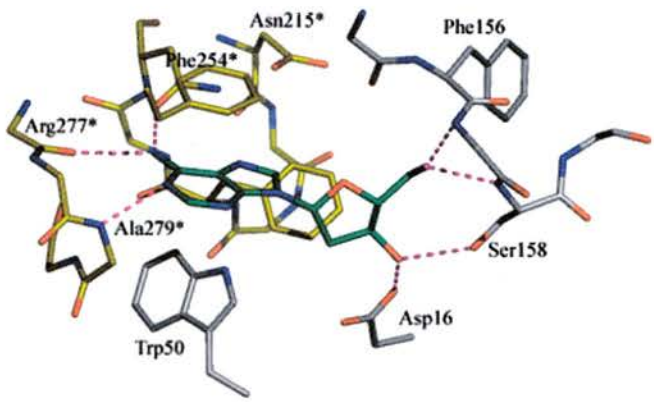
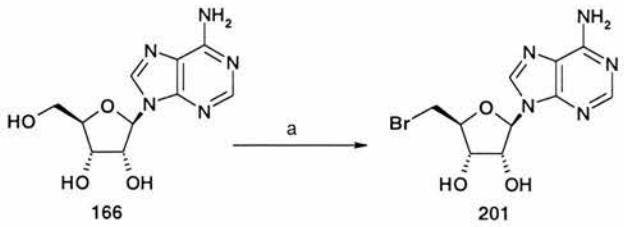


Figure 3.12 X-ray crystal structure of a 2'-deoxy-5'-FDA-fluorinase complex (Dr. D. Robertson and Prof. J. Naismith)

The conformation that 2'-deoxy-5'-FDA **181** adopts in the active site is almost identical to that exhibited by 5'-FDA **83** (compare with Figure 3.1). This implies that the mechanism for the fluorination of the 2'-deoxy substrates most likely occurs in an identical manner to the fluorination of SAM. Thus the fluoride ion must reside in the same binding pocket in the active site prior to the formation of the C-F bond in both cases. It is particularly interesting that the 3'-OH group of both 5'-FDA **83** and 2'-deoxy-5'-FDA **181** is involved in hydrogen bonding to both Ser158 and Asp16 of the protein. It is likely that these hydrogen bond interactions play a significant role in orientating the nucleosides in the correct conformation within the active site. This suggests that the removal of the 3'-OH may lead to a substantial or complete loss of enzymatic activity for 3'-deoxy-nucleoside substrates.

Attempts to convert 5'-FDA **83** into 5'-ClDA **165** using similar reaction conditions to those outlined in Table 3.6 were unsuccessful. Thus the fluorinase was not sufficiently reversible to convert organofluorine molecules to organochlorine products.

Having looked at both 5'-FDA **83** and 5'-ClDA **165** it was also of interest to investigate if 5'-bromo-5'-deoxy-adenosine (5'-BrDA) **201**[230] could also be used as a substrate. As bromine is considerable larger than fluorine it would not be expected that 5'-BrDA **201** would be so readily accommodated in the active site of the fluorinase. A synthetic sample of 5'-BrDA **201** was prepared in a relatively straightforward manner using the procedure outlined in Scheme 3.25.[231]



Scheme 3.25 Reagents and conditions: a) $P(Ph)_3$, CBr_4, pyridine, R.T, 4 h, 57 %.

The substrate analogue 5'-BrDA **201** was then explored as a potential substrate for fluorinase using the experimental protocol described in Table 3.7.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (μl)		
5'-BrDA **201** (6.0 mM)	10	10	10
L-methionine **84** (100 mM)	2	-	-
L-seleno-methionine **168** (100 mM)	-	2	-
KF (0.5 M)	5	5	5
Fluorinase (10 mg / ml)	100	100	100

All incubations were carried out at 37 °C for 16 h.

Table 3.7 Experimental protocol used to investigate if the fluorinase would catalyse the transhalogenation of 5'-BrDA **201** to 5'-FDA **83**.

HPLC analysis carried out after each of the experiments shown in Table 3.7 (Figure 3.13). The results confirmed the production of 5'-FDA **83** in all three samples. It was striking that 5'-FDA **83** was generated from 5'-BrDA **201** in the absence of **84** and **168** (Exp 3, Table 3.7, Figure 3.13 (i)).

Control experiments carried out in the absence of enzyme ruled out the possibility that the conversion of 5'-BrDA **201** to 5'-FDA **83** was occurring by a non-enzymatic process (Figure 3.13, (ii)), so clearly a different reaction mechanism is operating. Presumably the C-F bond in 5'-FDA **83** is being formed by fluoride ion directly displacing the bromine atom at C-5' and the fluorinase is activating fluoride as a nucleophile (Scheme 3.26).

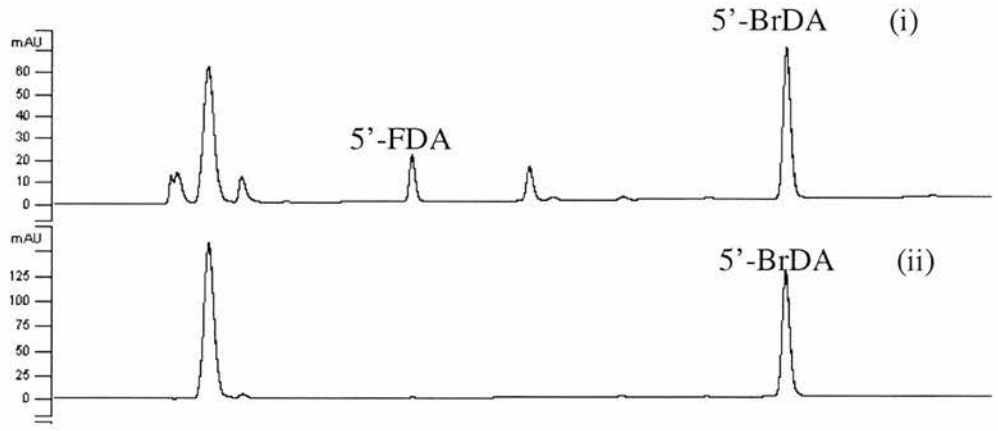
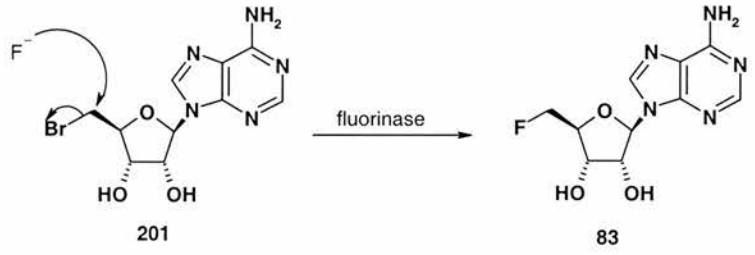


Figure 3.13 HPLC (UV, 0-20 min) analyses; (i) Exp 3 (Table 3.7) and control experiment (ii) 5'-BrDA + KF + buffer only.



Scheme 3.26 Direct fluorination of 5'-BrDA **201** catalysed by the fluorinase.

The X-ray crystal structures of the various fluorinase-complexes that have been determined so far suggest that it is unlikely that the C5'-Br bond of 5'-BrDA **201** can sit in the similar position to C5'-F bond of 5'-FDA **83** due to steric factors. Thus it can be tentatively proposed that the bromine atom of 5'-BrDA may sit where the sulfur atom of SAM normally resides. In this position 5'-BrDA **201** would act as a SAM **85** mimic and would be susceptible to fluoride ion attack to generate 5'-FDA. However without conclusive structural evidence this can only be a viewed as a working hypothesis.

5'-BrDA **201** was tested as a potential substrate for the enzymatic preparation of 5'-ClDA **165** using the experimental protocol shown in Table 3.8.

	Exp 1	Exp 2	Exp 3
	Volume (μl)		
5'-BrDA **201** (6.0 mM)	10	10	10
L-methionine **84** (100 mM)	2	-	-
L-seleno-methionine **168** (100 mM)	-	2	-
NaCl (0.5 M)	5	5	5
fluorinase (10 mg / ml)	100	100	100

Incubations were carried out at 37 °C for 16 h.

Table 3.8 Experimental protocol used to investigate if the fluorinase would catalyse the transhalogenation of 5'-BrDA **201** to 5'-ClDA **165**.

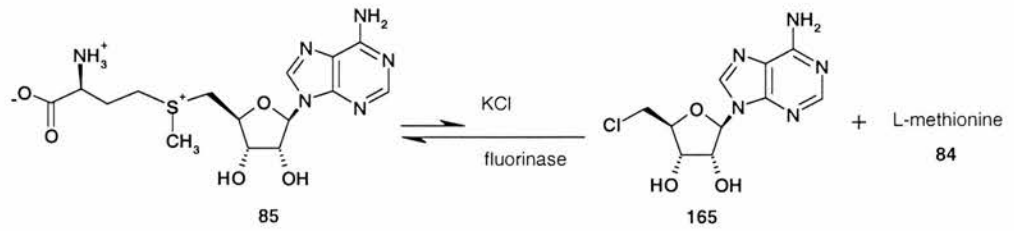
HPLC analysis was carried out on each of the experiments (1-3) shown in Table 3.8. The results showed that it was not possible to prepare 5'-ClDA **165** from 5'-BrDA **201** under any of the reaction conditions tested.

3.3.4 Is chloride ion a nucleophile for the fluorinase

In order to explore the level of specificity that the fluorinase shows for fluoride ion it was of interest to investigate if other halide ions could be accepted as substrates in the absence of fluoride ion. To this end chloride was investigated as a possible substrate for the fluorinase. Accordingly SAM (20 mM, 20 μl), excess chloride ion (KCl, 0.5 M, 10 μl), fluorinase (100 μl of 3 mg / ml, Tris-HCl buffer, 20 mM, pH 7.4) and Tris-HCl buffer (100 μl 20 mM, pH 7.4) were incubated for 16 hours at 37 °C. HPLC analysis gave no indication of 5'-ClDA **165** production. A synthetic reference was available for comparison. The experimental conditions were modified but neither an increase in the concentration of chloride ion or of the enzyme facilitated the formation of 5'-ClDA **165**. This experiment suggested that chloride ion is not a substrate for the fluorinase.

However the fact that the fluorinase catalyses the formation of SAM **85** from 5'-ClDA **165** and L-methionine **84** in the reverse direction raised an interesting question. If chloride ion is a leaving group it must have a binding site on the enzyme. Perhaps the equilibrium lies

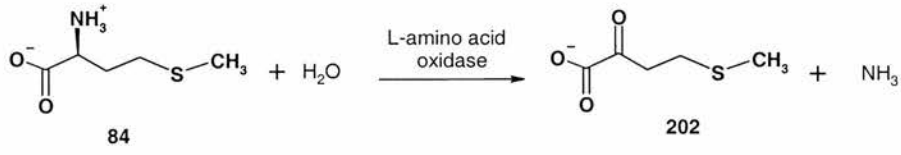
in favour of free chloride ion and not a organo-chloro product. Clearly if the reverse reaction could be inhibited then any 5'-ClDA **165** formed may accumulate. The strategies that were employed to try and encourage a shift in the equilibrium towards 5'-ClDA **165** are discussed in the following sections.



Scheme 3.27 Putative description of the enzymatic chlorination reaction mediated by the fluorinase.

3.3.4.1 Enzymatic preparation of 5'-ClDA from SAM

L-amino acid oxidase (EC 1.4.3.2)[232] from snake venom is a commercially available enzyme that converts L-methionine **84** to the α-oxo-acid **202** (Scheme 3.28). By coupling this enzyme to the fluorinase reaction any L-methionine formed will be consumed and thus unable to support the formation of SAM by the reverse reaction.



Scheme 3.28 Oxidation of L-methionine catalysed by L-amino acid oxidase (EC 1.4.3.2)

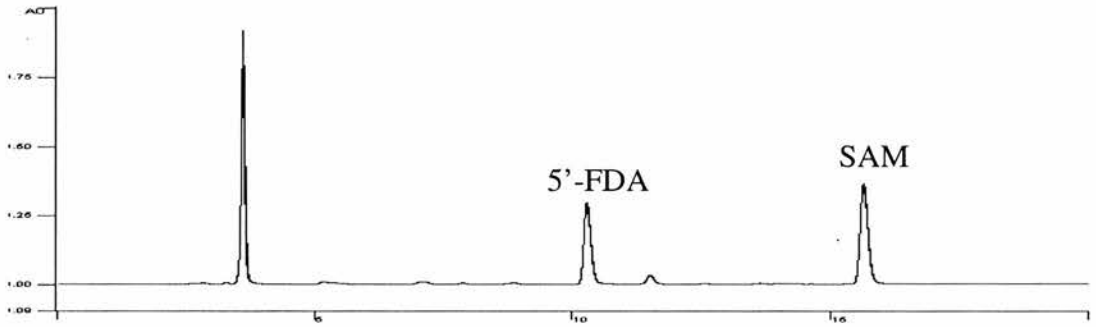
Thus an incubation of SAM **84** with chloride ion was carried out with both the fluorinase and the L-amino acid oxidase according to the procedure detailed in Table 3.9. A control experiment was also conducted where chloride was replaced with fluoride.

	Exp1	Exp 2	Exp 3	Exp 4
	Volume added (µl)			
SAM **85** (13 mM)	25	25	25	25
L-amino acid oxidase (10 mg / ml)	200	-	200	-
KF (0. 5 M)	10	10	-	-
KCl (0.5 M)	-	-	10	10
Fluorinase (2.6 mg / ml)	200	200	200	200
Tris-HCl Buffer pH 7.8 (50 mM)	100	300	100	300

Incubations were carried out for 16 h at 37 °C. Both fluorinase and the L-amino acid oxidase were prepared in Tris-HCl buffer, 50 mM, pH 7.8.

Table 3.9 Experimental protocol used to investigate the ability of the fluorinase / L-amino acid oxidase to catalyse the halo-nucleoside formation.

The production of 5'-FDA **83** was confirmed in the control reaction Exp 1 (Table 3.9) after HPLC and GC-MS analysis (Figure 3.14 (i) HPLC and (ii) GC-MS). This showed that the L-amino acid oxidase had no inhibitory effect on the fluorinase. Production of 5'-FDA **83** was also confirmed in Exp 2 in the absence of the L-amino acid oxidase. Significantly the production of 5'-ClDA **165** was confirmed by both HPLC and GC-MS analysis of Exp 3 (Figure 3.15 (i) HPLC and (ii) GC-MS) but not Exp 4.



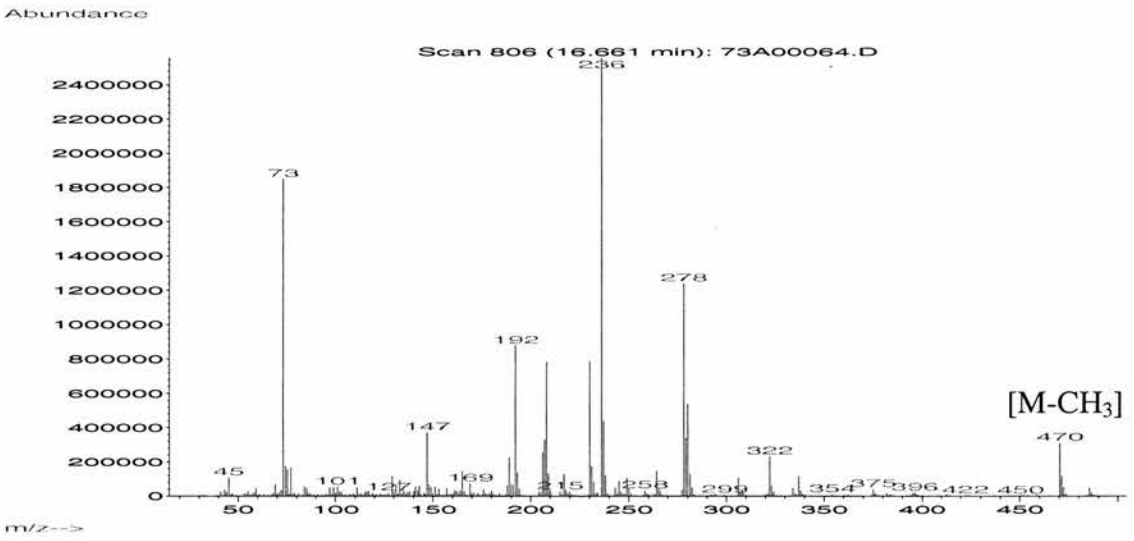


Figure 3.14 HPLC (UV) and GC-MS (after MSTFA derivatisation) confirmation of 5'-FDA **83** production in Exp 1 (Table 3.9).

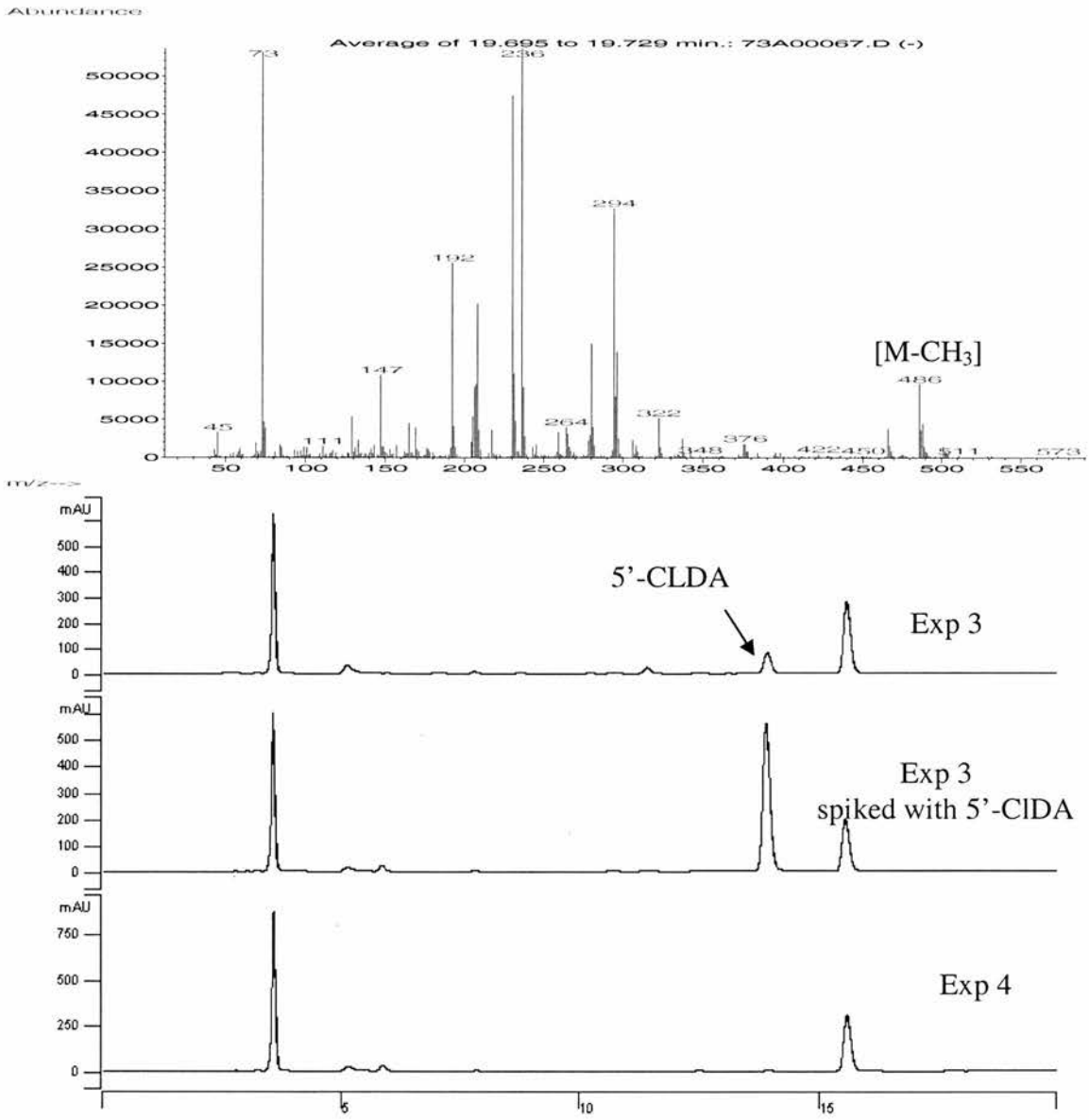
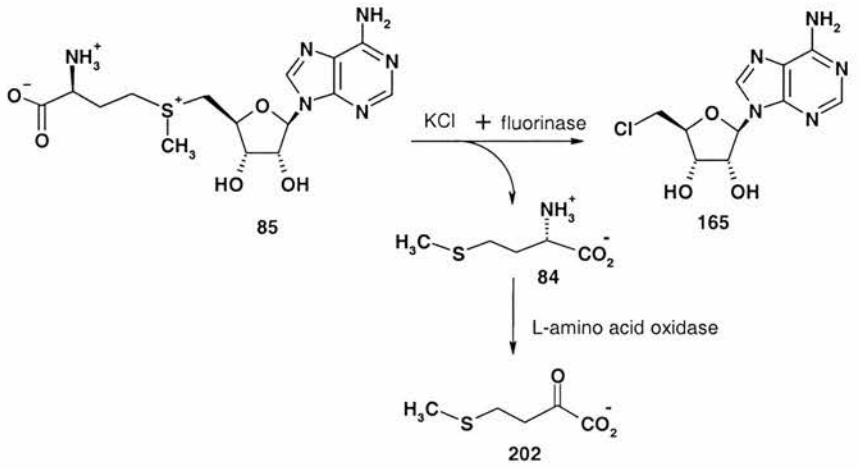


Figure 3.15 HPLC (UV) of Exp 3 and Exp 4 and GC-MS confirmation of 5'-ClDA **165** production in Exp 3 (Table 3.9).

This showed that for the first time, the fluorinase can catalyse the formation of a C-Cl bond in the absence of fluoride. Presumably chlorination proceeds by an S_N2 process in a similar manner to fluorination. The fact that no 5'-ClDA **165** was detected in the absence of L-amino acid oxidase indicates that under "normal" reaction conditions the equilibrium lies in favour of SAM **85**. However in the presence of L-amino acid oxidase, L-methionine **84** is removed from the reaction and thus the equilibrium shifts in favour of 5'-ClDA **165** formation (Scheme 3.29).

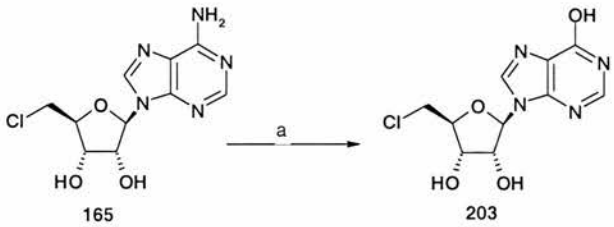


Scheme 3.29 Enzymatic preparation of 5'-ClDA **165** catalysed by FDAS in the presence of L-amino acid oxidase.

Fluorinase incubations carried out in combination with the oxidase using KBr in place of KCl did not lead to the formation of 5'-BrDA **201**. This is consistent with the results from the transhalogenation studies (see previous) that suggested that it would not be possible for the active site of fluorinase to accommodate both SAM **85** and a bromide ion at the same time.

3.3.4.2 Enzymatic preparation of 5'-ClDI

An alternative procedure to investigate the chlorination reaction involved the enzymatic conversion of 5'-ClDA **165** to an inert shunt product. One strategy to achieve this was to couple the fluorinase reaction with a commercially available deaminase enzyme (AMPDA). If AMPDA could be used to convert 5'-CDA **165** to 5'-CLDI **203** *in situ* this would prevent the reformation of SAM **85**. In order to explore this idea a reference sample of 5'-chloro-5'-deoxy-inosine **203** (5'-ClDI) was required. Accordingly 5'-ClDA **165** was treated with AMPDA (Scheme 3.30). The reaction was monitored by HPLC and was shown to be complete after 12 h. 5'-ClDI **203** was produced using this method in quantitative yield with a purity of greater than 95 % (determined by HPLC).



Scheme 3.30 Reagents and conditions: a) AMPDA, 25 °C, 12 h, 100%.

An incubation of SAM **85** with chloride ion was then carried out with both the fluorinase and AMPDA according to the procedure detailed in Table 3.10. A control experiment was also conducted in which SAM **85**, KF, fluorinase and AMPDA were incubated to confirm that the fluorinase remained active in the presence of AMPDA.

	Exp1	**Exp 2**
	Volume added (µl)	
SAM **85** (20 mM)	100	100
AMPDA (1 mg / ml)	20	20
KF (0. 5 M)	40	-
KCl (0.5 M)	-	40
Fluorinase (10 mg / ml)	100	-

Incubations were carried out for 16 h at 37 °C. The fluorinase was prepared in Tris-HCl buffer 50 m, pH 7.8. AMPDA was prepared as a solution in water.

Table 3.10 Experimental protocol used to investigate the enzymatic preparation of 5'-ClDI **203** and 5'-FDI **86**.

The ability of the fluorinase to function in the presence of AMPDA was confirmed by the detection of 5'-FDI **86** in Exp 1 (Table 10) by both GC-MS and HPLC analysis with a reference (HPLC, Figure 3.16) Control experiments confirmed that the production of 5'-FDI **86** only occurred in the presence of both the fluorinase and AMPDA. The production of 5'-FDI **86** in Exp 1 was assumed to occur by the reaction sequence outlined in Scheme 3.31.

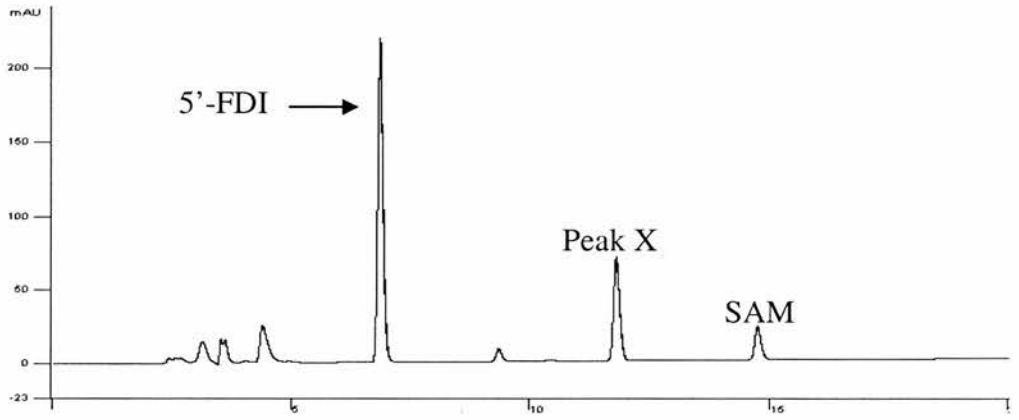
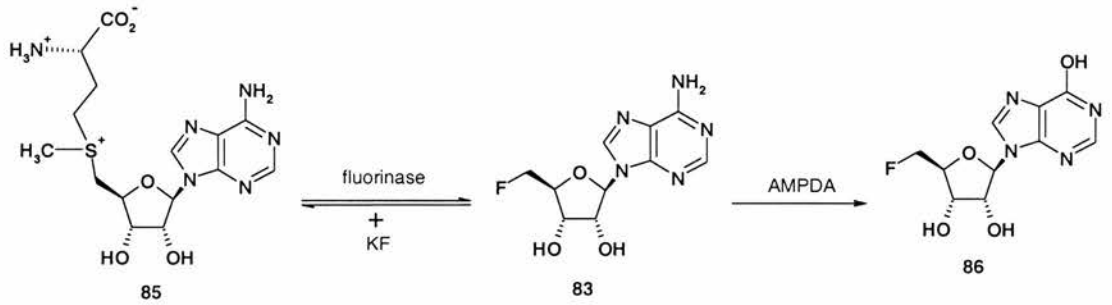


Figure 3.16 HPLC analysis of products from Exp 1 (Table 3.10).



Scheme 3.31 Enzymatic preparation of 5'-FDI **86** catalysed by the fluorinase / AMPDA coupled enzymatic process.

The successful production of 5'-ClDI **203** in Exp 2 (Table 3.10) was confirmed by both HPLC and GC-MS analysis (HPLC, Figure 3.17). As AMPDA cannot convert SAM **85** directly into 5'-ClDI **203**, 5'-ClDI must arise by the enzymatic deamination of 5'-ClDA **165** (Scheme 3.32). This observation therefore reinforces the amino-acid oxidase experiment and is further evidence that the fluorinase can catalyse enzymatic carbon – chloride bond formation in the absence of fluoride ion.

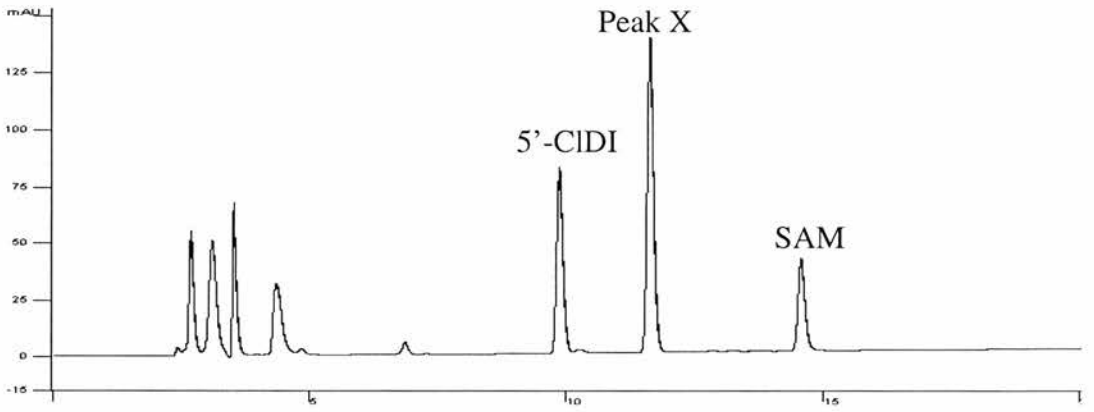
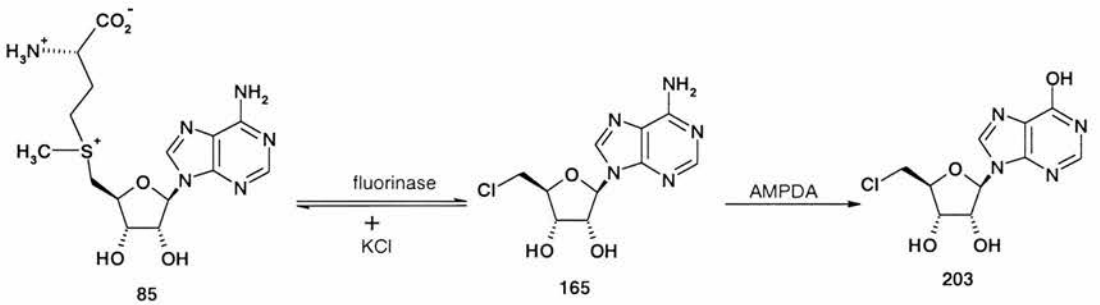
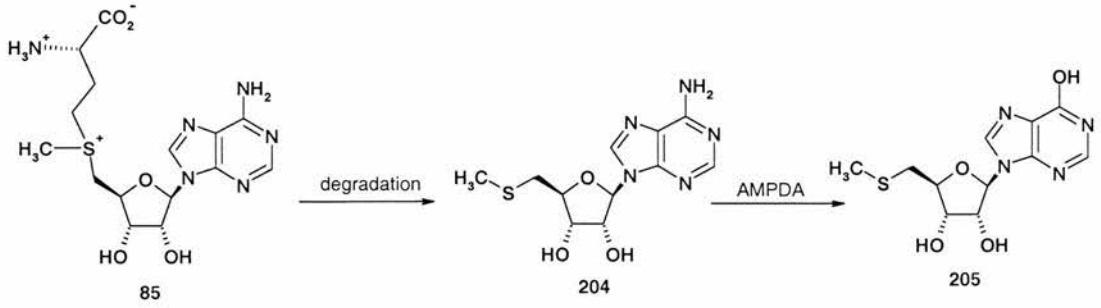


Figure 3.17 HPLC analysis of products from Exp 2 (Table 3.10).



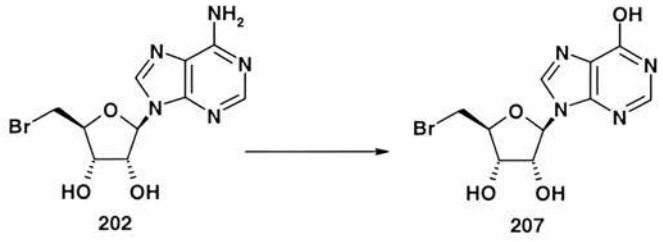
Scheme 3.32 Enzymatic preparation of 5'-ClDI **203** catalysed by the fluorinase / AMPDA coupled enzyme process.

The compound labelled "peak X" which was generated in both Exp 1 (Figure 3.15) and Exp 2 (Figure 3.16) was identified as 5'-thiomethyl-inosine **205**. A reference sample of **205** was prepared from 5'-thiomethyl-adenosine **204** using AMPDA. The production of **205** in Exp 1 and 2 is believed to arise from the *in situ* formation of **205** from the degradation of SAM **85**. Deamination of **204** by APMDA would thus give **205** (Scheme 3.33). It is interesting to note that control experiments showed that in the presence of AMPDA but without any halide ion, SAM **85** is completely degraded within 4 h at 37 °C.



Scheme 3.33 Process accounting for the formation of **205** in Exp 1 and Exp 2 (Table 3.10).

In order to investigate the possibility that bromide might be accepted as a substrate for the fluorinase a reference sample of 5'-bromo-5'-deoxy-inosine **206** was prepared from 5'-BrDA **201** using AMPDA (Scheme 3.34).



Scheme 3.34 Reagents and conditions: a) AMPDA, 25 °C, 12 h, 100%.

Using the same reaction conditions outlined (Table 3.10) but with bromide ion as the halide source, several attempts were made to prepare 5'-BrDI **206**. However this proved to be unsuccessful and reinforced the earlier conclusions for the reactions carried out in the presence of L-amino acid oxidase, that the fluorinase will not accept bromide as a substrate.

3.4 Conclusion

The fluorinase has been shown to catalyse a reversible reaction. It has the ability to catalyse the formation of SAM **85** from 5'-FDA **83** and L-methionine **84**. The reaction is thought to proceed by an S_N2 mechanism in which L-methionine acts as a nucleophile and

attacks the C-5' position of 5'-FDA. This leads to the cleavage of the C5'-F bond in 5'-FDA and the simultaneous formation of a new C5'-S bond. 5'-ClDA **165** can be used as an alternative substrate to 5'-FDA for the fluorinase catalysed production of SAM. The use of 5'-ClDA leads to a rate enhancement for the reaction consistent with the C5'-Cl bond being weaker than the C5'-F bond. Several commercially available L-methionine analogues (**168**, **171-173**, **175** and **176**) were investigated as alternative substrates but only L-seleno-methionine **168** and D-methionine **176** were accepted by the fluorinase.

To further explore the substrate specificity of the fluorinase, 2'-deoxy-5'-FDA **181** and 2'-deoxy-5'-ClDA **182** were prepared from the commercially available precursor 2'-deoxy-adenosine (2'dA) **185**. The preparation of 3'-deoxy-adenosine (3'dA) **190** was achieved from adenosine **166** using a literature procedure. However attempts to convert 3'dA **190** into 3'-deoxy-5'-FDA **183** and 3'-deoxy-5'-ClDA **184** using similar synthetic procedures to those utilised in the preparation of **181** and **182** were unsuccessful. Attempts to prepare 3'-deoxy-5'-FDA **183** and 3'-deoxy-5'-ClDA **184** by alternative synthetic routes were also unsuccessful. The difference in reactivity between the 5'-OH groups in 2'dA **185** and 3'dA **190** may be due to the fact that the molecules favour different conformations in solution. 2'dA **185** prefers a South (S) conformation whereas 3'dA **190** predominantly adopts a North (N) type conformation. The N conformation that 3'dA **190** adopts may result in unfavourable steric interactions between the base and the 5'-OH group. This steric hinderance may reduce the reactivity of the 5'-OH group.

The fluorinase was shown to convert both 2'-deoxy-5'-ClDA **182** and 2'deoxy-5'-FDA **181** into 2'-deoxy-SAM **177**. It was also possible to prepare 2-amino-SAM **198** from 2-amino-5'-ClDA **195** using the same strategy. This work highlighted that the fluorinase may

have a role to play in the chemo-enzymatic preparation of novel SAM analogues. These analogues may be useful in the study of other SAM dependent enzymes.

By exploiting the reversibility of the fluorinase it was found that it could be used to mediate a novel enzymatic transhalogenation reaction in which 5'-ClDA is converted to 5'-FDA. In addition to KF the transhalogenation reaction required either L-methionine **84** or L-seleno-methionine **168** in order to proceed. This is because the reaction mechanism involves a two step process. Firstly 5'-ClDA **165** is converted to SAM **85** or seleno-SAM **169** depending on whether the reaction is carried out with **84** or **168**. The second stage of the process involves the enzymatic fluorination of SAM or seleno-SAM to give 5'-FDA **83**. It was possible to convert 2'-deoxy-5'-ClDA **182** to 2'-deoxy-5'-FDA **181** using this technique. This confirmed that the 2'-OH group is not essential in order for the fluorinase to exhibit its catalytic activity.

It was not possible to convert 5'-FDA to 5'-ClDA using a similar reaction as the equilibrium disfavours this process. Intriguingly the fluorinase did catalyse the conversion of 5'-BrDA **201** directly to 5'-FDA **83** with fluoride ion without any L-methionine **84** or L-selenomethionine **168**. Thus in this case the enzyme is catalysing a direct halogen displacement. This was an unexpected result and implies that the bromine atom must be orientated in the active site in a different position to both the fluorine and chlorine atoms, most probably in the sulphur site.

Chloride ion was not accepted as a substrate by the fluorinase under normal reaction conditions. It emerged that this was because the products of the chlorination reaction 5'-ClDA **165** and L-methionine **84** acts as efficient substrates for the reverse reaction. Consequently the reformation of SAM **85** is favoured over the accumulation of 5'-ClDA **165**. However it was found that this equilibrium can be shifted to favour the formation of

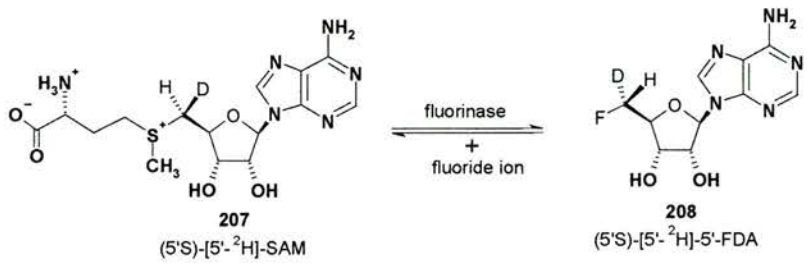
the chlorinated product 5'-ClDA **165** if the reverse reaction was prevented. It was possible to achieve this by carrying out the fluorinase catalysed chlorination reaction in the presence of an L-amino acid oxidase. This enzyme converts L-methionine **84** into its oxo-acid derivative **202** and thus prevents the enzymatic reformation of SAM **85**. It was also possible to convert SAM **85** to 5'-ClDI **203** if a deaminase enzyme (AMPDA) was added in addition to the fluorinase to deaminate 5'-ClDA **165** *in situ*. The production of both 5'-ClDA **165** and 5'-ClDI **203** showed that the fluorinase can catalyse the formation of a carbon-chloride bond and that chloride ion is a substrate.

Part B Enhancing the rate of the fluorination reaction

3.5 Introduction

3.5.1 Enzymatic fluorination occurs by an S_N2 mechanism

An early stereochemical study carried out with deuterium labelled glycerol indicated that the carbon – oxygen bond of glycerol was converted to the carbon fluorine bond in FAc **15** with an overall retention of configuration.[215] During this study it was assumed that the chirality of the labelled glycerol remained unchanged during its biosynthetic incorporation into ATP **82**. As the conversion of ATP **85** to SAM **85** is known to occur with an inversion of stereochemistry it was suggested that the fluorination of SAM **85** must also proceed with an inversion of stereochemistry. Hence two inversions give the observed retention of configuration. However this study relied on two assumptions. Firstly it relied on the correct interpretation of the metabolic and stereochemical incorporation of glycerol into ATP **82** and secondly it was FAc **15** and not the direct product of the fluorination event (5'-FDA) that was analysed. Thus it was assumed that the stereochemistry of the C-F bond does not change after it has been formed. The availability of purified fluorinase prompted a new stereochemical study to be carried out by Cadicamo *et al.*[216] They were able to prepare a chiral derivative of SAM that carried a deuterium at C-5'. The fluorination of (5'S)-[5'-^{2}H]SAM **207** generated (5'R)-[5'-^{2}H]5'-FDA **208** (Scheme 3.35). The absolute configuration of the C-5' carbon of the fluorinated product **208** was determined using chiral liquid crystal ^{2}H NMR analysis against a synthetically prepared standard. The results confirmed that the fluorinase catalysed reaction proceeds with an inversion of configuration at the C-5' position.



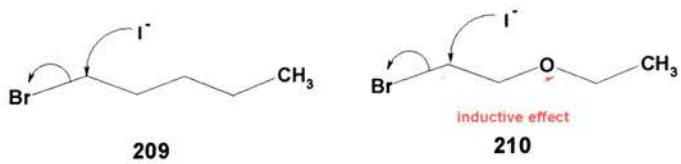
207
(5'S)-[5'-^{2}H]-SAM

208
(5'S)-[5'-^{2}H]-5'-FDA

Scheme 3.35 The fluorination of **207** catalysed by the fluorinase proceeds with an inversion of stereochemistry at C-5'.[216]

3.5.2 Stereoelectronics of enzymatic fluorination

Early investigations on S_N2 reactions revealed that an oxygen atom *beta* to the leaving group retards the rate of nucleophilic substitution. It was reported that the nucleophilic displacement of bromide with iodide in n-butyl bromide **209** occurs 10 times faster (at 25 °C) than the displacement of the bromine from β-bromo-ethyl ethyl ether **210**.[233] The difference in the reaction rates can be rationalised by considering the inductive effect (I-) that that oxygen imparts on the molecule. This gives rise to an increase in the bond strength of the C-Br bond which consequently results in a decrease in the rate of nucleophilic substitution. Also in the preferred conformation the nucelophile has to pass the oxygen atom (Figure 3.18).



209 210

Figure 3.18 The effect of a *beta* oxygen on the rate of nucleophilic substitution.

It was felt that a similar *beta* oxygen effect might be operating during the fluorinase catalysed nucleophilic fluorination of SAM **85**. This is because the O-4' atom in SAM sits in the *beta* position to the C5'-S bond that is to be cleaved during the fluorination reaction (Figure 3.19, (i)). It is also likely that any inductive (I-) effect exerted by the O-4' atom in this case will be increased by any anomeric effect (AE) exhibited by the nucleoside.[234,235] It is interesting to note that O-4' of SAM **85** does not form any hydrogen bonds with the protein when the molecule is bound in the active site of the fluorinase. This could be due to the fact that such interactions would further increase the inductive effect of the oxygen atom and may lead to the rate of the fluorination reaction being reduced further.

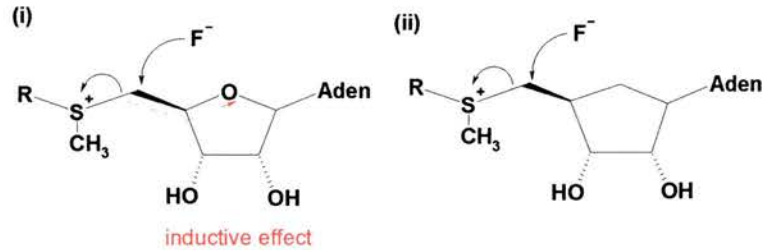
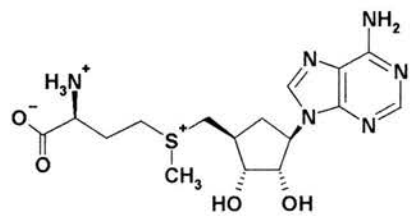


Figure 3.19 The influence of the O-4' atom on the rate of the fluorination reaction catalysed by the fluorinase.

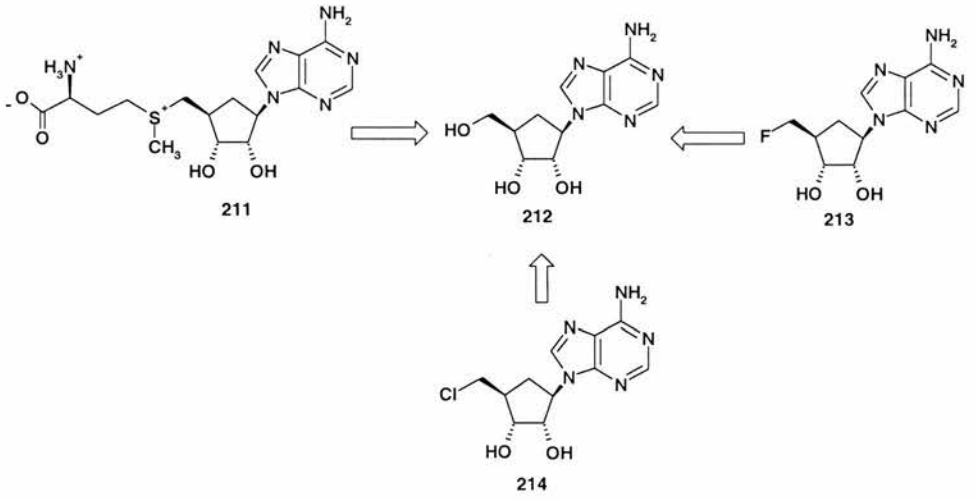
In theory replacement of the O-4' atom of SAM **85** (Figure 3.19 (ii)) by CH_2 should remove the electronegativity that may impede the nucleophilic reaction (Figure 3.19 (i)). In turn such a replacement may lead to an increase in the rate of the fluorination reaction. In order to investigate this hypothesis it became an objective to prepare a sample of carbocyclic SAM **211** in order to explore it as a substrate for the fluorination reaction.



211

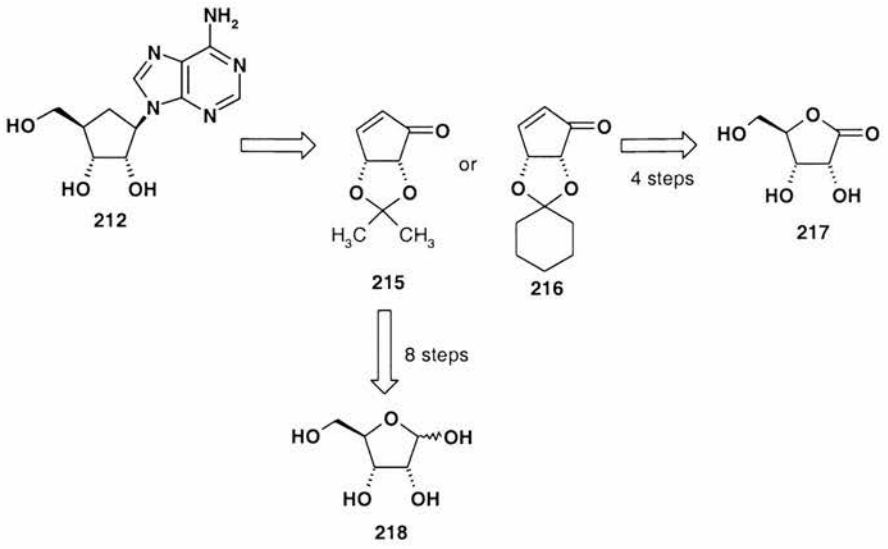
3.6 The synthesis (*2R, 3R*)-2,3-(cyclohexylidenedioxy)-4-cyclopentenone a key intermediate in the preparation of carbocylic SAM.

Retrosynthetic analysis revealed that carbocyclic SAM **211** could be prepared from (-)-aristeromycin **212** (Scheme 3.36). Aristeromycin **212** could also be used as a convenient starting point for the synthesis of **213** and **214**, compounds that would be a required as reference samples. As it happens **212** is a naturally occurring nucleoside[236] which has been extensively used as a building block in the preparation of carboxylic nucleosides.[237,238] Consequently an extensive range of literature exists detailing the synthesis of **212**.[239,240]



Scheme 3.36 (-)-Aristeromycin **212** the key building block for the synthesis of **211**, **213** and **214**.

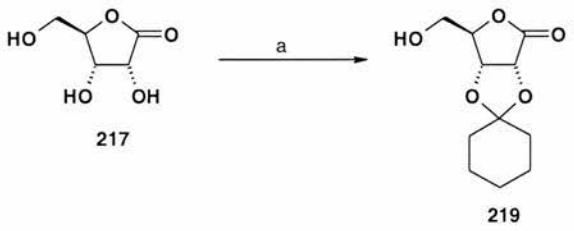
The key intermediates on the majority of the synthetic routes to **212** are the L-2-cyclopentenones, (*2R, 3R*)-2,3-*O*-isoproylidene-4-cyclopentenone **215** and (*2R, 3R*)-2,3-(cyclohexylidenedioxy)-4-cyclopentenone **216** (Scheme 3.37).



Scheme 3.37 Retrosynthetic analysis for the preparation of **212**.

The decision was made to use the synthetic procedure developed Brochardt and coworkers.[241,242] and to prepare the key intermediate **216**. This is shorter than the alternative route to the **215** developed by Chu and coworkers.[243]

Treatment of D-ribonolactone **217** with cyclohexanone in the presence of $FeCl_3$ afforded 2,3-cyclohexylidene-D-ribonolactone **219** in a good yield (Scheme 3.38).[244]

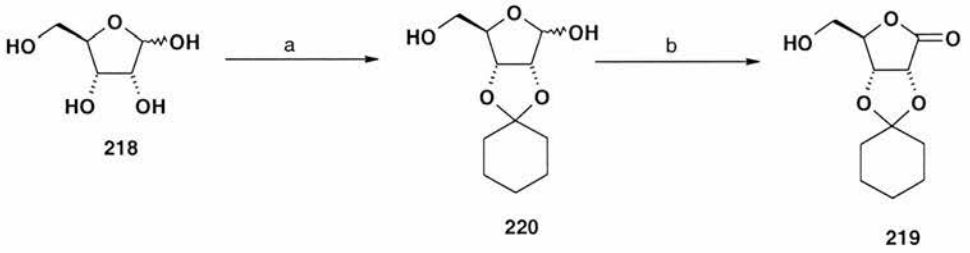


Scheme 3.38 Reagents and conditions: a) cyclohexanone. $FeCl_3$, 50 °C, 3 h, 87%.

Unfortunately the supply of **217** was found to be rather unreliable and therefore an alternative method to prepare **219** was sought. Liu and Caperelli reported it was possible to prepare **219** in two steps from D-ribose **218**.[245] This represented an attractive alternative route to **219** as D-ribose **218** is both inexpensive and commercially available. The treatment of **218** with cyclohexanone and conc. H_2SO_4 (cat) at R.T for 2 h was found to

yield **220** but in a poor yield.[245] Increasing the reaction time was found not to improve the yield of the reaction. However further investigations revealed that the production of **220** proceeded more efficiently if *p*-toluenesulfonic acid (*p*TSA) was used in place of H_2SO_4 as the acid catalyst.[246] Accordingly D-ribose **218** was reacted with *p*TSA (cat) and cyclohexanone for 12 h at R.T and afforded **220** in an excellent yield (Scheme 3.39). It was also possible to carry out the reaction on a large scale (up to 30 g) with out any detrimental effect on the product yield.

The oxidation of **220** to give 2,3-*O*-cyclohexylidene-D-ribonolactone **219** was achieved using both pyridinium dichromate (PDC) and pyridinium chlorochromate (PCC) under the reaction conditions detailed by Liu and Caperelli.[245] However in both cases the yield of **219** recovered from the reactions was lower than that reported in the literature (PDC, 33% lit.[245] 59%, PCC, 38%, lit.[245] 59%).



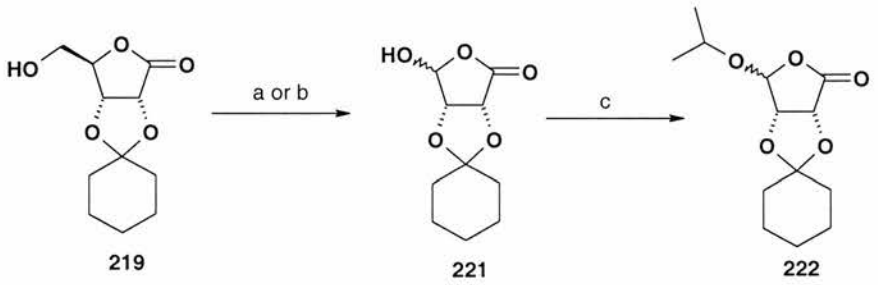
Scheme 3.39 Reagents and conditions: a) cyclohexanone, pTSA, R.T, 12 h, 82% b) PDC, DCM, R.T, 16 h, 33% c) PCC, DCM, R.T, 16 h, 38%.

TLC analysis of the crude reaction mixture showed that several by-products were produced during the PDC / PCC oxidation of **220**. Unfortunately it was not possible to isolate any of these from the crude reaction mixture but it was suspected that the unwanted oxidation of the free primary hydroxyl group (5-OH) may have caused problems during the reaction.

Conversion of **219** into 2,3-*O*-cyclohexylidene-L-erythruronolactone **221** was achieved in good yield (61%) using the procedure detailed by Vasella (Scheme 3.40, reagents and

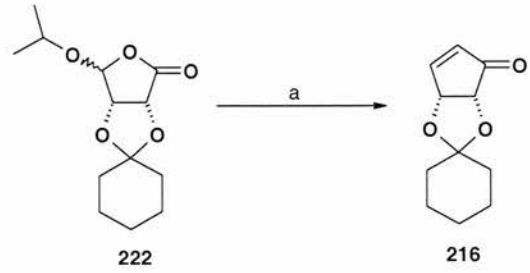
conditions: a).[244] However it was found that the reaction yield could be increased to 85% if 1,4-dioxane was used as a co-solvent (Scheme 3.40, reagents and conditions: b).[245]

Treatment of **221** with 2-propanol and *p*-toulenesulfonate at reflux for 2 h afforded 2,3-(cyclohexylidenedioxy)-4-hydroxy-4-(2-propyloxy)-butyrolactone **222** in a moderate yield (41%).[241] This was a considerably lower yield that that reported in the literature (80%) by Brochardt *et al.*[241] However several other groups have also reported problems in trying to obtain yields of greater than 50% for this reaction.[247] In a recent analysis carried out by Mariën *et al.*[248] they concluded that typically the yield achieved for the conversion of **221** to **222** was between 41%-51%. Using the "optimum" reaction conditions reported by Mariën *et al.* the yield of **221** was increased to 48% (Scheme 3.40).



Scheme 3.40 Reagents and conditions: a) $NaIO_4$, NaOH, 0 °C, 10 min, 61% b) 1,4-dioxane, $NaIO_4$, NaOH, 0 °C, 10 min, 85% c) 2-propanol, p-toluenesulfonate, 60 °C, 12 h, 48%.

Finally **222** was treated with dimethyl methylphosphonate and BuLi (2.5 M hexane) to give 2,3-(cyclohexylidenedioxy)-4-cyclopentenone **216** in a moderate yield (Scheme 4.41).[241] Once again the yield was consistent with that previously reported in the literature.[248, 249] The opening steps of the synthetic route to (-)-aristeromycin **212** have been accomplished. The route now continues in the laboratory as part of the research of a new PhD student within the group.



Scheme 3.41 Reagents and conditions: a) dimethyl methyl phosphonate, BuLi (2.5 M hexane), -78 °C – R.T, 4 h, 59%.

3.7 Conclusion

In order to investigate the effect of the O-4' atom in SAM **85** on the rate of the fluorination reaction, a synthesis of the carbocyclic SAM **211** was addressed. Retrosynthetic analysis of **211** revealed that it could be prepared from the well known carbocyclic nucleoside (-)-aristeromycin **212**. Several synthetic routes to **212** exist within the literature. A review of this material identified 2,3-(cyclohexylidenedioxy)-4-cyclopentenone **216** as the first key intermediate on the synthetic pathway to **212**. Thus the preparation of 2,3-(cyclohexylidenedioxy)-4-cyclopentenone **216** has been achieved using a combination of previously reported experimental procedures. The most problematic step on the synthetic route was the preparation of 2,3-cyclohexylidene-D-ribonolactone **219**. **219** can easily be prepared from D-ribonolactone **217** but the problems associated with the availability of this commercial starting material meant that an alternative route had to be investigated. It was possible to prepare **219** in two steps from D-ribose **218** using the synthetic methodology developed by Liu and Caperelli.[245] Unfortunately the maximum overall conversion of D-ribose **218** to **219** that could be achieved using this procedure was only 31%. It was however possible to carry out the reactions on a large enough scale such that enough **219** could be prepared to make this synthetic route viable.

4 Investigating the metabolic fate of 5'-fluoro-5'-deoxy-adenosine (5'-FDA) in *S. cattleya*

Part A: Identification of 5-fluoro-5-deoxy-D-ribose-1-phosphate (5'-FDRP) as an intermediate on the biosynthetic pathway.

4.1 Introduction

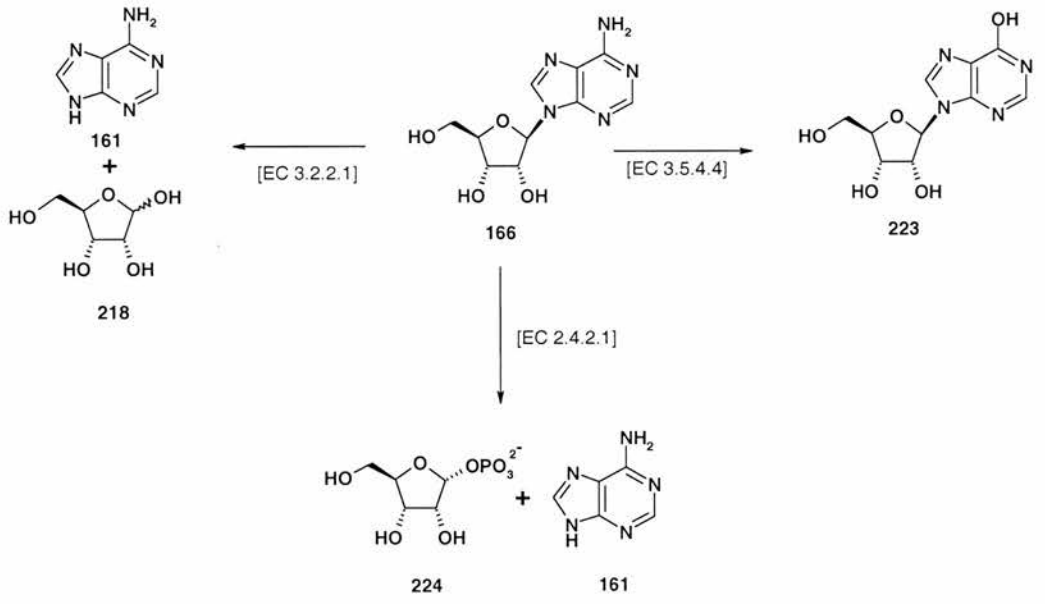
The nucleoside 5'-fluoro-5'-deoxy-adenosine (5'-FDA) **83** represents the first committed fluorinated intermediate on the biosynthetic pathway to the secondary metabolites fluoroacetate (FAc) **15** and 4-fluorothreonine (4-FT) **16** in *S. cattleya* (Chapter 2). Having established this, the next logical step was to try and identify the enzymes responsible for the metabolism of 5'-FDA **83** within *S. cattleya*. This would in turn allow the remaining fluorinated intermediates to be identified.

One of the strategies which could be utilised in the elucidation of the remaining pathway required the synthesis of potential intermediates. Such synthetic molecules would then be tested in a crude cell free extract (CFE) to see if they could support the fluorometabolite biosynthesis. The problem with this approach is guessing which molecules to prepare. In order to make an educated guess as to the nature of the next fluorinated intermediate on the pathway the metabolism of structurally similar nucleosides was reviewed.

4.1.1 A general review of adenosine metabolism in biological systems

Adenosine **166** contains a 5'-hydroxyl in place of the 5'-fluorine atom of 5'-FDA. It is an intermediate on several important biosynthetic pathways. Three of the most commonly occurring enzymes that can catalysis the metabolism of adenosine **166** are shown in

Scheme 4.1.



Scheme 4.1 The metabolism of adenosine **166**.

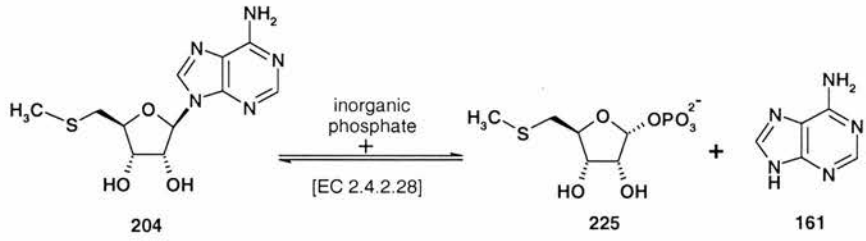
Adenosine deaminase (ADA) (EC 3.5.4.4) catalyzes the conversion of adenosine **166** to its 6-oxopurine analogue inosine **223**.[158,159] The two remaining enzymes shown in Scheme 4.1 have the ability to cleave the C-N glycosidic bond converting **166** into two components. The first of these is a purine nucleosidase (PNase) (EC 3.2.2.1),[250] an enzyme which mediates the hydrolytic cleavage of the glycosidic bond in **166** to give adenine **161** and D-ribose **218**. A second enzyme is a purine nucleoside phosphorylase (PNP) (EC 2.4.2.1).[251] PNP cleaves the C-N glycosidic bond by a phosphorolysis reaction and consequently the enzyme requires inorganic phosphate as a cofactor. The products of the reaction are ribose-1-phosphate **224** and **161**.[252,253] The enzymatic reaction is specific for β-nucleosides and the catalytic mechanism proceeds in a stereospecific manner to only give α-ribose-1-phosphate.[254]

It is noteworthy that PNP catalyses a reversible reaction where as PNase does not. The reversibility of the PNP renders ribose-1-phosphate **224** as a pivotal intermediate in

nucleoside and nucleotide metabolism and thus the enzyme is essential for normal cellular function. This has stimulated considerable biomedical interest in PNP and in particular the human form of the enzyme (hPNP). hPNP has been identified as a potential a target for chemical inhibition in an attempt to combat a variety of illness such as leukaemia.[251]

4.1.2 5'-Methylthio-adenosine (MTA) metabolism in biological systems

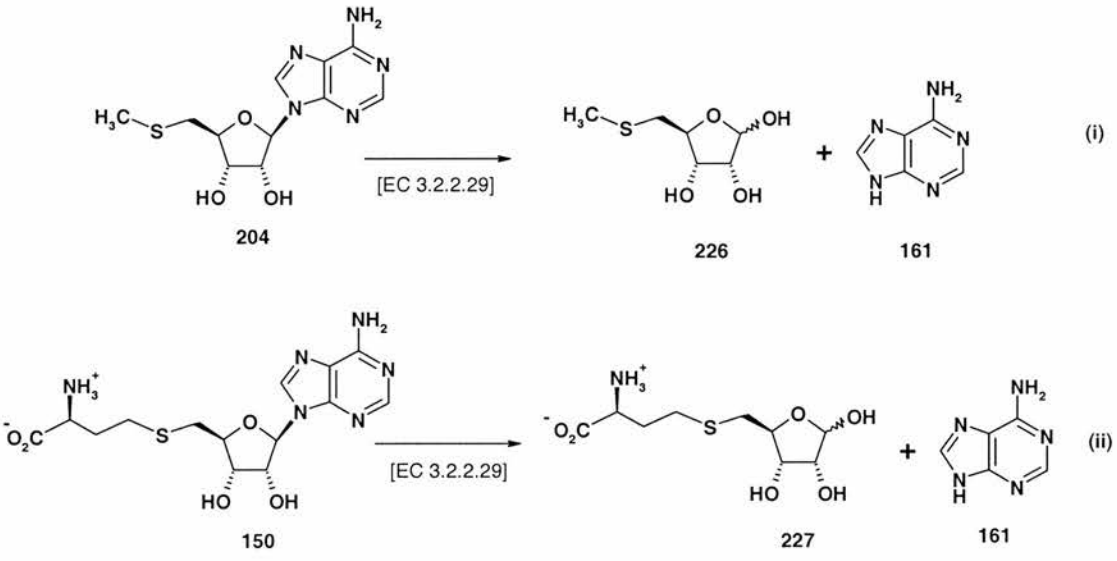
5'-Methylthio-adenosine (MTA) **204**[255] is formed from *S*-adenosyl-L-methionine (SAM) **83** by at least five independent pathways.[256] This nucleoside only differs structurally from adenosine **166** and 5'-FDA **83** at C-5' where it contains a 5'-methylthio group in place of a free hydroxyl or a fluorine atom. Unlike adenosine **166**, MTA **204** cannot be metabolised by enzymatic conversion to its corresponding inosine analogue via a deaminase. Thus the major pathways responsible for the metabolism of **204** occur by enzymatic cleavage of the C-N glycosidic bond. This can occur via a PNP phosphorylase reaction, catalysed by the enzyme 5'-methylthio-adenosine phosphorylase (MTAP) (EC 2.4.2.28).[251,257] The enzyme requires inorganic phosphate as a co-factor and leads the formation of 5'-methyl-thio-ribose-1-phosphate **225** and adenine **161**.



Scheme 4.2 Enzymatic cleavage of the C-N glycosidic bond of MTA **204** catalyzed by MTAP (EC 2.4.2.28).

Hydrolytic cleavage of the C-N glycosidic bond of **204** is mediated by the enzyme 5'-methylthio-adenosine (MTA) / *S*-adenosylhomocysteine (SAH) nucleosidase (MTAN, EC

3.2.2.9)[258,259] to give 5-methylthio-D-ribose **226**. Additionally MTAN is a dual substrate-specific enzyme and it also has the ability to hydrolysis SAH **150** to *S*-ribosylhomocysteine **227** (SRH) and **161**.

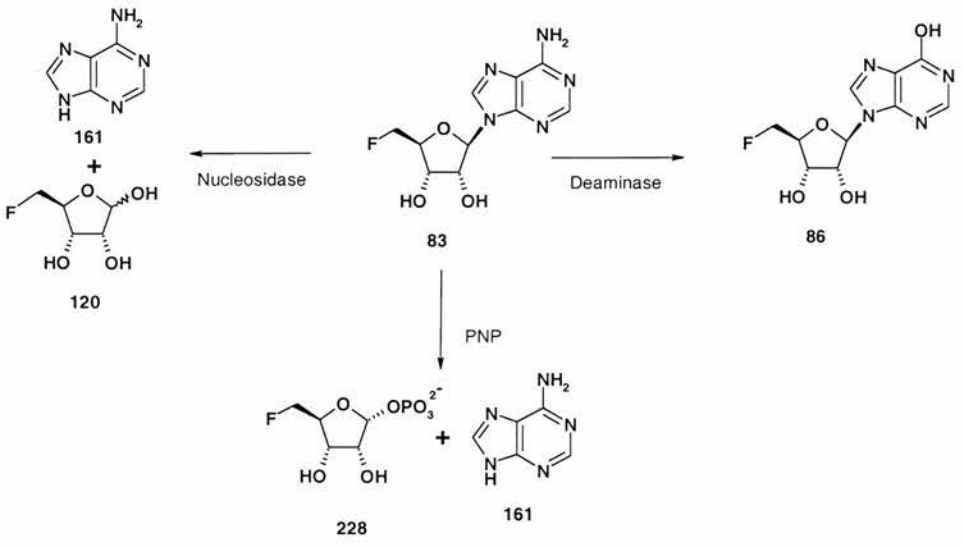


Scheme 4.3 The action of MTAN (EC 3.2.2.9) on its natural substrates MTA **204** (i) and SAH **150** (ii).

4.2 5-Fluoro-5-deoxy-D-ribose (5-FDR) as a potential intermediate on the biosynthetic pathway.

4.2.1 Introduction

If the metabolism of 5'-FDA **83** is assumed to proceed along a similar line to that of the **166** and **204** then there are three reactions that could represent the next enzymatic step on the fluorometabolite biosynthetic pathway (Scheme 4.4). Of these the deamination of 5'-FDA **83** to give 5'-FDI **86** can be immediately eliminated. This is due to the fact that as discussed in Chapter 2 a synthetic sample of 5'-FDI has already been shown to be metabolically inert in the crude cell free extract (CFE) from *S. cattleya* (Chapter 2).

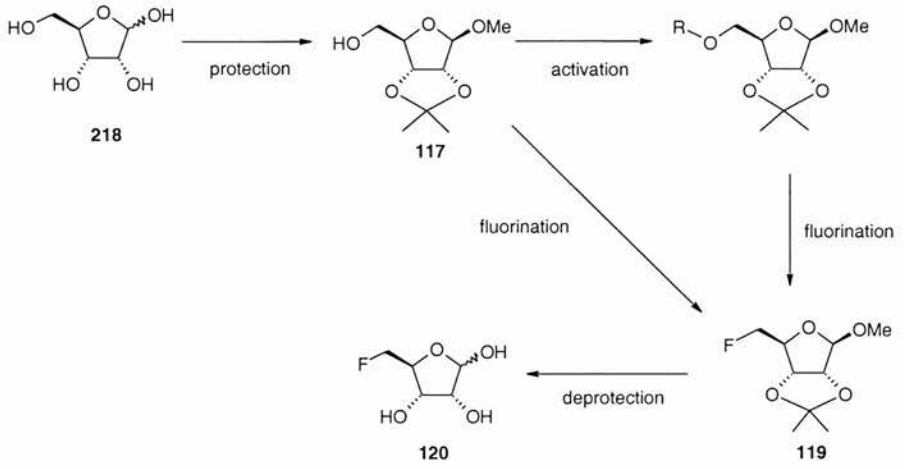


Scheme 4.4 Potential enzymatic conversions of 5'-FDA **83** in *S. cattleya*.

Consequently this leaves two enzymes both of which facilitate the cleavage of the C-N glycosidic bond of 5'-FDA **83**. A phosphorylase enzyme would be convert **83** to **161** and 5-fluoro-5-deoxy-D-ribose-1-phosphate (5-FDRP) **228**. A nucleosidase enzyme would also generate **161** but it would produce the free sugar 5-fluoro-5-deoxy-D-ribose (5-FDR) **120** in place of **228**. Both processes represent realistic options as it is likely that the metabolism of 5'-FDA **83** will at some stage result in the cleavage of the C-N glycosidic bond.

4.2.2 Synthesis of 5-fluoro-5-deoxy-D-ribose (5-FDR)

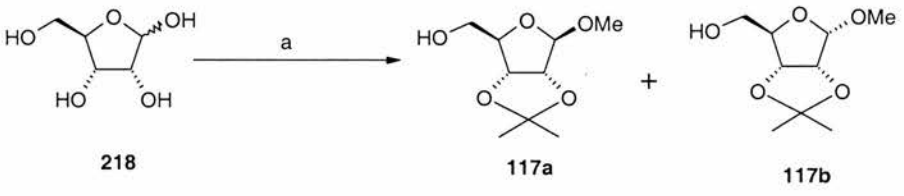
In order to investigate the potential of 5-FDR **120** as a biosynthetic intermediate a synthetic sample was required. Two potential routes to **120** have previously been discussed in Chapter 2 (Schemes 2.6 and 2.7).[133,134] The favoured route involved a combination of these synthetic strategies and is outlined Scheme 4.5.



Scheme 4.5 Proposed synthetic strategy to 5-FDR **120**.

The synthesis outlined utilises methyl-2',3'-*O*-isopropylidene–β-D-ribofuranoside **117** as a starting material.[260] **117** is a commonly used building block in carbohydrate chemistry and can be synthesised in one step from D-ribose **218**.[261]

Initial attempts to prepare **117** using both concentrated HCl and HCl_{gas} in methanol gave rise to the desired product **117** but in poor yields.[261,262] It was however found that by employing the Lewis acid BF_3 (in diethyl ether) as the catalyst and a combination of 2,2-dimethoxypropane and acetone it was possible to generate **117** in a good yield after purification by distillation.

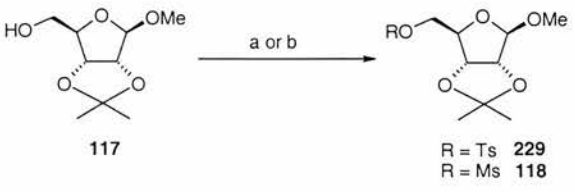


Scheme 4.6 Reagents and conditions: a) Acetone, 2,2-dimethoxypropane, methanol, BF_3/ether, R.T, 24 h, 60%.

Spectroscopic analysis of the product revealed that only one of the two possible anomeric forms (**117a** and **117b**) had been prepared during the reaction. The product was confirmed

to be the β anomer **117a** by comparison of the ^{1}H and ^{13}C NMR data with the literature.[262] With **117** in hand attention was turned to introducing the fluorine atom at C-5. Sharma *et al.* reported that the direct conversion of the 5-OH to fluorine with DAST proceeded but in only a 35% yield.[134] Despite this the reaction was attempted. Under the reaction conditions described and with a prolonged reaction time, the use of DAST did not furnish any fluorinated products.

Sharma *et al.* also reported that a more efficient process for the fluorination of **118** occurred *via* the corresponding 5'-*O*-mesylate which was then fluorinated in a nucleophilic displacement reaction with anhydrous TBAF. The fluorination step in this case was reported to proceed with a yield of 90%. Accordingly the treatment of the **117** with methanesulfonyl chloride in pyridine afforded methyl-2',3'-*O*-isopropylidene-5'-*O*-mesyl-β-D-ribofuranoside **118** in a good yield (61%).[262] However it was found that a combination of DCM and triethylamine improved the yield to 81%.[263] The analogous tosylate derivative, methyl-2',3'-*O*-isopropylidene-5'-*O*-toysl-β-D-ribofuranoside **229** was also synthesised as a possible substrate for the TBAF fluorination reaction. It was found that **229** could be easily be prepared under standard reaction conditions in an excellent yield (79%).[264]

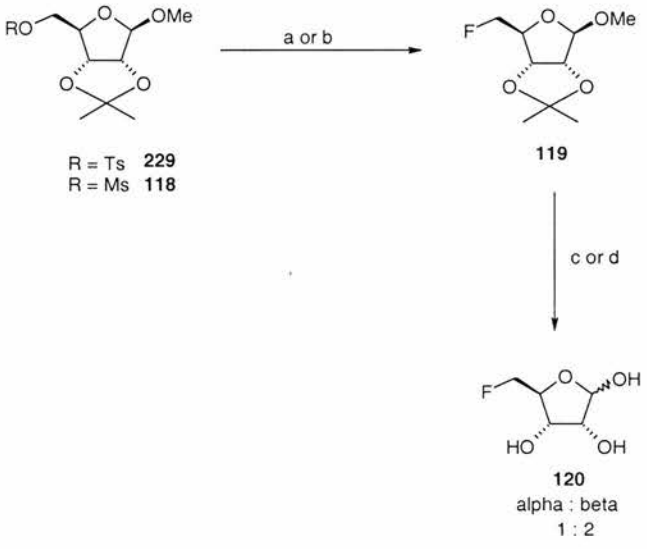


Scheme 4.7 Reagents and conditions: a) MsCl, DCM, triethylamine,0 °C - r.t, 4 h, 81% b) TsCl, pyridine, 0 °C - r.t, 12 h, 79%.

It was found that the reaction of **118** with TBAF.$3H_2O$ (1.5 molar eq.) in anhydrous acetonitrile at reflux resulted in the formation of the desired product methyl-2',3'-*O*-

isopropylidene-5'-fluoro-5'-deoxy-β-D-ribofuranoside **119** albeit in a low yield (40%) after purification. If the stoichometry of TBAF.$3H_2O$ was increased to 2.5 equivalents then it was possible increase the yield of the reaction to 74%. Alternatively the reaction of **229** with only 1.25 molar equivalents of TBAF.$3H_2O$ afforded **119** in a good yield (62%). Analytical and spectroscopic data for the product **119** in each case were in good agreement with the literature.[134]

It was found that cleavage of both the methoxy and isopropylidine protecting groups of **119** could be achieved in excellent yield by using either dilute sulfuric acid (89%)[262] or amberlite IR-120 [H^+] resin (75%).[263] As expected the product was the free fluorinated sugar **120**, which existed as an anomeric mixture in solution. The anomers are clearly visible by ^{19}F NMR (D_2O) which has two fluorine signals in a ratio of 2:1 (-228.4 ppm, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 25.6 and -230.6 ppm, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 26.8). The major anomer was determined by comparison of the ^{19}F and ^{13}C spectra with the literature to be the β-anomer.



Scheme 4.8 Reagents and conditions: a) TBAF.$3H_2O$ (1.25 mol. eq), MeCN, reflux, 16 h, 62% (from **118**) b) TBAF.$3H_2O$ (2.5 mol.eq), MeCN, reflux, 16 h, 72% (from **229**) c) dilute H_2SO_4, reflux, 89% d) Amberlite (H^+), water, reflux, 75%.

4.2.3 Incubation of 5-FDR in the cell free extract

Cells from *S. cattleya* were grown and used to prepare a cell free extract (CFE) using the method described in section 5.2. The CFE was assayed for activity by incubation with synthetic 5'-FDA **83**. ^{19}F NMR analysis confirmed the presence of FAc **15** indicating that all of the biosynthetic enzymes were active.

The synthetic sample of 5-FDR **120** (5 mM, final conc.) was incubated for 16 h with CFE (900 µl, 0.2g / ml 50 mM Tris-HCl buffer, pH 7.8), and the reaction was analysed by ^{19}F NMR spectroscopy. The resultant ^{19}F NMR spectrum recorded for the sample is shown in Figure 4.1.

There are only two ^{19}F NMR signals each a doublet of triplets, present at –228.4 ppm (beta anomer) and -230.6 ppm (alpha anomer), peaks which belong to the 5-FDR **120**. This result showed that **120** is metabolically inert in the CFE from *S. cattleya*. It cannot support the biosynthesis of FAc **15** or 4-FT **16** and is therefore does not appear to be an intermediate on the biosynthetic pathway to the fluorinated metabolites. This result indicates that the metabolism of 5'-FDA **83** in *S. cattleya* does not occur by a nucleosidase enzyme.

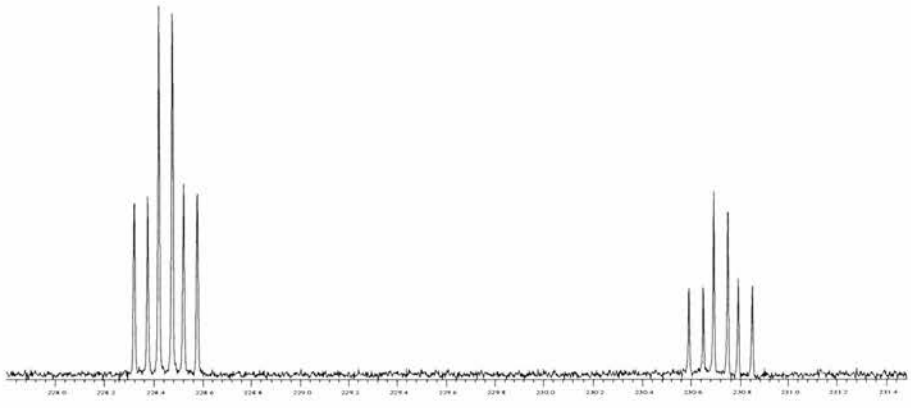


Figure 4.1 ^{19}F NMR (10% D_2O) analysis of the CFE after 16 h incubation with 5-FDR **120** at 37 °C.

4.3 Cell free extract metabolism of 5'-FDA

4.3.1 The use of ^{19}F NMR to follow the biosynthesis of fluorinated metabolites in a CFE

In addition to trying to elucidate the remaining intermediates on the biosynthetic pathway by synthesising potential intermediates, a second strategy that utilised ^{19}F NMR analysis was also undertaken. So far ^{19}F NMR spectroscopy has only been used for the final analysis of the crude CFE incubations, after the removal of the denatured protein. However ^{19}F NMR analysis can also be used to monitor "real time" metabolism of fluorinated molecules such as 5'-FDA within a CFE. This is a powerful technique as each ^{19}F NMR spectrum is a snap shot of the entire metabolic pathway at any given time.[264]

Until the availability of synthetic 5'-FDA it was not possible to fully exploit this technique. This is because a CFE incubation of SAM and KF only produce relativity low *in situ* concentrations of 5'-FDA. Hence all of the remaining fluorinated intermediates derived by the proceeding enzymes are generated in even lower concentrations. The availability of 5'-FDA however allows crude CFE to be overloaded (5 mM) with this initial fluorinated intermediate. This in turn increased the concentrations of all the fluorinated metabolites and has allowed the observation of molecules that were previously only present in concentrations below the ^{19}F NMR spectroscopy detection threshold.

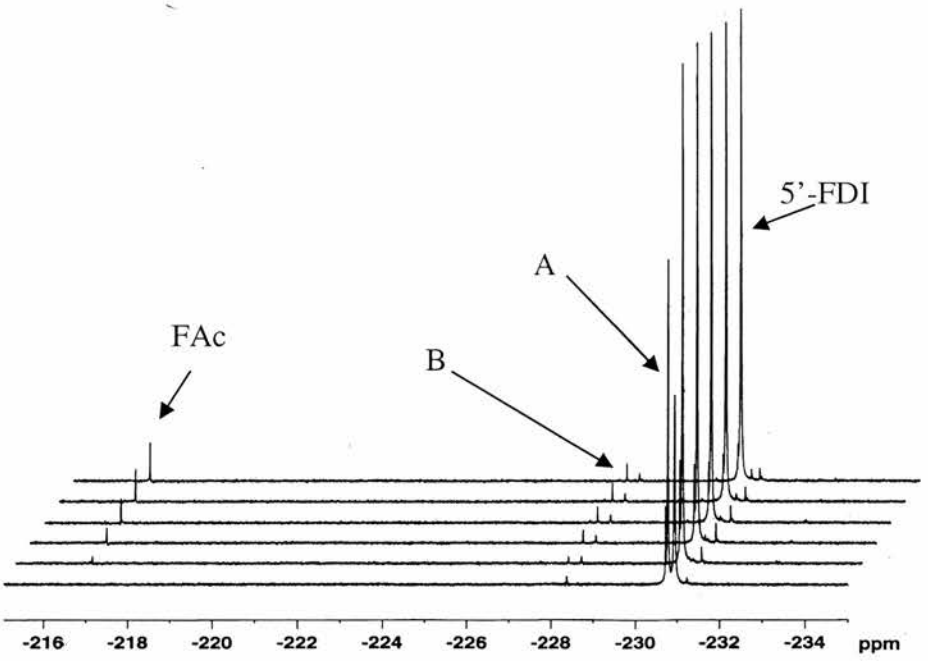
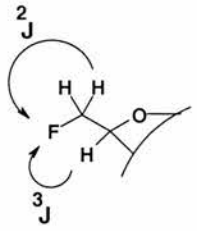


Figure 4.2 ^{19}F NMR (10 % D_2O, ^{1}H decoupled) analysis recorded every hour at 25 °C, monitoring the metabolism of 5'-FDA **83** (5 mM) in a Tris-HCl buffer CFE from *S. cattleya*.

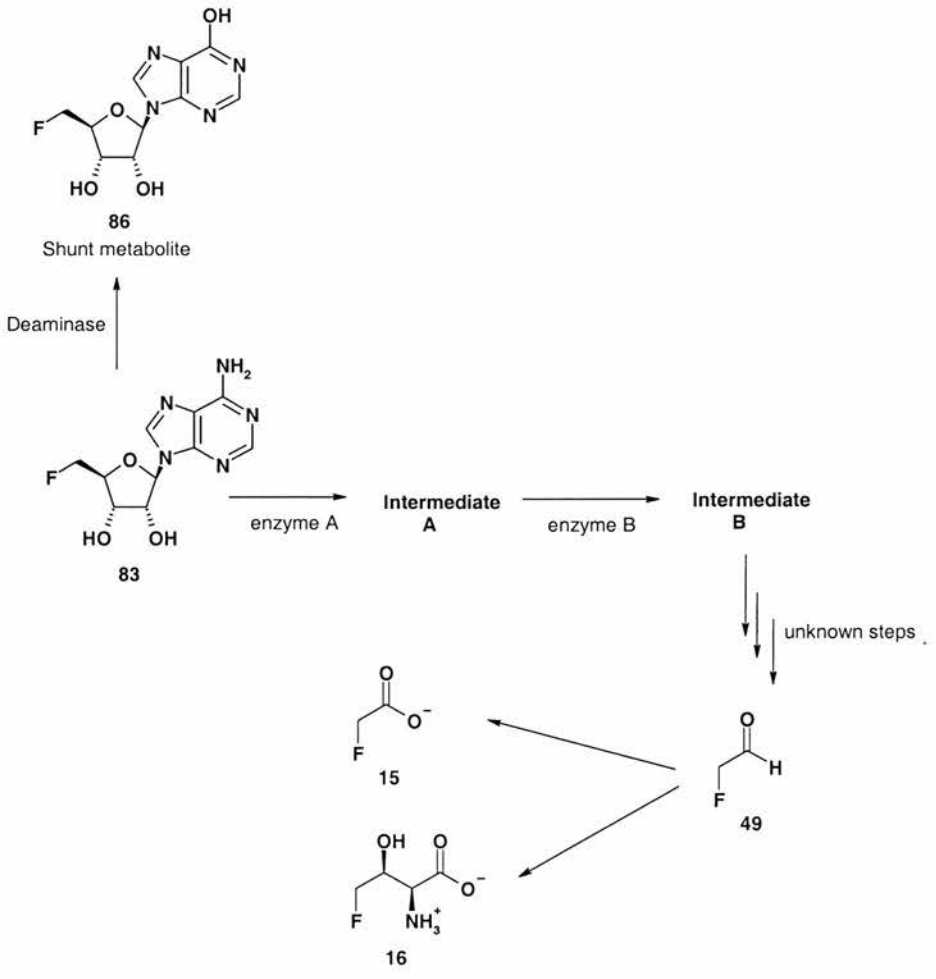
Figure 4.2 shows an example of the metabolism of 5'-FDA in a CFE. Clearly several fluorinated molecules are present. Some of these are identifiable based on our previous work (Chapter 2) and by comparison with reference standards which includes molecules such as FAc **15**, 4-FT **16** and 5'-FDI **86**. During the ^{19}F NMR time course experiments two new peaks consistently appeared during all the experiments carried out with 5'-FDA. The first of these was designated intermediate A (Figure 4.2, -230.5 ppm) because it was present after only one hour of the incubation. From 1 h onwards the concentration of A decreased as new fluorine signals emerged. The fact that no 5'-FDA is present after 1 h implies that the new fluorinated molecules are being biosynthesised as a result of the metabolism of A in the CFE suggesting that it is an intermediate on the pathway. A second potential intermediate termed intermediate B (Figure 4.2, -228.3 ppm) appears only after 2 - 3 h and the ^{19}F NMR profile suggested it was derived from A.

It was favourable to run the ^{19}F NMR time course experiments in ^{1}H decoupled mode (Figure 4.2). This improved the identification of the several different fluorine signals in the narrow ppm range that otherwise would have been difficult to resolve. More importantly it increased the intensity of the signals allowing even minor intermediates that were produced during the incubation to be monitored. It was also possible to obtain ^{1}H coupled ^{19}F NMR data for signals of both A and B and this revealed that both signals were a doublet of triplets (Table 4.1). Based on the coupling constants and splitting patterns it would appear that the fluorine atoms in compounds A and B reside in a very similar chemical environment to that of 5'-FDA (ie. as part of a fluoromethyl group). These results allowed a tentative hypothesis for the metabolic fate of 5'-FDA **83** to be constructed (Scheme 4.9).



Molecule	Signal (ppm)	Splitting pattern	^{2}J (Hz)	^{3}J (Hz)
5'-FDA **83**	-231.10	dt	47.0	28.7
5'-FDI **87**	-230.66	dt	47.0	28.9
Intermediate A	-230.46	dt	47.3	28.3
Intermediate B	-228.25	dt	46.2	15.3

Table 4.1 Comparison of the ^{19}F NMR (10% D_2O) data of 5'-FDA **83**, 5'-FDI **86** and molecules A and B.



Scheme 4.9 Putative pathway for the metabolism of 5'-FDA 83 in the CFE of *S. cattleya* based on results from initial ^{19}F NMR time course studies.

The proposed pathway involves the biosynthesis of potential intermediate A (-230.5 ppm) directly from 5'-FDA. Intermediate A would then undergo metabolism to give intermediate B (-228.3 ppm) which would eventually be converted to the known fluorinated intermediate fluoroacetaldehyde **49**.

One problem that was evident during these investigations was the production of the shunt metabolite 5'-FDI **86** via enzymatic deamination of 5'-FDA **83**. This side reaction reduces the concentration of the remaining fluorinated intermediates on the pathway. Therefore if the activity of the deaminase could be completely or partially suppressed, then more 5'-FDA would be available to progress down the actual biosynthetic pathway. This in turn

would lead to an increase in the concentration of the remaining fluorinated metabolites and may assist the isolation or further characterisation of such molecules. Our approaches to inhibiting the deaminase are detailed in the next section.

4.3.2 Attempts to inhibit 5'-FDI formation

4.3.2.1 Iodoacetamide experiments

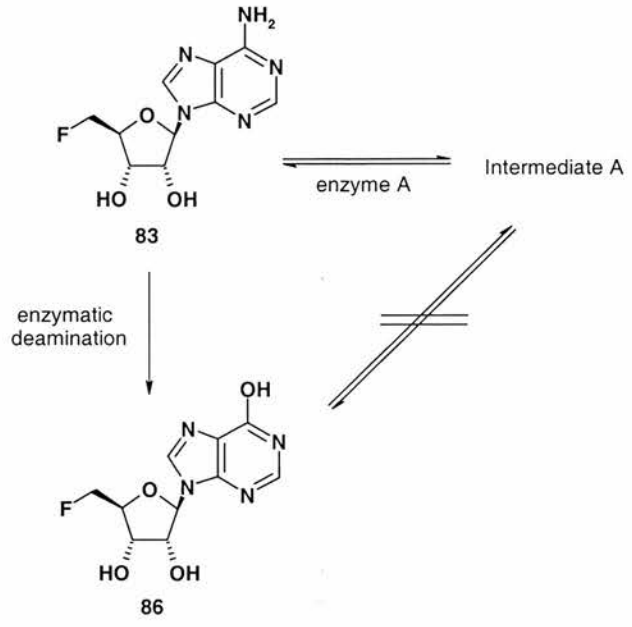
Fluoroacetaldehyde **49** is the known precursor to both of the fluorometabolites (FAc **15** and 4-FT **16**) produced in *S. cattleya*. In all of the previous CFE experiments significant levels of **49** were consumed by the fluoroacetaldehyde dehydrogenase (Chapter 1) leading to the formation of FAc **15**. Therefore if this enzyme could be inhibited this may lead to an increase in one or all of the earlier fluorinated intermediates on the biosynthetic pathway. It should be possible to achieve this by adding iodoacetamide to the CFE as this compound is a general inhibitor of the aldehyde dehydrogenase enzymes, and was shown to inhibit the fluoroacetaldehyde dehydrogenase.

Accordingly 5'-FDA **83** (3.7 mM, final concentration) was incubated in a CFE preparation (1 ml) that had been pre-incubated (30 min) with iodoacetamide (1 mM, final concentration) at 37 ^{o}C for 16 h. Subsequent ^{19}F NMR analysis revealed that the CFE contained only the shunt metabolite 5'-FDI **87**. Thus it appeared that iodoacetamide inhibited an enzyme further up the pathway in addition to the fluoroacetaldehyde dehydrogenase. To explore this further iodoacetamide and 5'-FDA **83** were added to cell free extracts and the incubations (37 ^{o}C) were terminated after 2 h, 4 h and 16 h. A summary of the results deduced from ^{19}F NMR analysis of these samples is shown in Table 4.2.

5'-FDA (mM)	Iodacetamide (mM)	Incubation period (h)	Compounds identified by ^{19}F NMR
3.7	1	2	5'-FDA, 5'-FDI and intermediate A
3.7	1	4	5'-FDI and intermediate A
3.7	1	16	5'-FDI

Table 4.2 ^{19}F NMR derived data from the incubation of 5'-FDA **83** and iodoacetamide in a CFE. Concentrations given are the final concentrations within the sample.

The results in Table 4.2 show that the production of intermediate A is not inhibited by the addition of iodoacetamide. Iodoacetamide appears to inhibit any further metabolism of intermediate A from occurring in the CFE. The fact that A disappears over time and that after 16 h only the shunt metabolite 5'-FDI **86** is observed in the CFE implies that the enzyme that mediates the transformation of 5'-FDA **83** to A is reversible. Consequently as A cannot be metabolised further its concentration in the CFE is gradually reduced as it reforms 5'-FDA **83** which is then deaminated to generate 5'-FDI **86** (Scheme 4.10). The possibility that intermediate A can be converted directly to 5'-FDI **86** has been ruled out on the basis that 5'-FDI is metabolically inert in the CFE. It is important however to recognise that this assumes that any enzyme that could mediate the transformation of A to 5'-FDI would operate reversibly.



Scheme 4.10 Metabolic relationships between 5'-FDA **83**, 5'-FDI **86** and intermediate A in the CFE of *S. cattleya*.

4.3.2.2 Incubations in partially purified extracts

Ammonium sulfate (AS) cuts are most commonly the first purification step after CFE preparation. Thus the preparation of various AS cuts from the CFE prepared from *S. cattleya* may allow the enzyme responsible for the deamination of 5'-FDA to be separated from the enzyme responsible for the conversion of 5'-FDA to intermediate A. Therefore AS cuts (0-40%, 40-60% and 60-100%) were prepared according to the procedure detailed in Section 5.2.

Each AS cut was tested for activity by incubating 5'-FDA **83** (3.7 mM, final concentration) for 4 h at 37 ^{o}C (Table 4.3, samples 1-3). The incubations were carried out only for 4 h such that the concentration of intermediate A did not decrease over time. In addition an experiment was conducted where 5'-FDA was incubated with all three AS cuts combined (Table 4.3, sample 4). ^{19}F NMR analysis of the resultant products are summarised in Table

4.3.

Sample	0-40%	40-60%	60-100%	Compounds identified by ^{19}F NMR
1	+	–	–	5'-FDI
2	–	+	–	5'-FDI
3	–	–	+	5'-FDA
4	+	+	+	5'-FDI

AS cuts were re-suspended in Tris-HCl buffer, 50 mM, pH 7.8. All incubations were carried out for 4 h at 37 °C.

Table 4.3 Results from the incubation of 5'-FDA **83** (3.7 mM, final concentration) for 4 h with various AS cuts.

Only the deaminase enzyme responsible for the production of 5'-FDI **86** appears to be active within the two of the ammonium sulfate cuts. No other intermediates were observed. This implied that a co-factor was required for further 5'-FDA **83** metabolism. In order to investigate this possibility standard co-factors were added to the AS cuts both individually and as part of a co-factor 'soup'.

Unfortunately it was not possible to produce FAc **15** or 4-FT **16** from 5'-FDA **83** with any of the co-factor / AS cut combinations that were examined. However it was found that when the AS cuts (0-60%) were resuspended in phosphate buffer (200 mM, pH 6.8) rather than Tris-HCl buffer the production of FAc **15** was re-established. This indicated that inorganic phosphate was an essential co-factor in the biosynthesis.

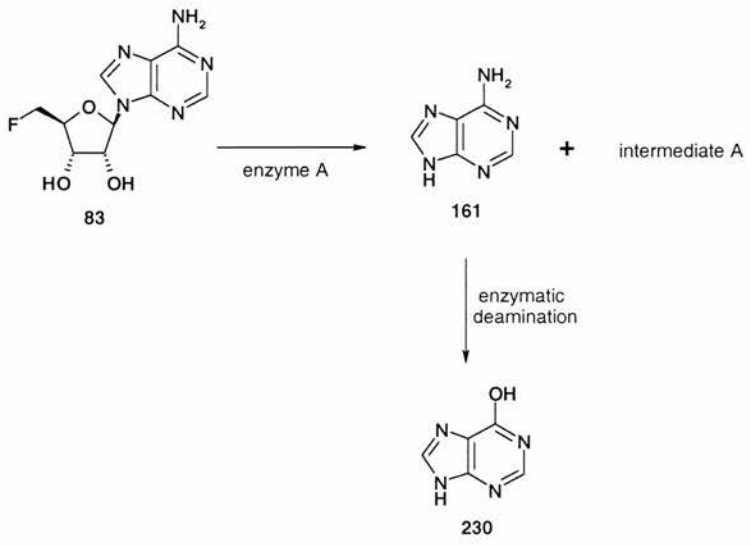
It is interesting to note that no other supplementary cofactors were required to produce FAc **15** during the incubation experiments with the AS cuts (0-60%) in phosphate buffer. Clearly co-factors such as NAD^+ that are required in order to produce FAc **15** from fluoroacetaldehyde **49** must still be present in the AS cuts to a sufficient extent. The production of 4-FT **16** which requires both pyridoxyl 5'-phosphate (PLP) and L-threonine was not observed. This is consistent with previous incubations carried out in the CFE

where the production of 4-FT **16** is only detected if the required cofactors are supplemented to the sample. The key discovery from these incubation experiments was that an enzyme involved at some stage in the metabolism of 5'-FDA requires inorganic phosphate as a cofactor. Further work was carried out to try and refine the AS cuts but unfortunately the deaminase activity could not be separated from that of the enzyme responsible for the production of A. Studies indicate that both of these enzymes appear to precipitate in an AS cut of 35-55%.

4.3.3 Attempted isolation of intermediate A from a crude CFE

^{19}F NMR time course experiments showed the level of intermediate A is at its highest during the first few hours (0 - 4 h) of the CFE incubation with 5'-FDA **83**. During this period the only other fluorinated entity that is present is the shunt metabolite 5'-FDI **87**. Therefore it was anticipated that if the incubation of 5'-FDA in a CFE was stopped after 1-4 h then it might be possible to isolate A by preparative HPLC.

It was possible to produce samples from a CFE that were shown by ^{19}F NMR analysis to contain only 5'-FDI **87** and intermediate A. This was achieved by reducing the 5'-FDA incubation times to between 2 - 4 h. The detection of 5'-FDI was achieved by HPLC (UV detection) analysis of the samples but there were no obvious peaks that could be attributed to intermediate A. The only two UV active peaks of a comparable size to 5'-FDI were confirmed to be adenine **161** and hypoxanthine **230**. The most logical explanation for the presence of these bases at the levels they were detected is that they are biosynthesised from the 5'-FDA **83** that was introduced into the CFE. This suggests that 5'-FDA has undergone C-N glycosidic bond cleavage to give **161** and intermediate A. **161** must then be enzymatically deaminated in the CFE to give **230** as shown in Scheme 4.11.



Scheme 4.11 Proposed biosynthetic pathway to account for the production of **161** and **230** in a CFE of *S. cattleya*.

It was concluded that intermediate A is not UV active. This is in keeping with the fact that although it can easily be detected by ^{19}F NMR it does not seem to be visible by UV when HPLC analysis is carried out on the same sample.

4.4 Investigating 5-fluoro-5-deoxy-D-ribose-1-phosphate as a potential biosynthetic intermediate

From the various experiments that have been carried out to try to establish more information about intermediate A, the following conclusions can be drawn;

1. Intermediate A is not the free sugar, 5-FDR **120**.
2. Intermediate A does not appear to exist as an anomeric mixture.
3. Inorganic phosphate is required as a cofactor for the enzymatic formation of A.
4. Intermediate A does not appear to be UV active. This suggests that C-N glycosidic bond has been cleaved.

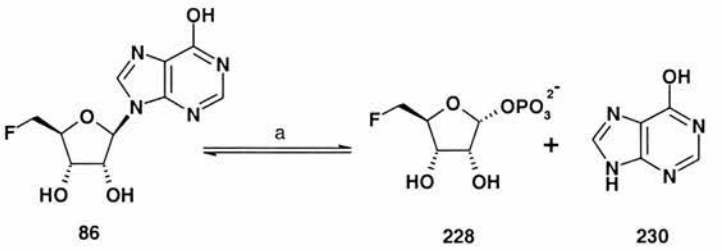
5. The enzymatic formation of A from 5'-FDA appears to be a reversible process.

6. ^{19}F NMR (^{1}H coupled) analysis implies that the fluorine is located at C-5'.

Based on these experimental observations it seems possible that 5'-FDA **83** could be metabolised in the crude cell free extract by a PNP enzyme to give 5-FDRP **230** (intermediate A) and adenine **161** (Scheme 4.4). Such an enzymatic transformation would be in keeping with all of the aforementioned experimental observations. Therefore it appeared appropriate to prepare a sample of 5-FDRP **228** for cross correlation.

4.4.1 Preparation of 5-FDRP

The chemical synthesis of ribose-1-phosphates are well documented in the literature.[265] However a more convenient method to prepare a sample of 5-FDRP **228** is to use an enzymatic approach as this overcomes the problems associated with trying to selectively produce only one anomeric form of this type of molecule. The enzymatic synthesis of **228** was achieved by utilising a commercially available PNP enzyme (EC. 2.4.2.1, Sigma Chemical Co. unknown bacterial source, N-8264). It was found that this PNP enzyme would not accept 5'-FDA **83** as a substrate but it would mediate the phosphorolyses of the C-N glycosidic bond of 5'-FDI **86** to afford the desired product 5-FDRP **228** (Scheme 4.12).



86 228 230

Scheme 4.12 Enzymatic preparation of 5-FDRP **228**. Reagents and conditions: PNP (EC. 2.4.2.1), 200 mM phosphate buffer pH 4.6, 37°C, 16 h, 60% conversion by ^{19}F NMR analysis.

Because the enzymatic preparation of **228** is a reversible process it was not possible to obtain a 100% conversion. The best conversion that could be achieved was only 60% as determined by ^{19}F NMR analysis. A practical HPLC assay was also developed that could be used to monitor the release of hypoxanthine **230** as the reaction progressed (Figure 4.3).

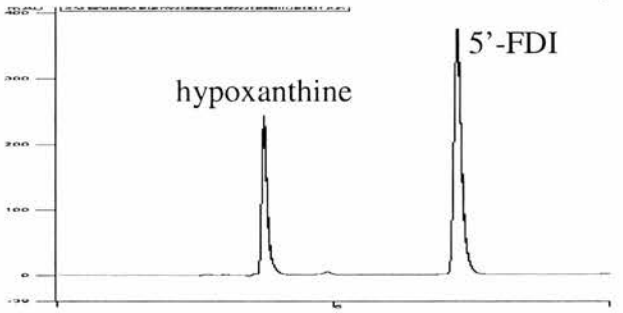
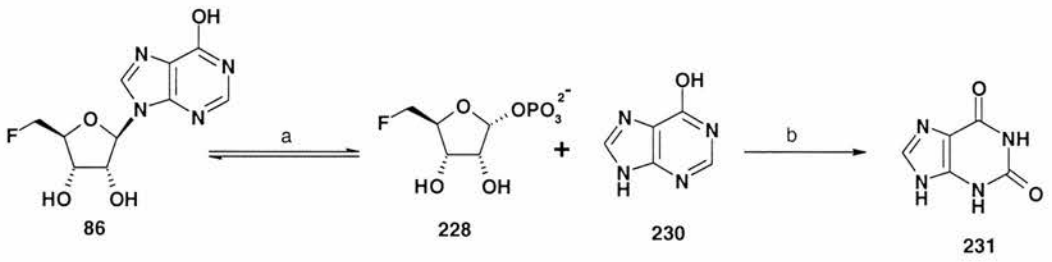


Figure 4.3 HPLC assay (0-10 min) for the enzymatic formation of 5-FDRP **228** from 5'-FDI **86** showing the release of hypoxanthine.

The enzymatic reaction was run at both 25 °C and 37 °C to investigate the effect of temperature on product formation. The progress of these reactions was monitored by HPLC and ^{19}F NMR was used to determine the ratio of hypoxanthine **230** to 5'-FDI **86**. Predictably the reactions performed at 37 °C showed the highest enzymatic conversion (60 % 5-FDRP **228** to 40% 5'-FDI **86**).

The use of the commercially available enzyme xanthine oxidase (E.C. 1.1.3.22) to prevent the reverse reaction from occuring was also investigated.[266] This particular enzyme catalyzes the irreversible formation of xanthine **231** from **230**. Thus if it was added in

addition to the PNP it may prevent the reformation of **86** and encourage an increase in the production of 5-FDRP **228** (Scheme 4.13).



Scheme 4.13 Preparation of 5-FDRP **228** using a PNP (EC. 2.4.2.1) and xanthine oxidase (E.C. 1.1.3.22) in a coupled enzymatic process.

The inclusion of xanthine oxidase (1 mg to 10 mg PNP with 1 mg of 5'-FDI) led to a slight enhancement in the overall conversion of the reaction. The increase in the production of 5-FDRP **228** when the reaction was carried out in the presence of xanthine oxidase was calculated to be around 15% based on ^{19}F NMR analyses of the samples. However on a larger scale (>1 mg of 5'-FDI) the inclusion of xanthine oxidase appeared to have little effect on the production of 5-FDRP with the maximum conversion achievable being ~60%. Presumably this was due to the fact that it was not possible to overload the system with enzyme as it is reasonably expensive to purchase and only available in small quantities. If a cheaper source of this enzyme could be located or if the enzyme could be over-expressed "in house" then the use of xanthine oxidase may be more applicable and merit further investigation.

4.4.2 Cell free extract incubations with 5-FDRP

Intermediate A prepared enzymatically from a CFE from 5'-FDA **83** had an identical ^{19}F NMR chemical shift and coupling constant to 5-FDRP **228**. In addition a CFE that contained A was spiked with **228** and re-analysis of the sample by ^{19}F NMR showed that

the fluorine signal corresponding to A had increased in intensity upon addition of **228**. An experiment was conducted to explore if 5-FDRP **228** was an intermediate on the biosynthetic pathway. Accordingly a sample of **228** was incubated in a CFE preparation. The sample of **228** contained residual 5'-FDI **86** but as this nucleoside had already been shown to be metabolically inert it was assumed that this would not present a problem.

Thus **228** (10 mM + residual **86**) was incubated in a crude CFE (500 µl of 0.2g / ml Tris-HCl buffer 50 mM, pH 7.8) for 16 h at 37 °C. The sample was then analysed by ^{19}F NMR spectroscopy, and the resultant spectrum is shown in Figure 4.4.

The production of FAc **15** confirms that 5-FDRP **228** can support the biosynthesis of this secondary metabolite in *S. cattleya*. This clearly reinforces a role for **228** as an intermediate on the fluorometabolite biosynthetic pathway. It is interesting to note that the compound previously identified as a potential intermediate "intermediate B" (-228.3 ppm) accumulated to only low levels in these experiments. Interestingly a previously unseen fluorine signal was observed at -231.4 ppm (Peak C, dt, $^{2}J_{F,H}$ 46.5 and $^{3}J_{F,H}$ 20.7). The splitting pattern caused by ^{1}H coupling (Figure 4.4 spectra (ii)) indicated that the fluorine atom was in a similar chemical environment to 5'-FDA **83** and 5-FDRP **228** and thus it might represent another intermediate on the biosynthetic pathway.

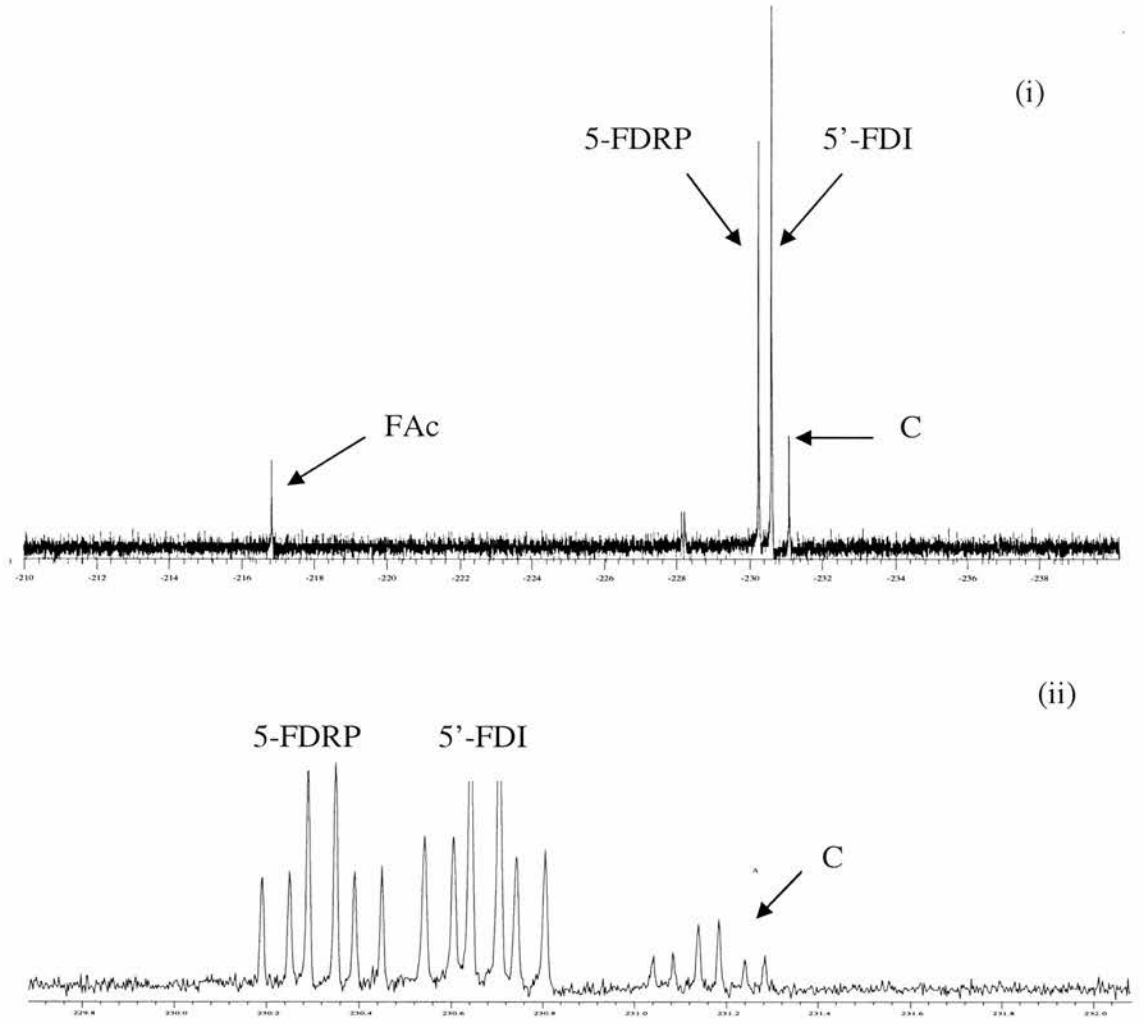


Figure 4.4 ^{19}F NMR (10 % D_2O) analysis of a crude cell free extract after 16 h incubation at 37 °C with synthetic 5-FRP **228** (10 mM). (i) {^{1}H decoupled} ^{19}F NMR spectrum (ii) ^{19}F NMR (^{1}H coupled) spectrum, expansion of region -228 ppm to -234 ppm.

4.4.3 CFE formation of 5-FDR from 5-FDRP

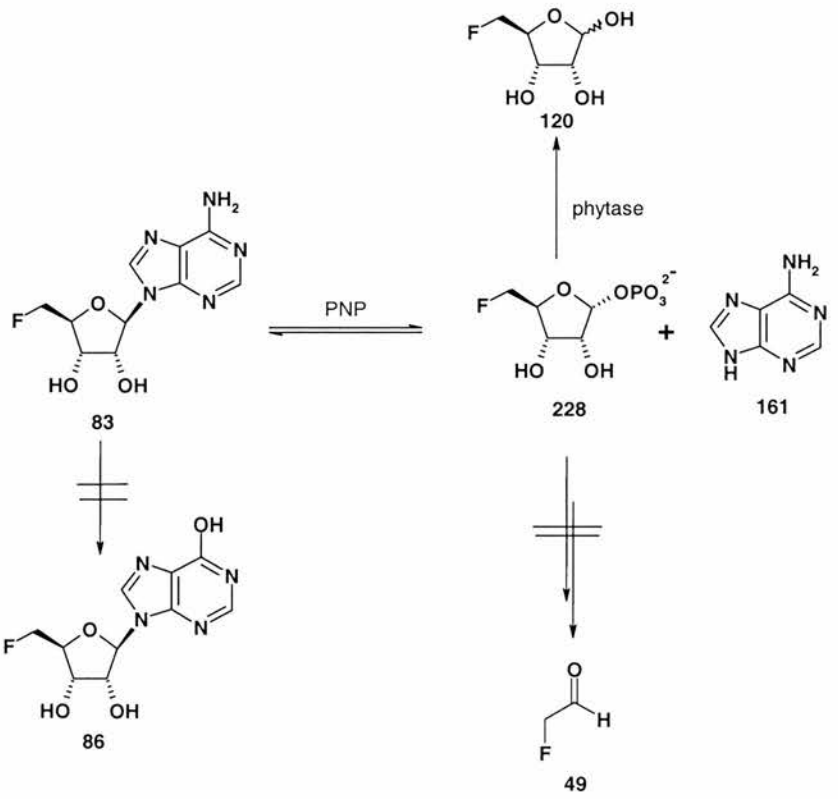
In order to establish more evidence for a role for **228** in fluorometabolite biosynthesis, it was decided to try and trap it as the free sugar, 5-FDR **120** in the cell free extract (CFE). Synthetic 5-FDR **120** has already been shown to be metabolically inert when incubated in the CFE. Additionally it is not possible to prepare 5-FDR **120** directly from 5'-FDA **83** in the CFE under normal conditions. Therefore if 5'-FDA **83** was incubated in the CFE with an added phytase it should be possible to accumulate 5-FDR **120** as a shunt metabolite.

Phytases act as dephosphorylation enzymes on a variety of substrates and the commercially available phytase from *Aspergillus ficuum* (EC. 3.1.3.8, Sigma Chemical Co., P-9792) was selected for this experiment. In nature this enzyme acts to convert 4-nitrophenyl phosphate to 4-nitrophenol but it has been shown to have a broad substrate tolerance. Unfortunately the phytase requires an operating pH of 4.6. Therefore prior to the use of the phytase enzyme within the CFE it was necessary to confirm that all of the enzymes on the biosynthetic pathway required for the metabolism of 5'-FDA **83** were active at pH 4.6. Furthermore it was essential to investigate whether or not 5-FDRP **228** could actually be converted to 5-FDR **120** with this phytase.

Hence a sample of 5-FDRP **228** (10 mM + residual 5'-FDI **86**) was incubated with phytase (4 mg in 1 ml of 200 mM phosphate buffer, pH 4.6) at 37 °C for 16 h. ^{19}F NMR (10% D_2O) analysis of the sample indicated the complete conversion of 5-FDRP **228** to 5'-FDR **120** (-230.6 ppm α-anomer and –228.4 ppm β-anomer). 5'-FDA **83** (3.7 mM) was then incubated with CFE (1 ml of 0.2 g / ml phosphate buffer 200 mM, pH 4.6) for 16 h at 37 °C. ^{19}F NMR analysis showed that FAc **15** had been produced. This confirmed that even at pH 4.6 all of the relevant enzymes in the CFE remained active. It was surprising to note that 5'-FDI **86** was not produced in this experiment. This indicates that the deaminase enzyme responsible for converting 5'-FDA to 5'-FDI is not active at this lower pH. It was also noticed that despite the shunt metabolite 5'-FDI not being formed the concentration of FAc **15** produced was less than when the incubations are carried out at pH 7.8. Additional control experiments were also carried out to confirm that both 5'-FDA and 5'-FDI were resistant to chemical hydrolysis at 37 °C in phosphate buffer (pH 4.6) over the 16 h period.

Thus with all of the appropriate control experiments carried out, 5'-FDA **83** (3.7 mM) was incubated with the CFE (1 ml of 0.2 g / ml 200 mM phosphate buffer, pH 4.6) with the

phytase (4 mg) added. HPLC analysis of the sample showed that all of 5'-FDA **83** had been consumed and there was no evidence of any 5-FDRP **228**. ^{19}F NMR (10% D_2O) analysis of the incubation products showed that only 5-FDR **120** was present, again as the characteristic anomeric mixture. As 5-FDR could only derive from 5-FDRP, this result confirms that it must have been transient in the incubation. An overview summarising the interrelationships in this experiment is outlined in Scheme 4.14.



Scheme 4.14 An overview of metabolism of 5'-FDA **83** in the CFE (Phosphate buffer pH 4.6) supplemented with a commercial phytase.

Further evidence for the presence of a PNP enzyme in *S. cattleya* was provided by CFE incubation with 5'-FDI **86**. This nucleoside has been shown to be biosynthetically inert in the CFE. However it is possible to convert 5'-FDI **86** to the fluorinated metabolites FAc **15** and 4-FT **16** if the CFE was supplemented with the commercial PNP. The ^{19}F NMR spectra generated for this experiment (Figure 4.5) show that 5'-FDA **83** is metabolised in

the CFE (spectrum (i)) but 5'-FDI **86** remains completely unchanged (spectrum (ii)). This is presumably because the PNP enzyme in *S. cattleya* only accepts adenine based nucleosides. This issue is further addressed later in this chapter 4. However if a PNP enzyme that can accept 6-oxopurine nucleosides is added to the CFE, then 5-FDRP **228** can be generated from 5'-FDI and is subsequently metabolised to produce FAc 15 (spectrum (iii)).

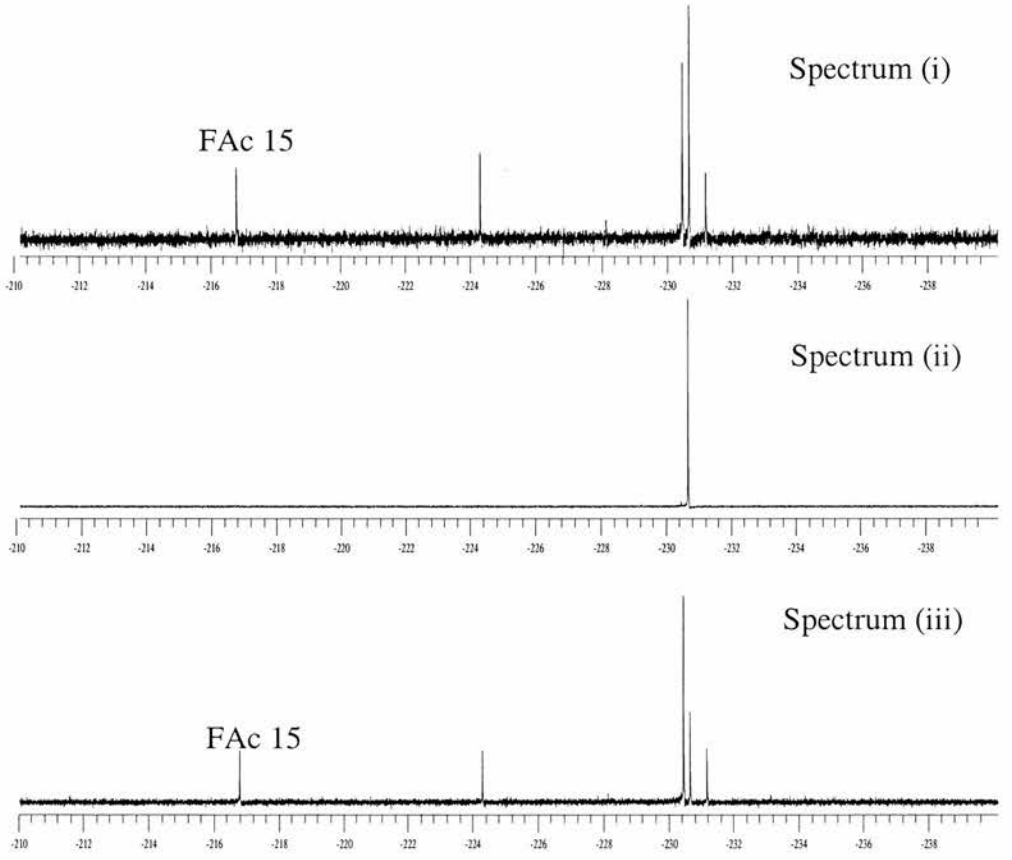


Figure 4.5 ^{19}F NMR (10% D_2O, ^{1}H decoupled) analyses of the following incubations (i) 5'-FDA + CFE (ii) 5'-FDI + CFE (iii) 5'-FDI + CFE + commercial PNP. All incubations were carried out at 37 °C for 16 h.

4.5 Investigating a PNP enzyme from *S. cattleya*

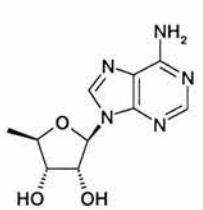
As already discussed 5'-FDA, **83** is metabolised to 5-FDRP **228** by a PNP enzyme in the CFE extract from *S. cattleya*. 5-FDRP **228** was also confirmed as the next intermediate on

the fluorometabolite pathway. The catalytic activity for PNP activity was shown to reside in the 30-55% AS fraction. Further purification was carried by Ryan McGlinchey (University of St. Andrews). However attempts to purify the PNP enzyme to homogeneity were unsuccessful. Therefore the experiments that were carried out in the following section were done so using partially purified PNP enzyme from *S. cattleya.*

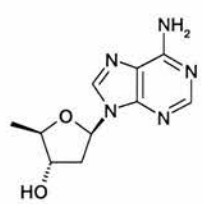
4.5.1 Synthesis of potential PNP substrates

The first committed enzyme on the fluorometabolite pathway is the fluorinase enzyme. It was important to establish if the PNP, the second enzyme on pathway had evolved specifically to deal with the fluorinated substrate 5-FDRP **228**. Some indication as to whether or not this is the case can be obtained by assessing the substrate specificity of the PNP with a variety of nucleosides.

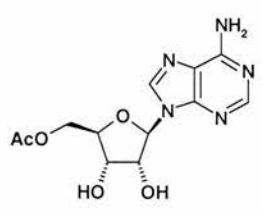
Several potential substrates were already available following our investigation of the properties of the fluorinase. The preparation of these nucleosides has already been discussed in chapters 2 and 3. To ascertain if the PNP was novel it was of particular interest to explore any preferences that the PNP enzyme might exhibit for nucleosides modified at C-5' position. To this end the synthesis of nucleosides **164**, **232** and **233** was undertaken. These analogues could be prepared either directly or in only two steps from commercially available or previously prepared precursors.



164

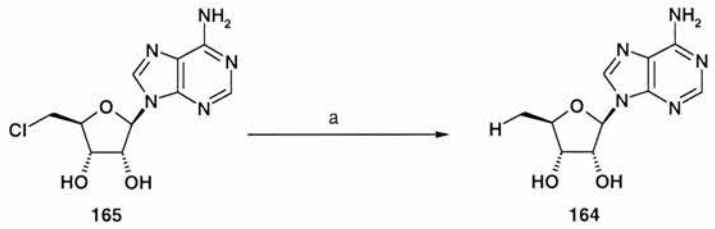


232



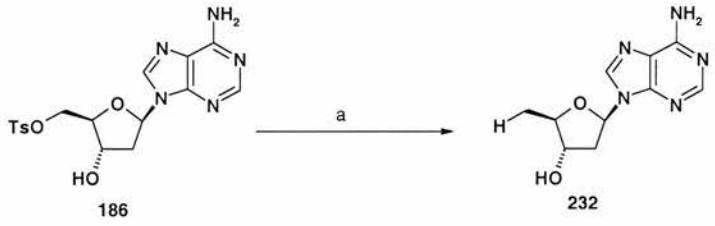
233

The preparation of 5'-deoxy-adenosine **164** was achieved by super hydride reduction of the corresponding 5'-chloro-nucleoside **165**.[221,267] The reaction did not run to completion but it did proceed cleanly affording only a mixture of unreacted starting material **166** and the desired product **164**. The two compounds were easily separated by column chromatography giving **164** in a moderate yield. Spectroscopic and analytical data were in good agreement with the literature.



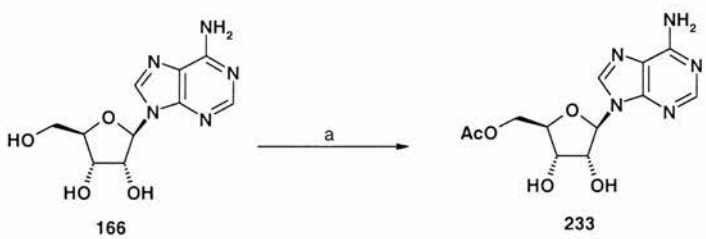
Scheme 4.15 Reagents and conditions: a) 1,4-Dioxane, super hydride (1M THF), 0 °C – R.T, 16 h, 54%.

The preparation of 2',5'-dideoxy-adenosine **232** was achieved by a similar reaction protocol involving the super hydride reduction of a C-5' activated nucleoside. It was more convenient to carry the reaction out on 5'-*O*-tosyl-2'-deoxy-adenosine **186** rather than the corresponding 5'-chloro-nucleoside **182** due to its ease of preparation. The reaction occurred in a good yield affording **232** with spectroscopic and analytical data that were in good agreement with the literature.[268]



Scheme 4.16 Reagents and conditions; a) 1,4-Dioxane, super hydride (1M THF sol.), 0 °C - RT, 16 h, 67%.

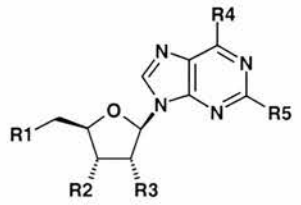
The preparation of 5'-acetyl-adenosine **233** was carried out using a selective *Candida antarctica* lipase mediated acylation of adenosine **166** as reported by Ciufrreda and Santaniello.[269] Spectroscopic and analytical data confirmed that **233** had been produced, consistent with the reported selectivity of the lipase enzyme.



Scheme 4.17 Reagents and conditions; a) Vinyl acetate, THF, *Candida antarctica* lipase, 60 °C, 4 h, 64%.

4.5.2 Substrate specificity of the PNP enzyme from *S. cattleya*

The synthesis of compounds **83**, **86**, **164**, **165**, **181**, **182**, **190**, **195**, **201**, **203**, **232** and **233** has already been discussed in Chapters 2, 3 and 4 and the remaining nucleosides **138**, **166**, **205** were obtained from commercial sources. All of the nucleosides were tested as substrates by incubating them with partially purified PNP from *S. cattleya*. The samples were then analysed by HPLC (UV detection) and the detection of the appropriate free base taken to indicate that the nucleoside has been accepted as a substrate.



	R1	R2	R3	R4	R5	Result
83	F	OH	OH	NH_2	H	+
86	F	OH	OH	OH	H	-
181	F	OH	H	NH_2	H	+
165	Cl	OH	OH	NH_2	H	+
182	Cl	OH	H	NH_2	H	+
203	Cl	OH	OH	OH	H	-
195	Cl	OH	OH	NH_2	NH_2	+
201	Br	OH	OH	NH_2	H	+
190	OH	H	OH	NH_2	H	-
205	SMe	OH	OH	NH_2	H	+
233	OAc	OH	OH	NH_2	H	-
164	H	OH	OH	NH_2	H	+
232	H	OH	H	NH_2	H	+
138	OPO_3	OH	OH	NH_2	H	-
166	OH	OH	OH	NH_2	H	-

(+) result indicates that the production of the corresponding purine base was detected by HPLC analysis. (–) result indicates that no base was detected by HPLC analysis.

Table 4.4 Summary of the substrate specificity of the partial purified PNP isolated from *S. cattleya.*

Table 4.4 shows that the partially purified PNP enzyme did not accept either of the 6-oxopurine nucleosides **86** or **203** that were tested. Some degree of tolerance was exhibited at the C-2' position of the ribose ring highlighted by the fact that all of the 2'-deoxy-

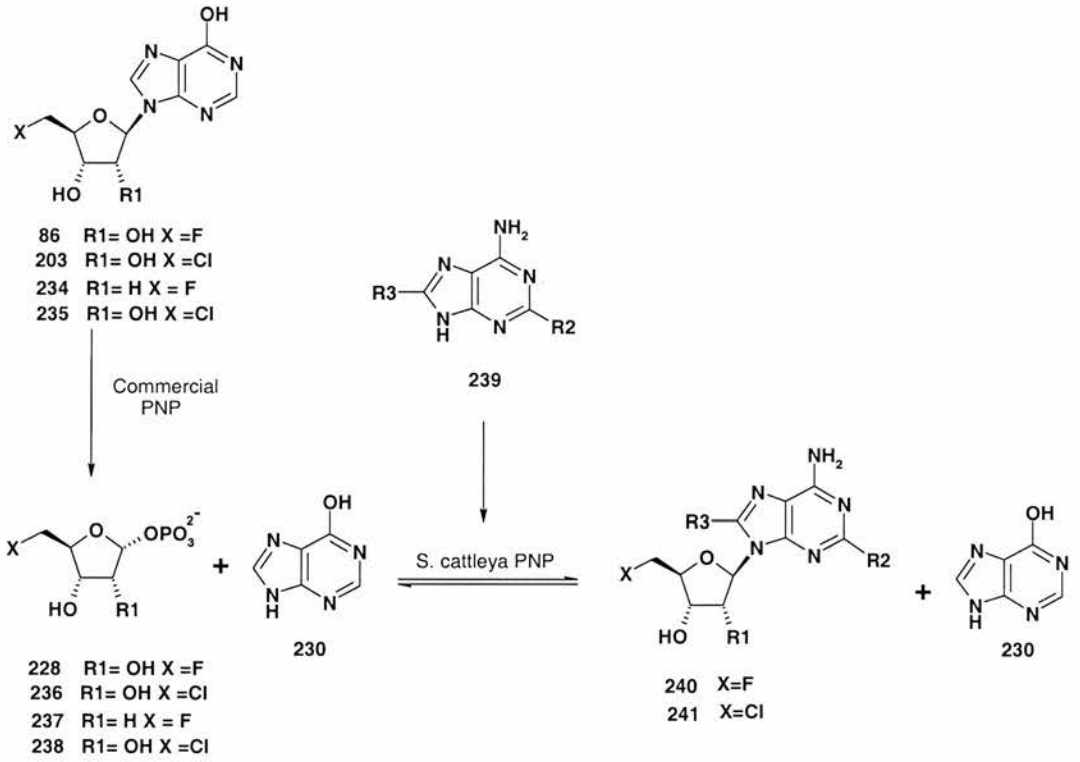
nucleosides (**181,182** and **232**) that were tested were shown to act as substrates. This is not uncommon as several PNP enzymes heave been reported to process 2'-deoxy-nucleosides.[270]

It is clear that the PNP permits certain degree of flexibility at the C-5' position. It was possible to substitute the fluorine atom at C-5' for H (**164** and **232**), Cl (**165, 182** and **195**) and Br (**201**) atoms and a SMe (**205**) group. The fact that **165** and **201** are tolerated implies that the pocket where the 5'-substituent must reside in the active site is not highly specific for a fluorine atom. It is interesting that although a -SMe group (**205**) is tolerated at C-5', a –OH (**166**) is not. This selectivity is clearly not a result of steric influence, as OH sits in size between F and –SMe. It is more likely that the enzyme has a hydrophobic binding pocket that accommodates the F at C-5' in the natural substrate. Consequently more polar C-5' substituents such a hydroxyl (**166**) or phosphate (**139**) that would prefer to participate in hydrogen bonding are not accepted by the enzyme. This rational is also consistent with the observation that **233** (5'-acyl) was not accepted as a substrate. Despite the fact that **195** has a substituent on the purine ring at the C-2 position it was accepted as a substrate. This showed that the C-2 H atom could be replaced by a $-NH_2$ group and the nucleoside would still be accepted as a substrate. This suggests that the enzyme may also accept a C-2 halo substituent (F or Cl) at this position a factor that might be exploited in the future to prepare novel biologically active nucleosides (see Section 4.5.3). Overall the substrate profile that can be deduced from Table 4.4 is one that closely resembles that of a MTAP enzyme.[271,272] The main difference with the PNP from *S. cattleya* is that it can not tolerate adenosine **166** as a substrate where as MTAP can.

4.5.3 Exploiting the reversibility of the PNP enzyme from *S. cattleya*

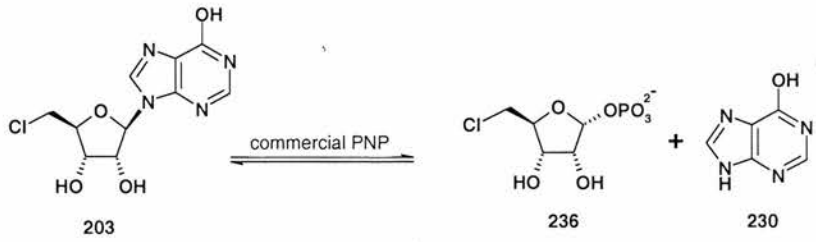
The reversibility of PNP enzymes was first investigated as early as 1947[273] as a means of forming a C-N glycosidic bond during the preparation of nucleosides. Despite the fact that this methodology presents a mild and stereoselective route to such compounds the number of reported applications of PNP enzymes in this capacity have been relatively limited.[273-275] One of the reasons for this might be that it is quite difficult to prepare the α-sugar-1-phosphates that are required as one of the building blocks for the enzymatic reaction. Also the variety of nucleosides that can be prepared using this technique is limited by the substrate tolerance of the PNP enzyme used. However it was felt that it might be possible to exploit the reversibility of the PNP enzyme from *S. cattleya* to prepare a variety of 5'-halogenated-nucloesides. These 5'-halogenated-nucleosides could then be tested as potential substrates for the fluorinase enzyme both to prepare novel SAM molecules and to identify compounds that may be of value in ^{18}F PET studies. This is an extremely attractive idea as in theory it should allow rapid access to a number of nucleosides the chemical synthesis of which would undoubtedly be more time consuming. In order to over come the problems associated with the synthesis of the required α-ribose-1-phosphates **228** and **236** it was reasoned that they could be prepared enzymatically *via* a commercial PNP from the previously synthesised precursors **86** (X = F) and **203** (X = Cl). It should also be possible to prepare the 2'-deoxy-α-ribose-1-phosphates **237** and **238** using this procedure and thus further increase the number of nucleosides that could be produced. The overall sequence by which this coupled enzymatic process was envisaged to occur is shown in Scheme 4.18. In theory the halogenated 6-oxopurine nucleosides **86, 203, 234** and **235** could be converted to their corresponding α-ribose-1-phosphates **228** and **236 - 238** by using a commercial PNP enzyme. As the PNP enzyme from *S. cattleya* is selective for only 6-amino-purine bases it should be possible to couple these sugar phosphates and with

new bases units (represented by generic compound **239**) to generate a series of 5'-halogenated-nucleosides carrying either a fluorine **240** or chlorine **241** at the C-5'. There should be no need to remove **230** during this process as it should not be accepted as a substrate by the PNP enzyme from *S. cattleya*. The new 5'-halogenated-nucleosides (series **240** and **241**) that are produced could then be tested as substrates for the fluorinase enzyme.



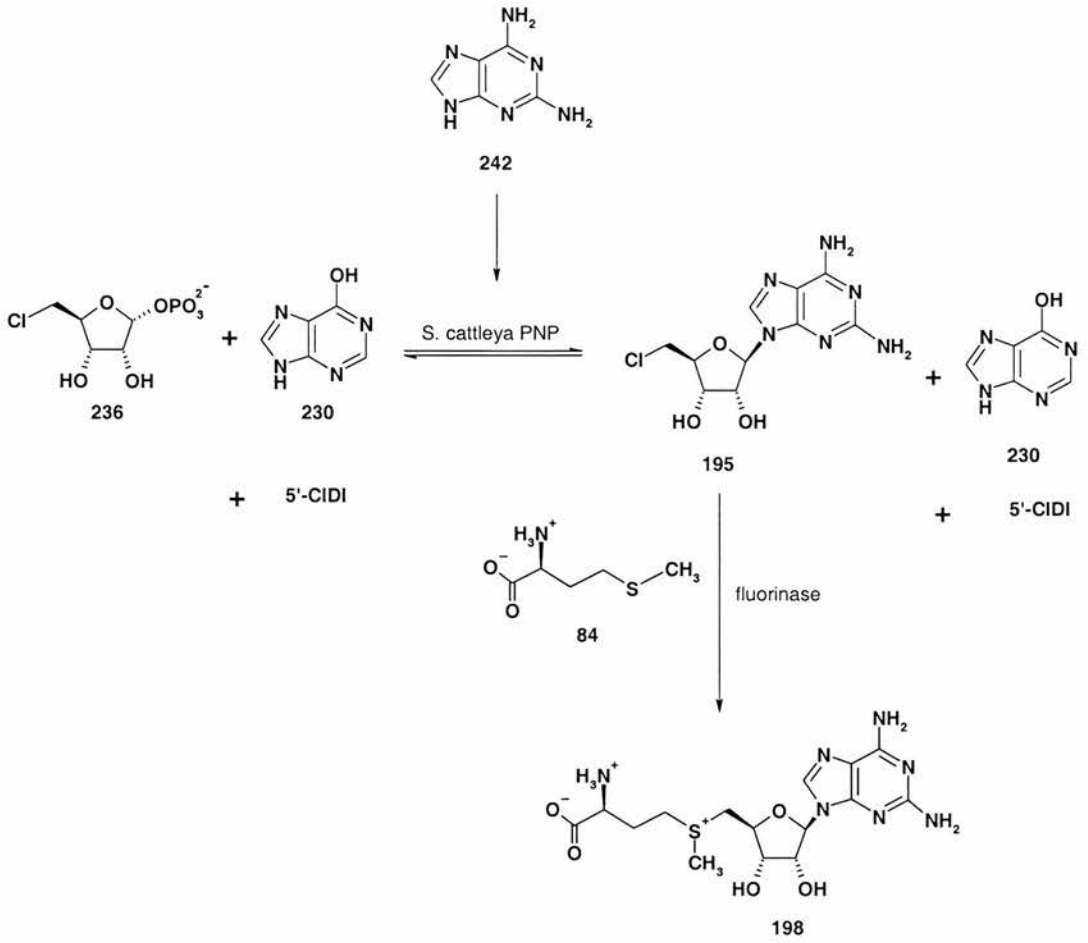
Scheme 4.18 Proposed coupled enzymatic synthesis of novel 5'-halogenated-nucleosides.

The preparation of 5-FDRP **228** using a commercial PNP enzyme has already been described thus it was of interest to see if a sample of 5'-chloro-5'-deoxy-D-ribose-1-phosphate (5'-ClDRP) **236** could also be prepared. This was easily achieved using the protocol that was utilised for the preparation of **228** (Scheme 4.19).



Scheme 4.19 Synthesis of 5-ClDRP **236**. Reagents and conditions: a) Phosphate buffer, 50 mM, pH 6.8, PNP 16 h, 37 °C, 60 % conversion by determined by HPLC (UV detection).

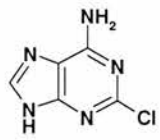
It terms of testing with the fluorinase enzyme it is more attractive to prepare the 5'-chloro-nucleoside derivates as they have been shown to react faster with L-methionine in the enzymatic preparation of SAM analogues. Accordingly **236** (containing un-reacted 5'-ClDI **203** and some **230**) was added to the PNP enzyme from *S. cattleya* along with a 2,6-diamino-purine **242** and was incubated for 16 h at 37 °C. HPLC analysis of the sample revealed the production of 5'-chloro-2-amino-adenosine **195** (confirmed against a reference) and by ES-MS. Subsequent incubation of the sample containing **195** in the fluorinase enzyme with L-methionine **84** revealed the production of 2-amino-SAM **198**. The production of 2-amino-SAM was carried out independently using synthetic **195** as previously described (Chapter 3) to confirm the result by HPLC. The preparation of 2-amino-SAM **198** confirmed that it was possible to use the enzymatic reaction sequence detailed in Scheme 4.20 to initially prepare a modified nucleoside (**195**) that could then be tested as a substrate for the fluorinase.



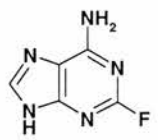
Scheme 4.20 Enzymatic preparation of 2-amino-SAM **198** from **203** using both the PNP and fluorinase enzymes isolated from *S. cattleya.*

Although only a preliminary study, it is clear that this coupled enzyme methodology could be very useful in preparing modified halo-nucleosides and modified SAM analogues for future studies. Two modified purines that may be of particular interest to test are 2-chloro-adenine **243** and 2-fluoro-adenine **244**. It is well documented that purine nucleosides that carry a halogen in the 2-position are resistant to enzymatic deamination. Consequently several important drug molecules that are currently in use such as Clofarabine **245** contain a 2-halo-substituent.[276-279] In relation to the studies that have been carried out into the biosynthesis of fluorinated intermediates in *S. cattleya* the preparation of a molecule such as **246** might be of considerable interest. This is because **246** should be resistant to

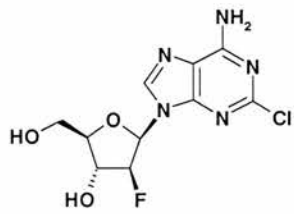
enzymatic deamination within the CFE and thus the main problem experienced when 5'-FDA **83** was incubated would be overcome.



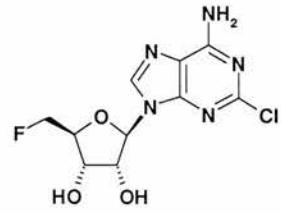
243



244



245



246

4.6 Conclusion

The synthesis of 5-fluoro-5'-deoxy-D-ribose **120** (5-FDR) was accomplished in four steps. Incubation of **120** in a crude cell free extract (CFE) from *S. cattleya* showed that it could not support the biosynthesis of any further fluorinated metabolites. This confirmed that 5'-FDR **120** is not an intermediate on the fluorometabolite biosynthetic pathway. In order to gain an insight into the metabolism of 5'-FDA **83** ^{19}F NMR time course experiments were carried out (Figure 4.2). This helped identify a molecule designated intermediate A that appeared to be produced directly from 5'-FDA **83** in the CFE. Further investigation suggested that "intermediate A" was 5'-FDRP **228.** This was confirmed by the enzymatic preparation of a reference sample of **228** from 5'-FDI **86** using a commercial PNP enzyme (EC. 2.4.2.1). 5-FDRP **228** was shown by incubation in the CFE to support the biosynthesis of FAc **15**. It was found that 5-FDR **120** could be isolated as a shunt metabolite if a commercial phytase enzyme was added to the CFE that was incubated with 5'-FDA **83**. This provided further evidence to support that 5-FDRP **228** was an intermediate on the biosynthetic pathway.

Partial purification of the PNP enzyme responsible for the production of 5-FDRP **228** in *S.*

cattleya was carried out by Ryan McGlinchey (University of St. Andrews). Initial investigations into the substrate specificity of this PNP enzyme were carried out using a variety of substituted nucleosides. Due to a lack of purified enzyme it was not possible to gain accurate kinetic data but an approximate substrate tolerance for the PNP was established. The enzyme appeared to be specific for 6-amino-purine nucleosides. The overall substrate specificity profile closely resembled that of MTAP enzymes. Since the original substrate specificity studies were carried out the gene sequence for the PNP enzyme from *S. cattleya* has been determined by Dr. J. Spencer's group (University of Cambridge). The enzyme has been shown to have a high homology with a MTAP which supports the experimental observations.

It was possible to use the reversibility of the PNP from *S. cattleya* to enzymatically prepared the nucleoside **195** from 5'-ClDRP **236** and 2,6-diaminopuirne **242**. Subsequent incubation of **195** with L-methionine **84** and over-expressed fluorinase resulted in the preparation of 2-amino-SAM **198**. This coupled enzymatic reaction approach (Scheme 4.20) offers a useful method for producing a variety of novel nucleosides that can be used to explore the substrate specificity of the fluorinase enzyme.

Part B: The metabolism of 5-FDRP in the cell free extract

4.7 Introduction

The second part of this chapter details the work that was carried out to investigate the subsequent metabolism of 5-FDRP **228** within *S. cattleya*.

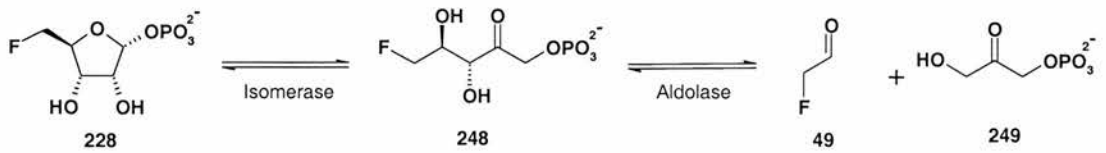
4.7.1 The metabolism of 5-methylthio-D-ribose-1-phosphate (5-MTRP)

In order to gain an insight into the potential biosynthetic pathway for the metabolism of 5-FDRP **228** it was appropriate to review the metabolism of 5-methylthio-D-ribose-1-phosphate (5-MTRP) **225** which is structurally similar to **228**. 5-MTRP **225** has been shown to be an intermediate on the methionine salvage pathway in several biological systems including humans. The metabolism of **225** is known to proceed *via* an aldose-ketose isomerase enzyme. The enzyme responsible is methylthioribose-1-phosphate isomerase and it catalysis the conversion of **225** to 5-methylthio-D-ribulose-1-phosphate **247**.[280,281]

Scheme 4.21 Conversion of 5-MTRP **225** to 5-MTRibP **247** mediate by methylthioribose-1-phosphate isomerase.

If a similar isomerase was present in *S. cattleya* then 5-FDRP **228** would be converted to 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248**. This is an attractive prospect as **248** would be an ideal substrate for a DHAP dependent aldolase enzyme. Such an aldolase could in theory mediate a reverse aldol reaction to give fluoroacetaladehyde **49** which has already been established as an intermediate. Such an isomerase and aldol reaction sequence represents a realistic metabolic pathway that could account for the conversion of 5-FDRP

228 to **49** (Scheme 4.22). There is some experimental evidence resulting from the ^{19}F NMR time course experiments to suggest that 5-FDRP **228** is converted to **49** *via* only one other fluorinated intermediate which could be **248**.



Scheme 4.22 Possible enzymatic pathway for the biosynthesis of **49** from 5-FDRP **228** in *S. cattleya*.

4.8 5-Fluoro-D-ribulose-1-phosphate as a biosynthetic intermediate.

4.8.1 CFE Incubation of 5'-fluoro-2',5'-dideoxy-adenosine

To explore further the possibility that an isomerase enzyme operates in *S. cattleya*, the ability of the previously synthesised 5'-fluoro-2',5'-dideoxy-adenosine **181** to support the CFE biosynthesis of fluoroacetaldehyde **49** was investigated. This compound has already been shown to act as a substrate for the PNP isolated from *S. cattleya*, to generate 5-fluoro-2,5-dideoxy-D-ribose-1-phosphate **237** as a product. Therefore one would expect that if **181** were to be incubated in the CFE then **237** would be produced. As there is no 2'-OH group present in **237** the isomerisation of this molecule would most likely be inhibited. Therefore it was of interest to see if **237** would be further metabolised or remain biosynthetically inert in the CFE.

Accordingly **181** (150 µl, 30 mM) was incubated in CFE (500 µl, 0.2 mg / ml phosphate buffer, pH 6.8) for 16 h at 37 °C (Section 5.2) and the resultant ^{19}F NMR spectra recorded are shown (Figure 4.6 (i)).

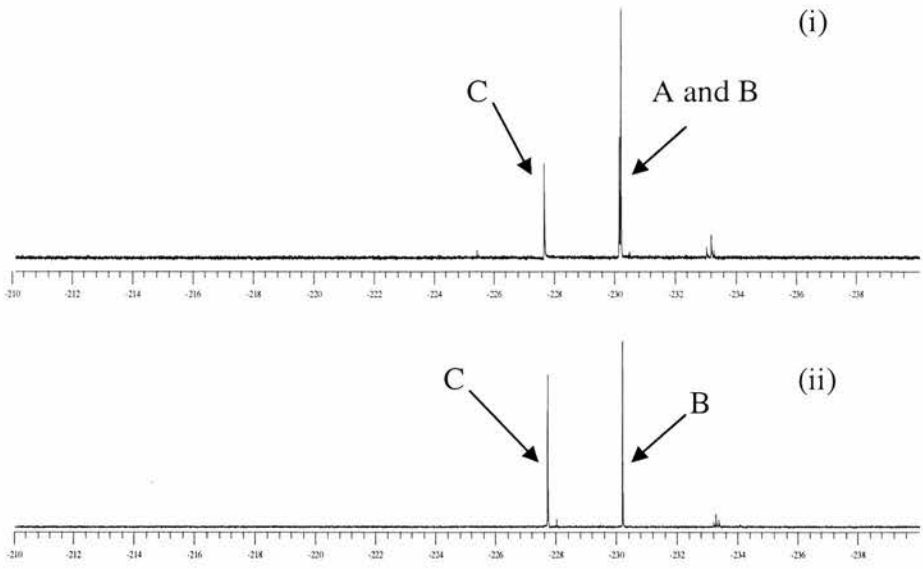


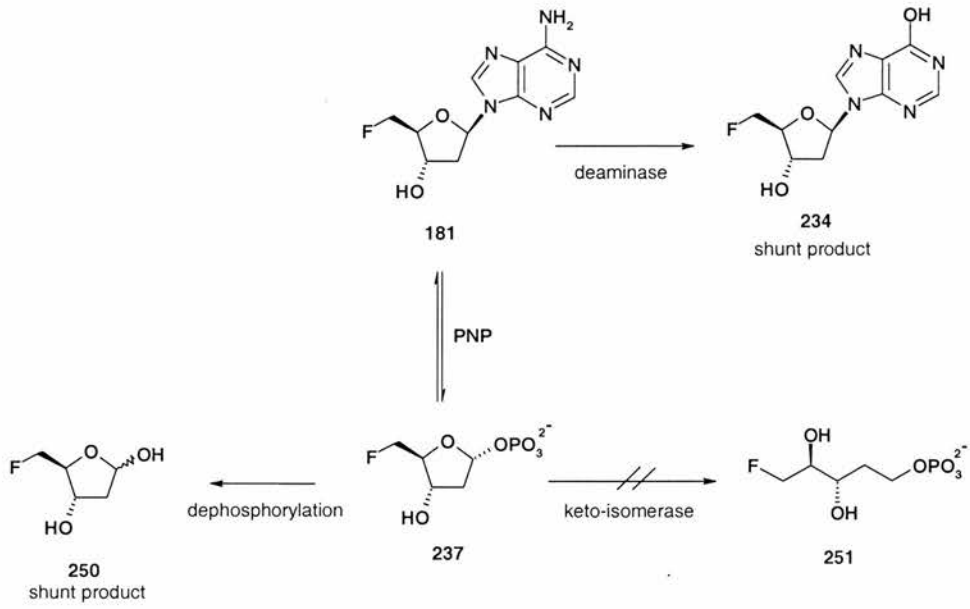
Figure 4.6 ^{19}F NMR (10% D_2O) analysis. (i) Spectra recorded after incubation 5'-fluoro-2',5'-dideoxy-adenosine **181** in a crude CFE from *S. cattleya* at 37 °C for 16 h. (ii) Spectra was recorded after treatment of original sample (i) with a phytase enzyme.

^{19}F NMR analysis (Figure 4.6, spectrum (i)) revealed that two main compounds were present in the sample. On the basis of previous work carried out with the PNP enzyme from *S. cattleya*, peak A was identified as **237**. Peak B was confirmed to be 5'-fluoro-2',5'-dideoxy-inosine **234** by HPLC analysis against a sample of **234** prepared independently by the direct deamination of **181** with AMPDA. The final molecule (peak C) was thought to be 5-fluoro-2,5-dideoxy-D-ribose **250**. Production of **250** could arise from the presence of a dephosphorylase enzyme operating within the CFE.

In order to confirm this assumption the sample was treated with a commercial phytase enzyme (EC 3.1.3.8) to remove the 1-phosphate group of **237**. When the sample was then re-analysed by ^{19}F NMR (Figure 4.6, spectrum (ii)) all of **237** (peak A) had been converted to peak C indicating that it was the free sugar 5'-fluoro-2',5'-dideoxy-D-ribose **250**.

The fact that **181** can not support the CFE biosynthesis of the fluorinated secondary metabolites Fac **15** and 4-FT **16** suggests **237** is not an intermediate on the biosynthetic pathway. From this it could be inferred that a 2-OH group on the ribose ring is essential for

the further metabolism of the fluorinated sugar phosphate intermediate, 5-FDRP **228**. However it is clear that this is only tentatively evidence to support the theory that an isomerase enzyme may be present in *S. cattleya*. The fact that **237** can not support fluorometabolite biosynthesis in the CFE could be due to of a variety of unforeseen factors.



Scheme 4.23 A summary of the metabolism of 5'-fluoro-2,'-5'-dideoxy-adenosine **181** within the CFE.

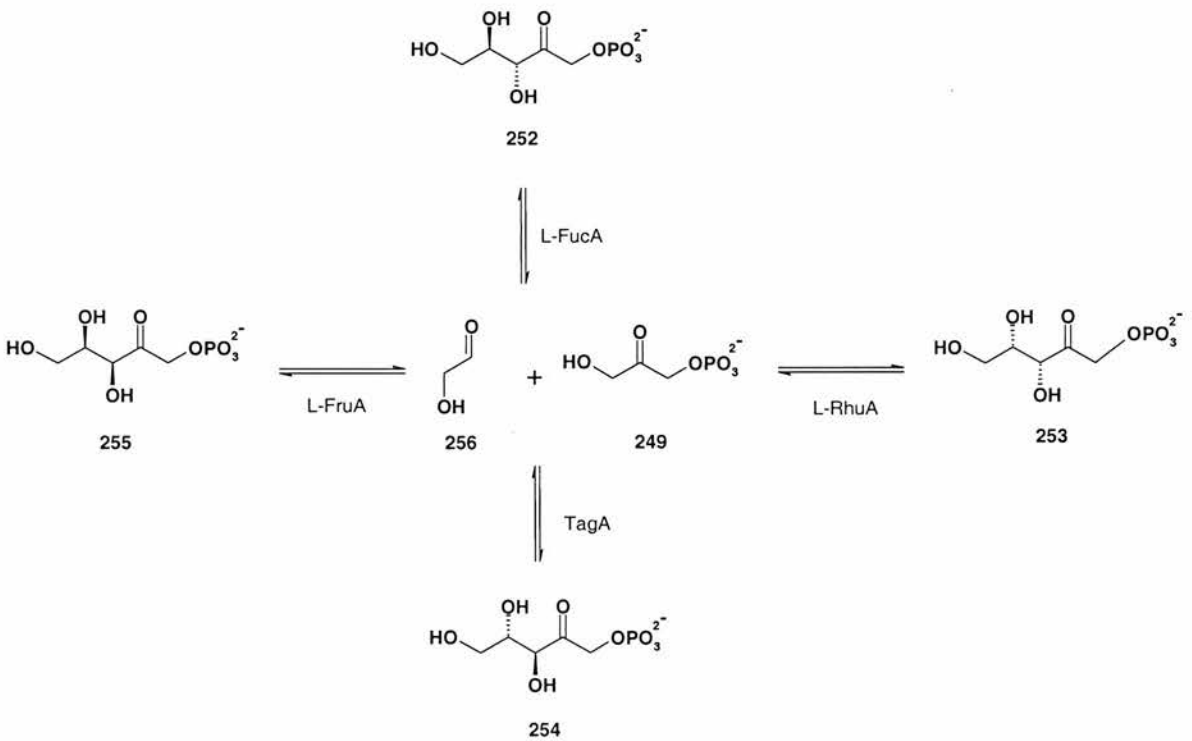
4.8.2 Enzymatic preparation of 5-fluoro-5-deoxy-D-ribulose-1-phosphate

Aldolases catalyze a retro-aldol reaction leading to cleavage of a carbon – carbon bond but when operating in the reverse direction they are capable of catalyzing carbon – carbon bond forming reactions. This has resulted in enzyme-catalyzed aldol additions becoming a useful synthetic tool for preparing carbohydrate derivatives.[282]

The synthesis of **248** was envisaged by this methodology in which a dihydroxyacetone (DHAP) dependent aldolase could be utilised. DHAP dependent aldolases catalyze the carbon-carbon bond formation between DHAP and an aldehyde.[283] There are four different

DHAP dependent aldolase enzymes and each one mediates the formation of a distinct stereoisomer.[283,284] The stereochemistry of the products formed during each enzymatic aldol reaction has been extensively studied and is shown in Scheme 4.24.

The components needed to prepare **248** by an aldolase catalyzed reaction can be readily obtained. DHAP **249** is commercially available and the synthesis of fluoroacetaldehyde **49** from fluoroethanol **149** has already been carried out (Chapter 2).



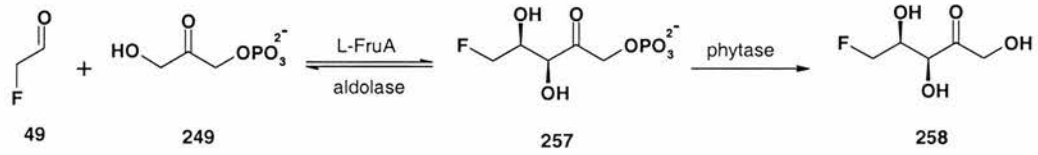
Scheme 4.24 The four DHAP dependent aldolases[283,284]

Unfortunately L-fuculose-1-phosphate (L-FucA)[285] aldolase, the enzyme that would give the desired stereochemistry for the production of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** is not commercially available. We had access to the vector containing the gene for L-FucA expressed in *E. coli* and from this it is possible to produce over-expressed aldolase.

However before the over-expression of L-FucA was attempted it was decided to investigate the suitability of this methodology using L-fructose-1,6-bisphosphate aldolase

(L-FruA from rabbit muscle)[286] as is it was commercially available.

Accordingly **49** (50 mM + residual fluoroethanol **149** and DHAP **149** were incubated with L-FruA in tris-HCl buffer (pH 7.8) at 37 °C for 16 h. The enzyme was denatured and the protein removed by centrifugation (Section 5.2). ^{19}F NMR analysis of the product mixture showed that only a small amount of the starting material fluoroacetaldehyde **49** remained. Along with residual fluoroethanol **149** two new signals were observed at -228. 25 ppm (dt, $^{2}J_{F,H}$ 46.2 Hz $^{3}J_{F,H}$ and 15.3 Hz) and -235.40 ppm. The peak at -235.40 ppm is due to a fluoroacetaldehyde tris-buffer complex. The peak at -228. 25 ppm was assumed to be the product of the aldol catalyzed reaction 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** but it was not possible to confirm this by ES-MS. It was possible, however, to remove any residual **49**, **149** and the fluoroacetaldehyde tris-buffer complex by lyophilisation of the sample leaving **257** as the only organo-fluorine product. A small amount of the freeze dried sample was treated with a commercial phytase enzyme. This resulted in a small shift in the fluorine signal from -228.25 ppm to -228.55 ppm consistent with the notion that that the product of the aldol reaction contained a phosphate group.



49 249 257 258

Scheme 4.25 Preparation of 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** and 5-fluoro-5-deoxy-D-xylulose **258**. Reagents and conditions: a) fluoroacetaldehyde **49**, DHAP **249**, Tris-HCl Buffer, ph 7.8, 16 h, 37°C b) Phytase, 16 h, 37 °C.

Satisfied that a DHAP dependent aldolase could be used to catalyse carbon - carbon bond formation between **49** and **249** attempts were made to examine an aldolase that would generate a product with the "correct" stereochemistry. Accordingly, over-expressed L-FucA aldolase was prepared by Dr. H. Deng (University of St. Andrews) from the plasmid.

The formation of the fluoroacetaldehyde tris-buffer complex must reduce the amount of **49** available to react in the L-FruA aldolase catalyzed reaction and this undoubtedly leads to a reduction in the yield of the desired product. Therefore in order to prevent this, the enzymatic preparation of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** using L-FucA was carried out in phosphate buffer (50 mM, pH 4.6). The procedure used was identical to that utilised in the formation of 5-fluoro-5-deoxy-D-xylulose **257** and is given in full detail in Section 5.2.

^{19}F NMR (^{1}H decoupled) analysis of the reaction products showed the production of two new fluorinated molecules which have been designated as peaks A and B in spectrum (i) Figure 4.7. Residual fluoroacetaldehyde **49** (peak C) was also identified in the sample. The ^{19}F NMR signals of both A and B were shown to be present as a doublet of triplets (dt) as shown in expansions (ii) and (iii) respectively. The chemical shift and coupling constants for peak A were identical to the product of the L-FruA aldolase reaction 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**. Co-injection with previously prepared **257** and subsequent reanalysis of the sample by ^{19}F NMR confirmed that peak A was indeed **257**.

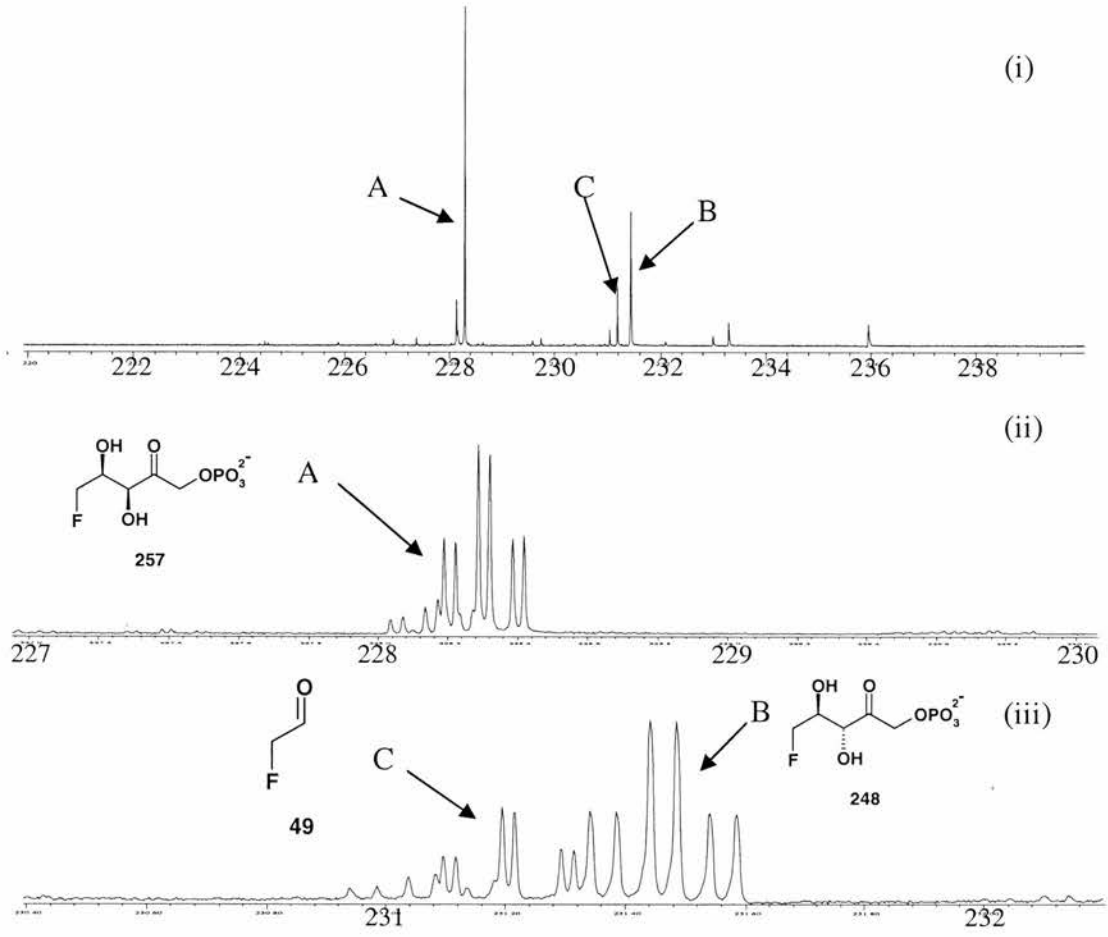
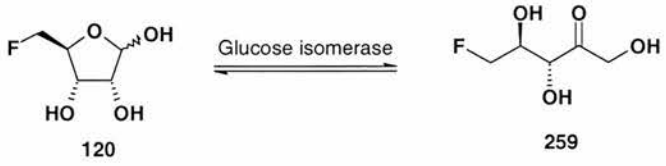


Figure 4.7 ^{19}F NMR (10% D_2O) analysis of the products produced by the enzymatic coupling of fluoroacetaldehyde **49** and DHAP **249** by L-FucA. (i) ^{1}H decoupled ^{19}F NMR spectrum of product mixture. (ii) ^{1}H coupled ^{19}F NMR spectrum expansion of peak A (dt). (iii) ^{1}H coupled ^{19}F NMR spectrum expansion of peaks B (dt) and C (dt).

Peak B was assumed to correlate the predicted stereochemical product of the L-FucA aldolase reaction 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248**. Unfortunately a synthetic sample of **248** was not available to confirm this unambiguously. It has been reported in the literature[287] that it is possible to prepare a sample of 5-fluoro-5-deoxy-D-ribulose **259** *via* enzymatic isomerisation of 5-FDR **120** (Scheme 4.26) using glucose isomerase. If 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** had been formed from fluoroacetaldehyde **49** and DHAP **249** by L-FucA then treatment of the reaction product mixture (Figure 4.7) with a phytase should give 5-fluoro-5-deoxy-D-ribulose **259**. The presence of **259** could then be confirmed by comparison with a reference sample of **259** produced independently *via*

the 5-FDR **120** / glucose isomerase enzyme protocol. In the event isomersation of synthetic prepared 5-FDR **120** was achieved using commercially available glucose isomerase and the procedure outlined by Stütz *et al.* (Scheme 4.26).[287]



Scheme 4.26 Preparation of **259** *via* enzymatic isomerisation of 5-FDR **120**. Reagents and conditions: Glucose isomerase, 4 A mol. sieves, 60 °C, 6 h.

The isomerisation reaction is reversible and does not proceed to completion (see Figure 4.8, 5-FDR **120** indicated by peaks A and B). The product of the isomerisation reaction **259** was identified as peak C (Figure 4.8).

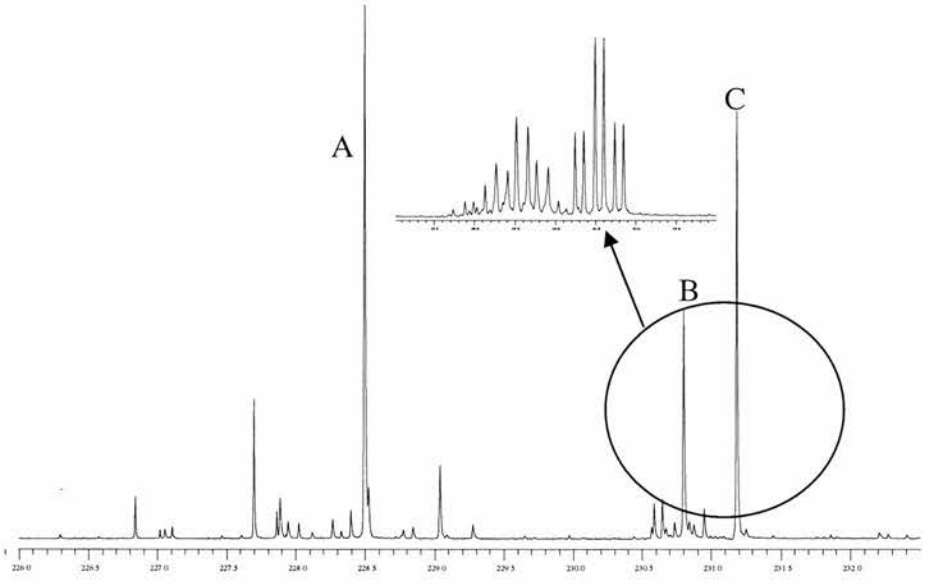


Figure 4.8 ^{19}F NMR (10% D_2O) analysis of the reaction solution from the glucose isomerase reaction of 5-FDR **120**

Having prepared 5-fluoro-5-deoxy-D-ribulose **259** the product sample that was suspected to contain 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** which was prepared from L-FucA (Figure 4.7) was treated with a phytase. The resulting product mixture prepared by this enzymatic transformation was then mixed with some of the original product mixture

material prior to it being treated with the pyhtase and the resulting ^{19}F NMR spectrum is shown in Figure 4.9.

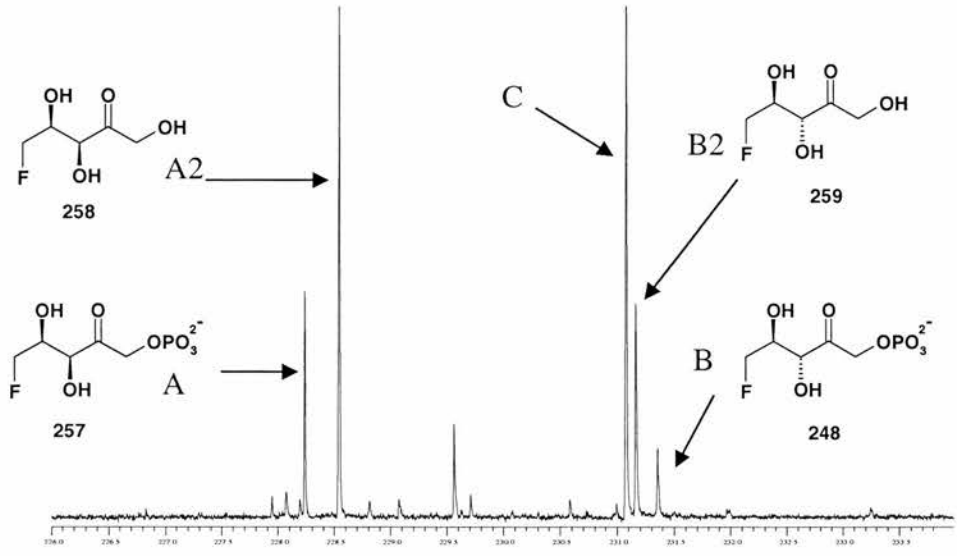
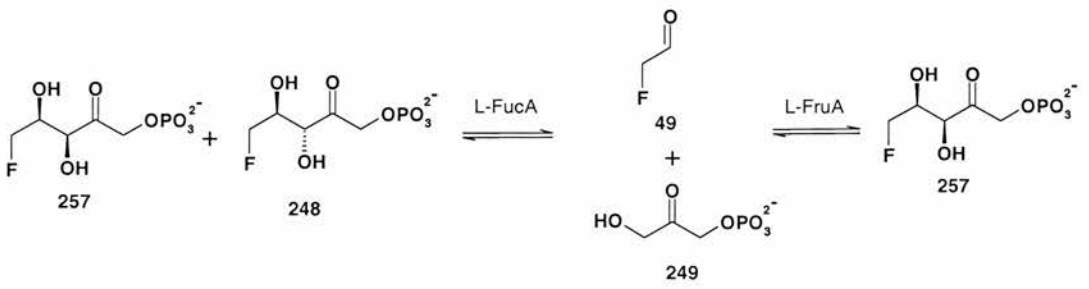


Figure 4.9 ^{19}F NMR (10% D_2O) showing the spectrum recorded from mixing of L-Fuc aldolase products before (Peaks A and B) and after treatment with the pyhtase enzyme (Peaks A2 and B2).

Peaks A and A2 were identified as **257** and **258** respectively based on the work that had been carried out with the commercial L-FruA aldolase. Peak C was identified as residual fluoroacetaldehyde **49** which was also detected in the original ^{19}F NMR analysis (Figure 4.8). Peak B2 was confirmed to be 5-fluoro-5-deoxy-D-ribulose **259** by comparison with the sample of **259** prepared *via* enzymatic isomerisation of 5-FDR **120** (Figure 4.9, peak C). 5-Fluoro-5-deoxy-D-ribulose **259** could only have come from the presence of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** in the original sample. Therefore it was concluded that 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** is formed by L-FucA and is represented in the ^{19}F NMR spectra (Figure 4.9) by peak B. Investigations at both pH 6.8 (phosphate buffer) and pH 7.8 (Tris-HCl buffer) showed that the epimerisation of **248** to **257** would not occur chemically at 37 °C. This confirmed that unlike the L-FruA aldolase the over-expressed L-FucA aldolase does not catalyze a streoselective aldol reaction. It gives rise to

the production of two diastereoisomers **248** (predicted) and **257** in a ratio of approximately 1:3 (Scheme 4.27). This is not an unusual occurrence as previously reported studies have shown that the use of certain aldehyde substrates can lead to a reduction in the daistereoselectivity of the products produced by an aldolase.[288]



Scheme 4.27 Stereoisomers produced from the aldol reactions mediated by the DHAP dependent aldolases L-FucA and L-FruA.

4.8.3 The potential roles of 248 and 257 in fluorometabolite biosynthesis

CFE studies with 5'-FDA **83** have suggested that a molecule identified at -228.25 ppm (intermediate B) might be an intermediate on the biosynthetic pathway. Comparison of the chemical shift and splitting pattern (^{19}F NMR spectroscopy) of this suspected intermediate with 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**, and mixed sample analysis confirmed that they were the same molecules. Therefore despite the fact that **257** is not the product that would be expected if 5-FDRP **228** was metabolised by an isomerase enzyme (Scheme 4.22) it was important to establish if **257** has any role to play in fluorometabolite biosynthesis. Subsequent CFE incubation studies carried out with **257** prepared from the L-FucA aldolase confirmed that it had the ability to support the biosynthesis of the FAc **15** which was detected by ^{19}F NMR spectroscopy (Figure 4.10). The presence of fluoroethanol (-223.4 ppm, Figure 4.10) has previously been observed in a crude CFE and arises from the enzymatic reduction of fluoroacetaldehyde.[107] The result strongly implicates a role for **257**

on the fluorometabolite biosynthetic pathway (Scheme 4.28). It is worth noting that the phosphate group at the C-1' position would appear to be essential for activity as 5-fluoro-5-deoxy-D-xylulose **258** was shown to be biosynthetically inert under the same incubation conditions.

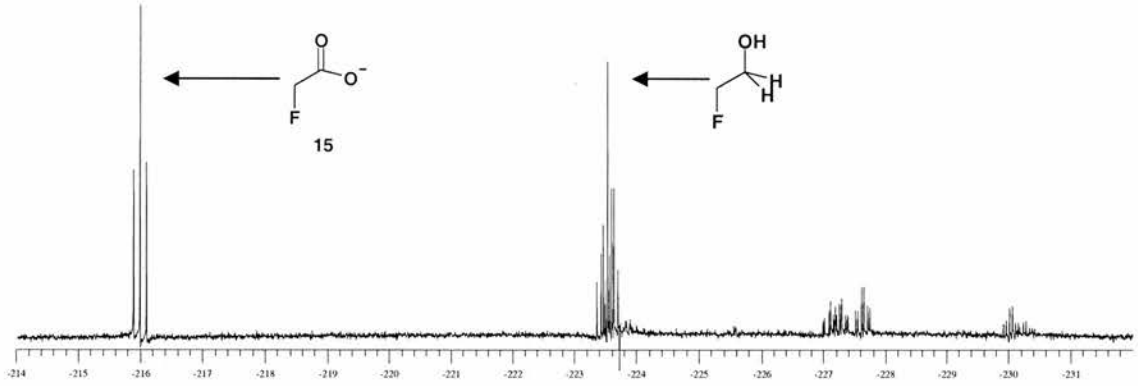
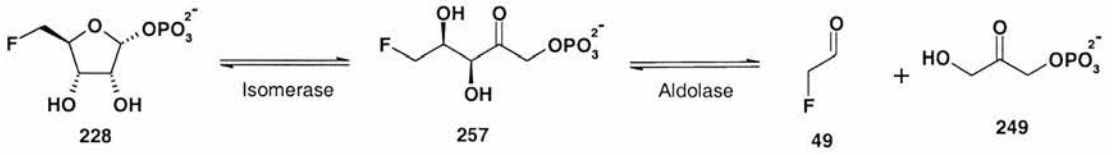


Figure 4.10 ^{19}F NMR (10% D_2O) spectrum showing the CFE production of FAc **15** from 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**.



Scheme 4.28 Hypothetically metabolic route for the production of **49** from 5-FDRP **228** incorporating as an intermediate **257**.

If this proposed pathway is correct then *S. cattleya* should posses a DHAP dependent aldolase that could catalyze the formation of 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** from the known fluorinated intermediate fluoroacetaldehyde **49** and DHAP. In order to test this idea **49** and **249** were incubated in the CFE (37 °C, 4-16 h). ^{19}F NMR analysis of the resultant sample confirmed that **257** was produced, which again provided strong evidence to support the theory that a DHAP dependent aldolase was operating in *S. cattleya*. In order to establish if it was an enzyme on the biosynthetic pathway a link between 5-FDRP **228** and 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** would have to be

established. In an attempt to establish this link 5'-FDA **83** (3.7 mM) was incubated in the CFE and its metabolism was monitored by ^{19}F NMR spectroscopy (Figure 4.11).

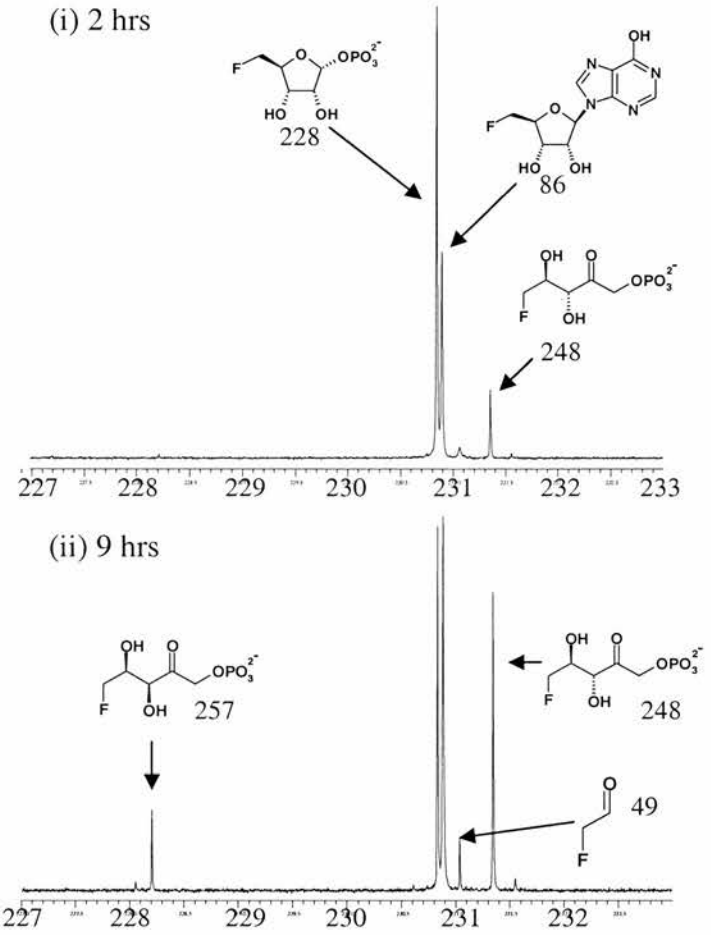
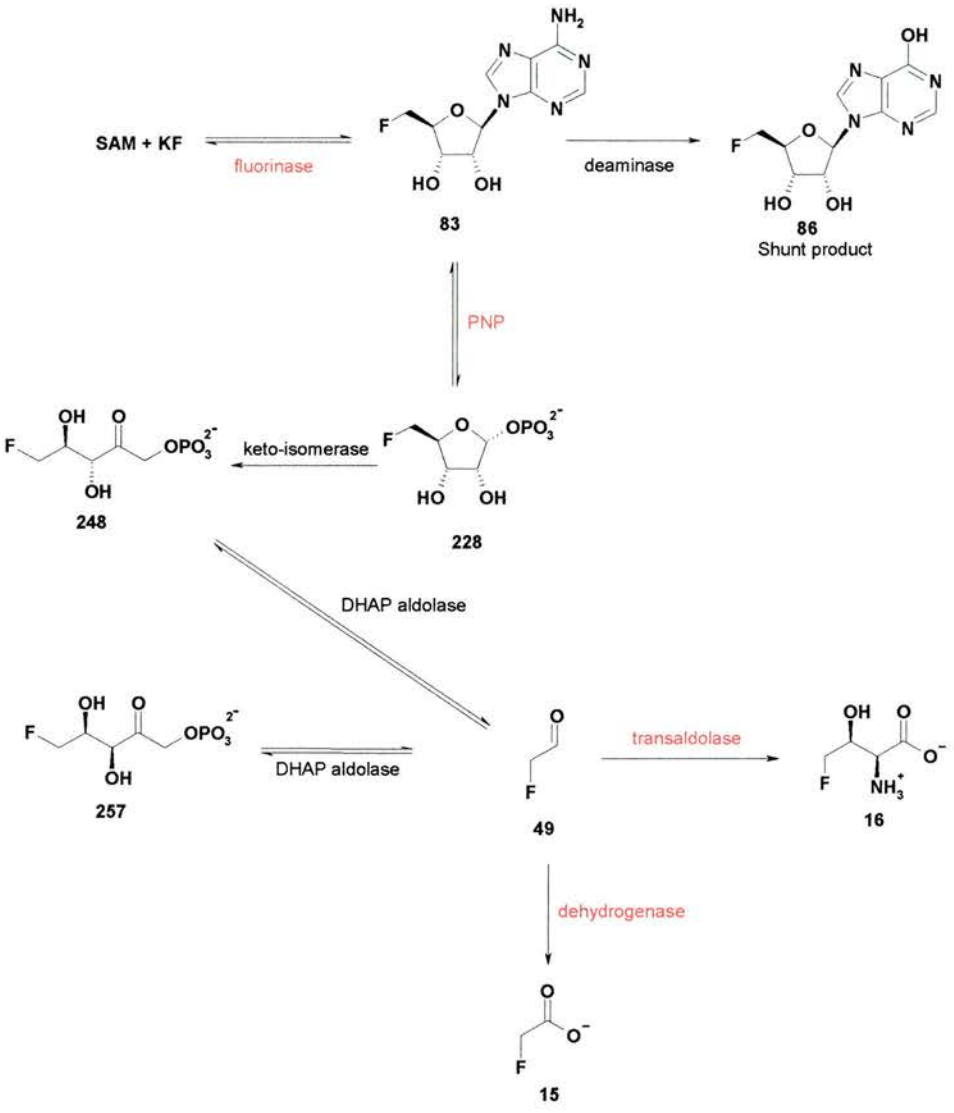


Figure 4.11 ^{19}F NMR spectra of the CFE extract (prepared in phosphate buffer pH 6.8) incubated at 37 °C with 5'-FDA **83** (3.7 mM) recorded at 2 h (i) and 9 h (ii).

The ^{19}F NMR analysis revealed that 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** is produced prior to the formation of its diastereoisomer 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** and before the production of **49**. This result implied that it is **248** and not **257** that is actually derived directly from the metabolism of 5-FDRP **228** *via* an isomerase enzyme. This is more in keeping with the predicted and reported stereochemical course of this type of enzymatic reaction. The production of 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** within the CFE can be rationalised by two different metabolic pathways.

Firstly, it is possible that **248** undergoes enzymatic epimerization to afford **257** which is then metabolised *via* a DHAP dependent aldolase to give **49**. The fact that **257** is produced from the reverse aldol reaction mediated by the CFE and that it has been shown to support the biosynthesis of FAc **15** adds weight to this hypothesis. However a second alternative pathway assumes that **257** is merely a shunt metabolite and not involved in the fluorometabolite biosynthetic pathway. In this case **257** would be formed from **248** by the action of a non-specific epimerase enzyme operating in the CFE. If this biosynthetic pathway is in operation in *S. cattleya* then two major experimental observations that support the first biosynthetic pathway must be rationalised. The first is the fact that **257** has been shown to support the biosynthesis of FAc **15**. This result can be explained by the substrate promiscuity that can be exhibited by DHAP dependent aldolase enzymes. These enzymes have the ability to cleave the carbon – carbon bond of isomers of the products which they naturally form. Therefore it is possible that if a DHAP dependent aldolase is present in *S. cattleya* it could produce **49** and ultimately FAc **15** from both **248** and **257**. The second experimental observation that must be considered is why reverse aldol reaction mediated by the CFE results in the formation of **257** and not **248**. The fact that L-FucA was shown to exhibit a poor diastereoselectivity casts doubt over the validity of this result. It might be that the aldol catalyzed production of **257** in the CFE happens simply due to the aldolase enzyme exhibiting a poor diastereoselectivity and actually favouring the formation of the "wrong" stereoisomer as the aldolase L-FucA does. The biotransformations that are believed to be responsible for the production of all the fluorinated metabolites observed and discussed throughout chapters 2,3 and 4 are summarised in Scheme 4.29.



Scheme 4.29 An overview of the entire fluorometabolite biosynthetic pathway operating in *S. cattleya*. The enzymes that have been isolated either previously or on the basis of the work reported in this thesis are shown in red.

4.9 Conclusion

Part B of this chapter has focused on the work that was carried out to try and elucidate the final steps on the fluorometabolite biosynthetic pathway in *S. cattleya*. Experimental evidence strongly indicates that an isomerase and a DHAP dependent aldolase are involved in the metabolism of 5-FDRP **228** to fluoroacetaldehyde **49**. Some confusion has arisen

due to the fact that two diastereosiomers 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** and 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257** are biosynthesised within the CFE. Unfortunately as **248** could not be prepared stereoselectively from the L-FucA aldolase it has not been possible to investigate whether or not **248** in the absence of **257** could support the biosynthesis of fluoroacetaldehyde **49** and FAc **15**. This is really the key experiment that would hopefully allow the exact nature of the final stages of the fluorometabolite biosynthetic pathway in *S. cattleya* to be determined.

5 Chemical and biological experimental

5.1 Chemical syntheses

5.1.1 General methods

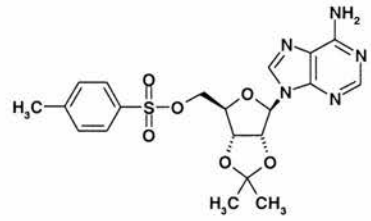
All reagents of synthetic grade were used as supplied. If further purification or drying was required the procedures used are detailed in Armarego and Perrin, "Purification of laboratory chemicals" 4th Ed.

Room temperature (R.T) refers to 20-25 °C. Air and moisture sensitive reactions were carried out under an inert atmosphere using oven-dried glassware (>150 °C). Reaction progress was monitored by thin layer chromatography (TLC) performed using Merck, Kieselgel 60 plates. Compounds were detected by either UV or by the use of an appropriate staining agent. Column chromatography was performed using Merck Kieselgel 60 silica gel (230 - 400 nm mesh) or Dowex (OH^-) resin. $MgSO_4$ was used as a drying agent.

Nuclear magnetic resonance (NMR) spectra were measured using a Bruker Av-300 or a Varian Unity Plus 300 operating at 300 MHz for ^1H, 75 MHz for ^{13}C, 282 MHz for ^{19}F. All chemical shifts (δ) are reported in parts per million (ppm) and are quoted relative to the residual proton peak of $CDCl_3$, d_6-DMSO, D_2O, CD_3CN or CD_3OH. Coupling constants (J) are given in Hertz (Hz) and represent $^3J_{H,H}$ unless otherwise stated. Spectral coupling patterns are designated as follows; d: doublet; t: triplet; q: quartet; m: multiplet and br: broad signal. GC-MS analysis was performed on an Agilent 5890 plus gas chromatograph equipped with a 5973 N mass selective detector (EI mode) and 7863 series injector. EI and CI mass spectrometry was carried out on a VG AutoSpec instrument and electrospray (ES) mass spectrometry on a Micromass time of flight LCT instrument. Melting points were determined in Pyrex capillaries using a Gallenkamp Griffin MPA350.BM2.5 melting point apparatus. High pressure liquid chromatography (HPLC) analysis was carried out using on

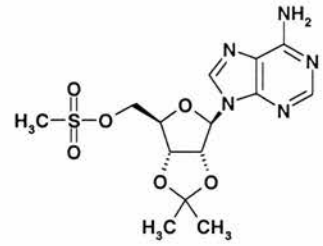
a Varian Prostar instrument fitted with a reverse phase C-18 column. The method used for all HPLC analyses is detailed in Appendix II. All infra red (IR) spectra were recorded in the range 4000-440 cm^{-1} on a Nicolet Avatar 360 FT-IR as KBr pellets or as a thin film on PTFE plates.

5.1.2 *2',3'-O-Isopropylidene-5'-O-tosyl-adenosine* **131**[146]



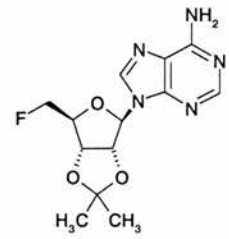
p-Toluenesulfonyl chloride (4.8 g, 25 mmol) was added in three portions to a solution of 2',3'-*O*-isopropylidene-adenosine **130** (5.1 g, 20 mmol) in anhydrous pyridine (40 cm^3) at 0 °C. The reaction solution was allowed to stir at 5 °C for 5 h after which time water (20 cm^3) followed immediately by an ice-cold saturated sodium bicarbonate solution (200 cm^3) was added. The resulting mixture was extracted with chloroform (2 x 100 cm^3). The organic layer was washed with water (2 x 150 cm^3) dried, filtered and concentrated under vacuum. The product was purified over silica using $CDCl_3$:EtOH (10:1.5) as the eluent to give **131** (5.0 g, 66%) as a white solid; mp 215-220 °C softens, 255 °C dec. (lit.,[289] 252 °C dec.); δ_H(300 MHz; d_6-DMSO) 1.30 (3 H, s, CC*H*$_3$), 1.52 (3 H, s, CC*H*$_3$), 2.33 (3H, s, ArOC*H*$_3$), 4.27 (2 H, m, 5'-H, 5'-H), 4.48 (1 H, m, 4'-H), 5.06 (1 H, dd, *J* 6.0 and 2.7, 3'-H), 5.32 (1 H, dd, *J* 6.0 and 2.0, 2'-H), 5.79 (2 H, br s, NH_2), 5.99 (1 H, d, *J* 2.0, 1'-H), 7.20-7.63 (4 H, m, AB, B'A' system, Ar-H), 7.87 (1H, s, 2-H) and 8. 25 (1 H, s, 8-H); δ_C(75 MHz; d_6-DMSO) 21.5 (Ar-*C*H$_3$), 25.4 (CH_3), 27.2 (CH_3), 70.2 (CH_2), 81.2 (CH), 83.6 (CH), 84.2 (CH), 89.5 (CH), 113.7 (C), 119.5 (C), 127.7 (Ar-CH), 130.1 (Ar-CH), 131.9 (C), 140.4 (C-2), 145.4 (C), 148.6 (C), 152.8 (C-8) and 156.5 (C); ***m/z*** (ES) 484 (M^+ + Na, 90%), 462 (M^+ +H, 100).

5.1.3 *2',3'-O-Isopropylidene-5'-O-mesyl-adenosine* **132**[147]



Methanesulfonyl chloride (1.9 cm^3, 24 mmol) was added dropwise to a stirred solution of 2',3'-*O*-isopropylidene-adenosine **130** (3.8 g, 12 mmol) in anhydrous pyridine (30 cm^3) at 0 °C. The reaction was warmed to R.T and stirred for 4 h. Ice-cold water (15 cm^3) and 2M KOH were then added until a neutral. The reaction mixture was concentrated to give a yellow oil, which was partitioned between chloroform (200 cm^3) and water (40 cm^3). The organic layer was washed with water (2 x 25 cm^3) dried, filtered and concentrated. Purification over silica eluting with $CHCl_3$:EtOH (10:1) gave **132** (3.4 g, 73%) as a white amorphous powder; mp 235-236 °C dec.; δ_H(300 MHz; $CDCl_3$) 1.41 (3 H, s, CCH_3), 1.63 (3 H, s, CCH_3), 2.92 (3 H, s, SO_2CH_3), 4.40-4.42 (3 H, m, 4'-H and 2 x 5'-H), 5.16 (1 H, dd, *J* 6.2 and 2.9, 3'-H), 5.47 (1 H, dd, *J* 6.2 and 2.0, 2'-H), 5.87 (2 H, br s, NH_2), 6.13 (1 H, d, *J* 2.0, 1'-H), 7.92 (1 H, s, 2-H) and 8. 36 (1 H, s, 8-H); δ_C(75 MHz; $CDCl_3$) 27.5 (CH_3), 25.7 (CH_3), 21.5 (SO_2-CH_3), 68.8 (CH_2), 81.8 (CH), 84.4 (CH), 85.2 (CH), 91.3 (CH), 115.2 (C), 119.1 (C), 140.5 (C-2), 150.0 (C), 153.2 (C-8) and 155.7 (C); ***m/z*** (CI) 386 (M^+ +H, 7%), 341 (6), 257 (10), 191 (10), (47), 58 (93) and 56 (100).

5.1.4 *2',3'-O-Isopropylidene-5'-fluoro-5'-deoxy-adenosine* **133**



Procedure A

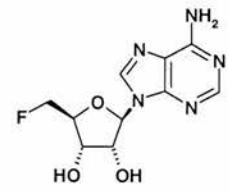
TBAF.$3H_2O$ (5.13 g, 16.3 mmol) in anhydrous MeCN (20 cm^3) was added to a solution of 2',3'-*O*-isopropylidene-5'-*O*-tosyl-adenosine **132** (3.00 g, 6.5 mmol) in anhydrous MeCN (60 cm^3) at R.T and the solution was refluxed for 16 h. The solvent was removed under reduced pressure to give a brown oil that was dissolved in chloroform (100 cm^3) and washed with water (2 x 25 cm^3). The organic layer was dried, filtered and concentrated to give a brown residue. Purification over silica eluting with $CHCl_3$:EtOH (10:1) gave **133** (1.15 g, 57%) as an off white powder; mp 159-160 °C; δ_H(300 MHz; $CDCl_3$) 1.40 (3 H, s, CC*H*$_3$), 1.64 (3 H, s, CC*H*$_3$), 4.52 (1 H, dm, 4'-H), 4.63 (2 H, dm, $^2J_{H,F}$ 46.7, 2 x 5'-H), 5.10 (1 H, dd, *J* 6.3 and 3.8, 3'-H), 5.37 (1 H, dd, *J* 6.3 and 1.9, 2'-H), 5.68 (2 H, brs, NH_2), 6.19 (1 H, d, *J* 1.9, 1'-H), 7.93 (1 H, s, 2-H / 8-H) and 8.36 (1H, s, 2-H / 8-H); δ_F(282 MHz; $CDCl_3$); -228.86 (dt, $^2J_{F,H}$ 46.7 and $^3J_{F,H}$ 23.6); δ_C(75 MHz; $CDCl_3$) 25.7 (CH_3), 27.5 (CH_3), 81.1 (d, $^3J_{C,F}$ 6.8, C-3'), 83.3 (d, $^1J_{C,F}$ 171.8, C-5'), 84.9 (C-2'), 85.9 (d, $^2J_{C,F}$ 19.5, C-4'), 91.3 (C-1'), 115.0 (C), 120.5 (C), 139.7 (C-8), 149.8 (C), 153.7 (C-2) and 156.0 (C); ***m/z*** (CI) 310 (M^+ + H, 100%), 290 (-HF, 10), 251 (8), 207 (5), 175 (7) and 75 (40); ***m/z*** (ES) 310.1321 (M^+ + H. $C_{13}H_{17}N_5O_3F$ requires 310.1315).

Procedure B

TBAF.$3H_2O$ (9.31 g, 29.5 mmol) in anhydrous MeCN (20 cm^3) was added to a solution of 2',3'-*O*-isopropylidene-5'-*O*-mesyl-adenosine **133** (4.54 g, 11.8 mmol) in anhydrous

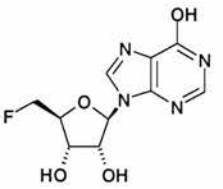
MeCN (80 cm^3) at R.T and the solution was left to reflux for 16 h. The reaction was cooled to R.T and concentrated under vacuum to afford a brown oil, that was partitioned between chloroform (150 cm^3) and water (50 cm^3). The organic layer was washed with water (2 x 50 cm^3), dried, filtered and concentrated. Purification over silica eluting with $CHCl_3$: EtOH (10:1) furnished **133** (1.67 g, 46%) as a light brown powder. Physical and spectroscopic properties were identical to that previously given for **133**.

5.1.5 *5'-Fluoro-5'-deoxy-adenosine (5'-FDA)* **83**[133]



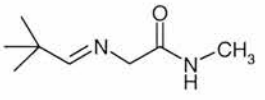
2',3'-*O*-Isopropylidene-5'-fluoro-5'-deoxy-adenosine **133** (2.25 g, 7.27 mmol) was suspended in 0.02 M sulfuric acid (15 cm^3) with stirring. The reaction slurry was then heated (100 °C) and the reaction was monitored by ^{19}F NMR until such time that no starting material was detected (4–5 h). The reaction mixture was cooled and the solvent co-evaporated several times with ethanol to give a pale yellow solid. Recrystallization from ethanol / water furnished **83** as a white amorphous powder (1.66 g, 85%); mp 203-204 °C (lit.,[133] 204-205 °C); δ_H(300 MHz; d_6-DMSO) 4.11 (1 H, dm $^3J_{H,F}$ 23.8, 4'-H), 4.26 (1 H, m, 3'-H), 4.60 (1 H, m, 2'-H), 4.64 (2 H, dm, $^2J_{H,F}$ 47.8, 2 x 5'-H), 5.46 (1 H, brs, OH), 5.65 (1 H, brs, OH), 5.94 (1 H, d, *J* 4.9, 1'-H), 7.33 (2 H, brs, NH_2), 8.16 (1 H, s, 2-H / 8 H) and 8.27 (1 H, s, 2-H /8-H); δ_F(282 MHz; d_6-DMSO) -230.00 (dt, $^2J_{F,H}$ 47.6, $^3J_{F,H}$ 23.8); δ_C(75 MHz; d_6-DMSO) 69.7 (d, $^3J_{C,F}$ 6.1, C-3'), 73.4 (C-2'), 82.6 (d, $^2J_{C,F}$ 18.0, C-4'), 83.3 (d, $^1J_{C,F}$ 171.8 , C-5'), 88.0 (C-1'), 119.4 (C), 139.7 (C-2 / C-8), 149.7 (C), 153.1 (C-2 / C-8) and 156.4 (C); ***m/z*** (GC-MS, EI, after MSTFA derivatisation) 485 (M^+, 11%), 470 (M^+ -CH_3, 3), 236 (100).

5.1.6 *5'Fluoro-5'-deoxy-inosine (5'-FDI)* **86**



5'-Fluoro-5'-deoxy-adenosine **83** (500 mg, 1.86 mmol) was suspended in purified water (10 cm^3) at R.T. 5'-Adenylic acid deaminase (AMPDA, EC 3.5.4.6 from *Aspergillus species*, Sigma Chemical. Co., A-907, 50 mg) was added and the mixture stirred at R.T for 12 h. The reaction solution was heated (100 °C / 3 min) and centrifugation (14,000 rpm / 15 min) was used to remove the denatured enzyme. Lypohilisation of the supernatant afforded **86** (497 mg, 99%) as an off white solid mp 156-159 °C softens, 190 °C dec.; δ_H(300 MHz; d_6-DMSO) 4.13 (1 H, dm, $^3J_{H,F}$ 24.3, 4'-H), 4.20 (1 H, m, 3'-H), 4.51(1 H, t, *J* 4.8, 2'-H), 4.64 (2 H, dm, $^2J_{H,F}$ 47.5, 2 x 5'-H), 5.92 (1 H, d, *J* 4.8, 1'-H), 8.09 (1 H, s, 2-H / 8-H) and 8.23 (1 H, s, 2-H / 8-H); δ_F(282 MHz; d_6-DMSO) -227.71 (dt, $^2J_{F,H}$ 47.5 and $^3J_{F,H}$ 24.2); δ_C(75 MHz; d_6-DMSO) 69.3 (d, $^3J_{C,F}$ 6.1, C-3'), 73.5 (d, $^4J_{C,F}$ 1.6, C-2'), 82.5 (d, $^2J_{C,F}$ 18.2, C-4'), 84.3 (d, $^1J_{C,F}$ 168.6, C-5'), 87.6 (C-1'), 124.4 (C), 138.5 (C-2 / C-8), 146.0 (C-2 / C-8), 148.2 (C), 156.6 (C); ***m/z*** (ES) 269 (M^+ - H. 100%); ***m/z*** (ES) 293.0668 (M^+ + Na. $C_{10}H_{11}N_4O_4FNa$ requires 293.0.662, 100%).

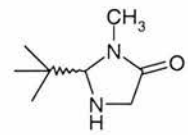
5.1.7 *N-(2,2-Dimethylpropylidene)-glycine-(N-methylamide)* **142**[163]



Glycine methyl ester **140** (12.56 g, 0.1 mol) was added slowly to a cooled solution of 8M ethanol / $MeNH_2$ (37 cm^3) with stirring. The reaction was then left to stir at R.T for 15 h. The resulting suspension was concentrated under vacuum, DCM (20 cm^3) was added and the solution was concentrated again to afford a white solid. A solution of this product,

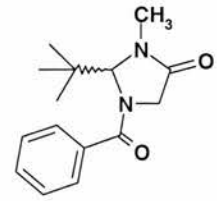
pivaldehyde (23 cm^3, 0.15 mol) and triethylamine (20.9 cm^3, 0.15 mol) in anhydrous DCM (100 cm^3) was heated under reflux for 16 h. The cooled reaction solution was filtered and the filtered cake was washed with diethyl ether (50 cm^3). The resulting white foamy filtrate was filtered and the solvent removed to give **142** (12.2 g, 78 %) as a yellow oil; ν_{max} (PTFE)/cm^{-1} 3330, 2957, 2870, 1682 (CO), 1542, 1483 and 1209; δ_H(300 MHz; $CDCl_3$) 1.03 (9 H, s, $C(CH_3)_3$), 2.82 (3 H, d, *J* 5,1, NCH_3), 3.95 (2 H, s, CH_2), 6.82 (1H, br s, N*H*) and 7.53 (1 H, d, *J* 1.53, *CH*NCH_2); δ_C(75 MHz; $CDCl_3$) 25.7 (*t*-butyl), 26.5 (NCH_3), 37.0 ($C(CH_3)_3$), 62.2 (CH_2), 171.51 (CO) and 174.9 (*C*HN); ***m/z*** (GC-MS, EI) 99 (M -C_4H_9, 100%), 73 (25) and 28 (100).

5.1.8 *2-(tert)-Butyl-3-methyl-imidazolidin-4-one* **143**[164]



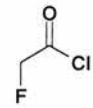
A solution of N-(2,2-dimethylpropylidene)-glycine-(N-methylamide) **142** (11.3 g, 72. 3 mnol) in anhydrous MeOH (30 cm^3) was treated with an solution of MeOH that had been saturated with HClg (60 cm^3) at 0 °C. After stirring at 0 °C for 30 min the solution was allowed to stir at R.T for 4 h. The solution was concentrated under reduced pressure to a give thick yellow oil which was redissolved in DCM (80 cm^3) and was washed with 3M aqueous NaOH (70 cm^3). Removal of the solvent under vacuum afforded **144** (8.62 g, 76%) as a yellow oil; ν_{max}(PTFE)/cm^{-1} 3329, 2959, 1745, 1682 (CO), 1558 1429, 1101 and 1003; δ_H(300 MHz; $CDCl_3$) 0.82 (9 H, s, $C(CH_3)_3$), 2.15 (1 H, br s, N*H*), 2.80 (3 H, s, NCH_3), 3.16 (1 H, d, $^2J_{H,H}$ 16.0, 5-H), 3.24 (1 H, d, $^2J_{H,H}$ 16.0, 5-H) and 3.98 (1 H, d, *J* 1.3, 2-H); δ_C(75 MHz; $CDCl_3$) 25.2 (*t*-butyl), 30.8 (NCH_3), 37. 2 ($C(CH_3)_3$), 48.9 (CH_2), 84.7 (CH) and 174.5 (CO); ***m/z*** (CI) 157.1339 (M^+ + H. $C_8H_{17}N_2O$ requires 157.1341) and 99 (10%).

5.1.9 *DL-Benzoyl-2-(tert)-butyl-3-methyl-imidazolidin-4-one* **144**[290]



Benzoyl chloride (4.01 g, 3.30 cm^3) and 1 M NaOH (17.5 cm^3) were added to a stirred solution of 2-(tert)-butyl-3-methyl-imidazolidin-4-one **143** (4.50 g, 28.8 mmol) in DCM (50 cm^3) at 0 °C. The reaction was allowed to warm to R.T and was stirred for 4 h. The solvent was removed under reduced pressure and the resulting yellow oil was dissolved in Et_2O (100 cm^3) and washed with 1 M NaOH (25 cm^3). The organic layer was separated, washed with H_2O (50 cm^3) and dried. Removal of the solvent gave a solid that was recrystallised from EtOH to afford **144** (6.53 g, 87%) as an off-white powder; mp 143 °C (lit.,[290] 143-144 °C); ν_{max}(PTFE)/cm^{-1} 2957, 1702 (CO), 1647 (CO), 1375, 1201, and 1142; δ_H(300 MHz; $CDCl_3$) 1.08 (9 H, s, C(CH_3)$_3$), 3.04 (3 H, s, NCH_3), 3.84 (1 H, d, $^2J_{H,H}$ 15.2, 5-H), 4.12 (1 H, d, $^2J_{H,H}$15.2, 5-H), 5.59 (1 H, s, 2-H) and 7.35 -7.58 (5 H, m, 5 x Ar-H); δ_C(75 MHz; $CDCl_3$) 26.0 (*t*-butyl), 31.6 (NCH_3), 39.7 (*C*-(CH_3)$_3$), 53.0 (C-5), 80.8 (C-2) and 128.0 (Ar-C), 128.6 (Ar-C), 131.5 (Ar-C), 134.5 (Ar-C), 169.3 (C-4) and 174.0 (CO); ***m/z*** (CI) 261.1611 (M^+ +H. $C_{15}H_{21}N_2O_2$ requires 261.1603, 100%) 203 (7), 89 (15), 75 (23) and 58 (46).

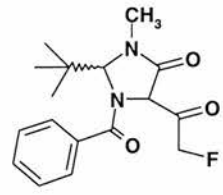
5.1.10 *Fluoroacetyl chloride* **145**[262,19]



***Fluoroacetate and fluoroacetyl chloride are extremely toxic nature and must be handled with great caution.**

Sodium fluoroacetate **15** (2.00 g, 20 mmol) and excess phthaloyl chloride (20 cm^3) were stirred vigorously and heated under reflux. Continuous distillation furnished **146** (1.54 g, 80%) as a clear oil; bp 70-71 °C (lit.,[291] 70-71 °C); δ_H(300 MHz; $CDCl_3$) 5.06 (2 H, d, $J_{H,F}$ 46.6, CH_2); δ_F(282 MHz; $CDCl_3$) -210.55 (t, $^1J_{F,H}$ 46.6); δ_C(75 MHz; $CDCl_3$) 82.1 (d, $^1J_{C,F}$ 197.4, CH_2) and 169.4 (d, $^2J_{C,F}$ 23.2, CO).

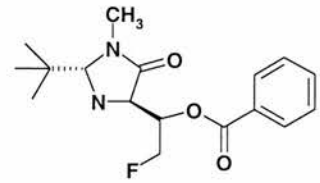
5.1.11 *5-(2'-Fluoroacet-1'-one)-2-(tert-butyl)-3-methylimidazolidin-4-one* **146**[262,19]



A solution of DL-benzoyl-2-(*tert*)-butyl-3-methyl-imidazolidin-4-one **144** (2.30 g, 8.83 mmol) in THF (45 cm^3) was slowly added (*via* cannula) to solution of diisopropylamine (1.25 cm^3, 8.9 mmol) and 2.5M butylithium (3.6 cm^3, 9.0 mmol) at -78 °C. The solution became red and was left to stir at -78 °C for 15 min. The temperature was lowered to -100 °C and was held for 30 min at this temperature before fluoroacetyl chloride **145** (2.5 cm^3, 45 mmol) was syringed slowly into the mixture. After 5 min the reaction was quenched sequentially with sat. NH_4Cl (50 cm^3) and Et_2O (45 cm^3) and allowed to warm to R.T. The aqueous layer was then extracted with Et_2O (3 x 40 cm^3) and the combined organic extracts were washed with H_2O (40 cm^3), dried ($MgSO_4$), filtered and evaporated to give a yellow oil. Purification on silica eluting with EtOAc:hexane (2:1) furnished **146** (2.82 g, 53%) as a white powder; mp 197- 198 °C dec. (lit.,[262] 197 °C dec.); ν_{max}(PTFE)/cm^{-1} 2928, 1749, 1702, 1648, 1364, 1202, 1110, and 1022; δ_H(300 MHz; $CDCl_3$) 1.08 (9 H, s, $C(CH_3)_3$), 3.05 (3 H, s, NCH_3), 4.46 (2 H, m, CH_2F), 5.38 (1 H, brs, 5'-H), 5.74 (1 H, brs, 2'-H) and 7.36 - 7.55 (5 H, m, 5 x Ar-H); δ_F(282 MHz; $CDCl_3$) -230.8 (t, $^1J_{F,H}$ 46.9); δ_C(75 MHz; $CDCl_3$) 26.1 (*t*-butyl), 32.3 (NCH_3), 40.2 ($C(CH_3)_3$), 66.2 (C-5), 80.4 (C-2),

83.8 (d, $^{1}J_{C,F}$ 186.7, C-2'); 127.7 (Ar-C), 128.9 (Ar-C), 131.7 (Ar-C), 135.8 (Ar-C), 165.0 (C-4), 170.6 (CO), 197.7 (d, $^{2}J_{C,F}$ C-1'); ***m/z*** (GC-MS, EI) 263 (M -C_4H_9, 10%), 105 (25).

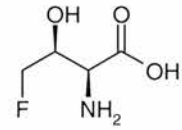
5.1.12 *5'-(1'-Benzoyloxy-2'-fluoroethyl)-2-(tert-butyl)-3-methylimidazolidin-4-one* **147**[262]



(racemic)

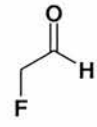
Sodium borohydride (0.12 g , 3.15 mmol) was added to a solution of 5'-(2'-fluoroacet-1'-one)-2-(tert-butyl)-3-methylimidazolidin-4-one **146** (1.00 g, 3.13 mmol) in MeOH (80 cm^3) and the reaction was left to stir at R.T for 10 min. Removal of the solvent left a yellow residue which was suspended in H_2O (40 cm^3) and extracted with Et_2O (3 x 30 cm^3). The solution was concentrated and the oil was purified over silica eluting with EtOAc:hexane (2:1) to afford **147** (0.63 g, 65%) as a yellow oil; mp 198 °C dec.; ν_{max}(PTFE)/cm^{-1} 3359, 2961, 1694 (CO), 1601 (CO), 1451, 1400, 1267 and 1109; δ_H(300 MHz; $CDCl_3$) 0.96 (9 H, s, C(CH_3)$_3$), 3.07 (3 H, s, NCH_3), 4.00 (1 H, dd, *J* 4.9 and $^{4}J_{H,H}$ 2.0, 5-H), 4.20 (1 H, d, $^{4}J_{H,H}$ 2.0, 2'-H), 4.81 (2 H, dm, $^{2}J_{H,F}$ 46.9, CH_2F), 5.47 (1 H, dm, $^{3}J_{H,F}$ 20.9, 1'-H) and 7.40 – 7.59 (5 H, m, 5 x Ar-H); δ_F(282 MHz; $CDCl_3$) -232.50 (dt, $^{2}J_{F,H}$ 46.9 and $^{3}J_{F,H}$ 20.8); δ_C(75 MHz; $CDCl_3$) 25.8 (*t*-butyl), 31.2 (NCH_3), 41.0 (C(CH_3)$_3$), 58.3 (d, $^{3}J_{C,F}$ 4.4, C-5), 72.4 (d, $^{2}J_{C,F}$ 19.9, C-1'), 81.9 (d, $^{1}J_{C,F}$ 172.5, C-2'), 83.7 (C-2), 128.4 (Ar-C), 129.5 (Ar-C), 129.7 (Ar-C), 133.3 (Ar-C), 165.4 (C-4) and 172.2 (C=O); ***m/z*** (GC-MS, EI) 265 (M -C_4H_9, 50%), 143 (100), 105 (72) and 77 (14) .

5.1.13 *(±)-4-Fluorothreonine (4-FT)* **16**[262,19]



A solution of 5'-(1'-benzoyloxy-2'-fluoroethyl)-2-(*tert*-butyl)-3-methylimidazolidin-4-one **147** (0.97 g, 3.06 mmol) in 10M HCl (45 cm^3) was heated (100 °C) in a steel bomb for 72 h. The cooled solution was extracted into Et_2O (3 x 25 cm^3) and the aqueous phase evaporated to give a residue that was absorbed on to Dowex-50-W X8 ion exchange resin. The resin was washed with water until neutrality followed by dilute HCl. The product was eluted with 1.5 M ammonia solution (1 L) which was evaporated to give **16**. Recrsytallisation from EtOH:H_2O gave **16** as an off white powder (0.27 g, 65%) 183 °C (lit.,[262] 182-183°C); ν_{max}(KBr)/cm^{-1}; 3542, 3069, 2784, 1467 and 1216; δ_H(300 MHz; D_2O) 4.05 (1 H, d, *J* 4.0, 2-H), 4.29 (1 H, dq, $^3J_{H,F}$ 24.8 and *J* 4.0, 3-H), 4.47 (2 H, ddd, $^3J_{H,F}$ 46.6 and *J* 10.4 and 4.0, 2 x 4-H); δ_F(282 MHz; D_2O) -232.57 ($^2J_{F,H}$ 46.7 and $^3J_{F,H}$ 24.9); δ_C(75 MHz; D_2O); 55.3 (C-2), 67.4 (d, $^2J_{C,F}$ 16.8, C-3), 84.9 (d, $^1J_{C,F}$ 168.0, C-4) and 170.4 (C-1); ***m/z*** (GC-MS, after MSTFA derivatisation) 236 (M –COOSi(Me_3), 56%), 218 (C1+C2 fragment, 100) and 73 (73).

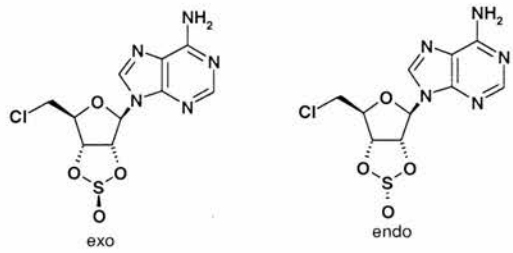
5.1.14 *Fluoroacetaldehyde* **49**[107]



Fluoroethanol **149** (5.0 cm^3, 85 mmol) was added to a suspension of PDC (10 g, 26.5 mmol) in DCM (60 cm^3) and the reaction was heated under reflux for 16 h. The reaction solution was cooled and was distilled into a separating funnel containing water (25 cm^3). The organic layer was separated and extracted with a further volume of water (15 cm^3). The aqueous layers were combined and analysed by ^{19}F NMR (282 MHz, D_2O). The

sample was shown to contain fluoroethanol **149**; -224.54 ppm (70% of total signal, tt, $^{2}J_{F,H}$ 47.6 and $^{3}J_{F,H}$ 32.1) and fluoroacetaldehyde **49** -231.01 ppm (30% of total signal, $^{2}J_{F,H}$ 46.7 and $^{3}J_{F,H}$ 10.0).

5.1.15 *5'-Chloro-5'-deoxy-2',3'-O-sulfinyl-adenosine* **167a** and **167b**[221]



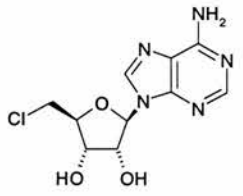
A stirred suspension of adenosine **166** (5.00 g, 18.7 mmol) in anhydrous MeCN (20 cm^3) was cooled to 0 °C and thionyl chloride (4.2 cm^3, 57.3 mmol) followed by pyridine (3 cm^3) were added. The reaction solution was left to stir at 0 °C for 4 h and for 16 h at R.T. H_2O (80 cm^3) was added to dissolve the precipitate that had formed and the solution was adjusted to ~ pH 6 by slow addition of solid $NaHCO_3$. The solution was extracted with EtOAc (200 cm^3 then 2 x 100 cm^3) and the organic layer was washed with sat. $NaHCO_3$ solution (100 cm^3) and finally H_2O (50 cm^3). Toluene (50 cm^3) was added to the organic layer and the solution was concentrated under vacuum (temperature <30 °C) to a volume of approx. 150 cm^3. Toluene (50 cm^3) was again added and the solution was further concentrated under vacuum to ~ 75 cm^3. Upon standing for 24 h at 5 °C **167** (4.74 g, 76%, was recovered as a white powder; ***m/z*** (EI) 331.0153 (M, $C_{10}H_{10}N_5O_4SCl$ requires 331.0142); The ratio of the isomers was determined by ^{1}H NMR spectroscopy to be *exo* 5:1 *endo*;

Major isomer (*exo*)[221] δ_H(300 MHz; $CDCl_3$) 3.91 (2 H, m, 2 x 5'-H), 4.53 (1 H, dt, *J* 6.4 and 3.8, 4'-H), 5.91 (1 H, dd, *J* 6.4 and 3.8, 3'-H), 6.35 (1 H, dd, *J* 6.4 and 3.1, 2'-H), 6.43 (1 H, d, *J* 3.1, 1'-H), 7.46 (2 H, brs, NH_2), 8.20 (1 H, s, 2-H / 8-H) and 8.37 (1 H, s, 2-H /

8-H); δ_C(75 MHz; $CDCl_3$) 43.3 (C-5'), 83.6 (C-4'), 85.4 (C-3'), 85.9 (C-2'), 87.2 (C-1'), 118.9 (C), 139.7 (C-8), 148.7 (C), 152.9 (C-2) and 156.2 (C).

Minor isomer (*endo*)[221] δ_H(300 MHz; $CDCl_3$) 4.02 (2 H, m, 2 x 5'-H), 4.73 (1 H, dt, *J* 6.4 and 3.8, 4'-H), 5.80 (1 H, dd, *J* 7.7 and 3.8, 3'-H), 6.22 (1 H, dd, *J* 7.6 and 3.1, 2'-H), 6.66 (1 H, d, *J* 3.1, 1'-H), 8.19 (1 H, s, 2-H / 8-H) and 8.42 (1 H, s, 2-H / 8-H); δ_C(75 MHz; $CDCl_3$) 46.3 (C-5'), 86.1 (C-4'), 88.0 (C-3'), 89.2 (C-2'), 89.2 (C-1'), 118.9 (C), 139.7 (C-8), 148.7 (C), 152.9 (C-2) and 156.2 (C).

5.1.16 *5'-Chloro-5'-deoxy-adenosine* **165**[220,221]



Procedure A

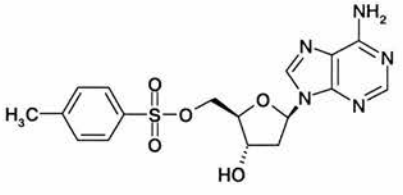
Adenosine **166** (1.00 g, 3.74 mmol) was taken up in hot (40 °C) dry pyridine (70 cm^3). The solution was cooled to R.T and $P(Ph)_3$ (2.0 g, 7.61 mmol) and CCl_4 (6.4 g, 4.0 cm^3, 48 mmol) were added. The reaction was left to stir for 2 h before being quenched with MeOH (20 cm^3). The reaction solution was partitioned between $CHCl_3$ (100 cm^3) and H_2O (100 cm^3). Evaporation of the aqueous layer followed by chromatography over silica using EtOAc:EtOH:H_2O (45:5:3) as the eluent afforded **165** (0.59 g, 55%) as a white powder; mp 101-102 °C (lit.,[220] 104-106 °C); δ_H(300 MHz; d_6-DMSO) 3.84 (1 H, dd, $^2J_{H,H}$ 11.6 and *J* 6.4, 5'-H), 3.95 (1 H, dd, $^2J_{H,H}$ 11.6 and *J* 5.1, 5'-H) 4.06 (1 H, m, 4'-H), 4.23 (1 H, m, 3'-H), 4.73 - 4.79 (1 H, m, 2'-H), 5.46 (1 H, d, *J* 5.1, 3'-OH), 5.60 (1 H, d, *J* 6.1, 2'-OH), 5.93 (1 H, d, *J* 5.3, 1'-H), 7.30 (2 H, brs, NH_2), 8.16 (1 H, s, 2-H / 8-H) and 8.34 (1 H, s, 2-H / 8-H); δ_C(75 MHz, d_6-DMSO) 45.2 (CH_2), 71.6 (CH), 73.0 (CH), 84.0 (CH), 87.7 (CH), 119.0 (C), 140.1 (C-2 / C-8), 149.8 (C), 153.1 (C-2 / C-8) and 156.4 (C); ***m/z*** (CI)

288 (M $(Cl^{37})^+$ + H, 30%), 286 (M $(Cl^{35})^+$ + H, 100); ***m/z*** (ES) 286.0707 (M^+ + H. $C_{10}H_{13}ClN_5O_4$ requires 286.0713).

Procedure 2

The isomers of 5'-chloro-5'-deoxy-2',3'-*O*-sulfinyl-adenosine **167a** and **167b** (350 mg, 1.06 mmol) were suspended in MeOH / H_2O (5 cm^3/ 1 cm^3). Concentrated NH_3 / H_2O (1.5 cm^3) was added and the solution was stirred at R.T for 4 h. The reaction solution was evaporated and the resulting semi-solid was recrystallised from H_2O and dried *in vacuo* over P_2O_5 to give **165** (274 mg, 91%) as a white amorphous powder. Physical and spectroscopic data was identical to that previously given for **165**.

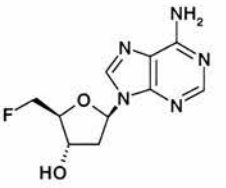
5.1.17 *5'-O-Tosyl-2'-deoxy-adenosine* **187**[291]



2'-Deoxy-adenosine **185** (0.500 g, 2.00 mmol) was dissolved in anhydrous pyridine (10 cm^3). TsCl (1.14 g, 6.00 mmol) was added and the reaction solution was left to stand at -20 °C with occasional shaking for 3 days. The solvent was removed to give a yellow residue that was purified over silica eluting with DCM:MeOH (80%:20%) gave **187** (0.470 g, 58%) as an amorphous white powder; mp 145-147 °C (lit.,[291] 146-148 °C); δ_H(300 MHz; CD_3CN) 2.37 (1 H, ddd, $^2J_{H,H}$ 14.5, *J* 7.1 and 4.8, 2'-H), 2.39 (3 H, s, CH_3), 2.84 (1 H, ddd, $^2J_{H,H}$ 14.5 and *J* 7.1 and 5.7, 2'-H), 4.07 (1 H, dt, *J* 5.6 and 4.6, 4'-H), 4.23 (1 H, dd, $^2J_{H,H}$ 10.7 and *J* 5.6, 5'-H), 4.29 (1 H, dd, $^2J_{H,H}$ 10.7 and *J* 3.8, 5'-H), 4.56 (1 H, dt, *J* 6.4 and 4.0, 3'-H) and 6.16 (2 H, brs, NH_2), 6.20 (1 H, t, *J* 7.1, 1'-H), 7.26 (2 H, d, *J* 7.9, 2 x Ar-H), 7.54 (2 H, d, *J* 8.2, 2 x Ar-H), 7.95 (1 H, s, 2-H) and 8.15 (1 H, s, 8-H); δ_C(75 MHz; CD_3CN) 20.3 (CH_3), 38.5 (C-3'), 69.5 (C-5'), 70.3 (CH), 84.0 (CH), 84.3 (CH),

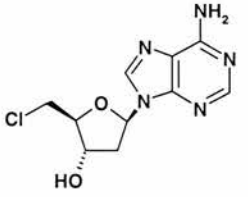
119.1 (C), 127.4 (Ar-C), 129.5 (Ar-C), 132 (C) 139.9 (C), 141.0 (C-2 / C-8), 145.2 (C), 147.2 (C-2 /C-8), 149 (C) and 152.6 (C); ***m/z*** (ES) 428 (M^+ + Na, 35%), 406 (M^+ + H, 10), 234 (M^+ - OTs, 100).

5.1.18 *5'-Fluoro-2',5'-dideoxy-adenosine* **181**[292]



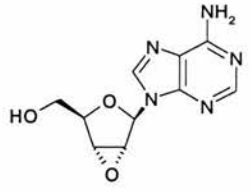
TBAF.$3H_2O$ (486 mg, 1.54 mmol) was added to a solution of 5'-*O*-tosyl-2'-deoxy-adenosine **187** (250 mg, 0.62 mmol) in MeCN (15 cm^3) and the reaction was then heated under reflux for 16 h. The reaction solution was cooled and the solvent removed to give a thick brown oil. Purification over silica eluting with EtOAc:EtOH: H_2O (45:5:3) furnished **184** (69 mg, 44 %) as a white amorphous powder; mp 216 °C (lit.,[292] 215-217 °C); δ_H(300 MHz; D_2O) 2.30 (2 H, m, 2 x 2'-H), 4.03 (1 H, dm, $^3J_{H,F}$ 29.0, 4'-H), 4.43 (1 H, m, 3'-H), 4.43 (2 H, dm, $^2J_{H,F}$ 47.1, 2 x 5'-H), 6.09 (1 H, t, *J* 6.7, 1'-H), 7.77 (1 H, s, 2-H / 8-H) and 7.90 (1 H, s, 2-H / 8-H); δ_F(282 MHz; D_2O) -231.64 (dt, $^2J_{F,H}$ 47.2 and $^3J_{F,H}$ 29.0); δ_C(75 MHz; D_2O) 39.4 (C-3'), 70.6 (d, $^3J_{C,F}$ 6.1, C-3'), 84.0 (C-1'), 84.2 (d, $^1J_{C,F}$ 168.1, C-5'), 85.6 (d, $^2J_{C,F}$ 17.7, C-4'), 120.3 (C), 139.7 (C-8), 146.6 (C), 152.8 (C-2) and 155.5 (C); ***m/z*** (ES) 276 (M^+ + Na, 100%) and 254 (M^+ +H, 57).

5.1.19 *5'-Chloro-2',5'-dideoxy-adenosine* **182**[293]



$P(Ph)_3$ (2.10 g, 8.00 mmol) followed immediately by CCl_4 (4 cm^3 , 48 mmol) were added to a solution of 2'-deoxy-adenosine **185** (1.00 g, 4.00 mmol) in anhydrous pyridine (40 cm^3) at R.T. The reaction solution was left to stir at R.T for 11 h. Removal of the solvent followed by purification over silica eluting with EtOAc:EtOH: H_2O (45:5:3) furnished **182** (0.53 g, 49%) as a white amorphous powder, mp 168-170 °C dec. (lit.,[293] 120 °C softens, 162-168 °C dec.); δ_H(300 MHz; D_2O) 2.37 (1 H, ddd, $^2J_{H,H}$ 14.1, *J* 6.5 and 4.4, 2'-H), 2.58 (1 H, m, 2'-H), 3.58 (1 H, dd, $^2J_{H,H}$ 12.2 and *J* 4.8, 5'-H), 3.60 (1 H, dd, $^2J_{H,H}$ 12.2 and *J* 4.5, 5'-H), 4.08 (1 H, dd, *J* 4.6 and 8.5, 3'-H), 4.45 (1 H, dt, *J* 6.0 and 4.0, 4'-H), 6.12 (1 H, t, *J* 6.4, 1'-H), 7.84 (1 H, s, 2-H / 8-H) and 8.01 (1 H, s, 2-H / 8-H); δ_C(75 MHz; D_2O) 38.8 (CH_2, C-3'), 44.4 (CH_2, C-5'), 71.8 (CH), 83.8 (CH), 85.9 (CH), 118.6 (C), 139.8 (C-2 / C-8), 148.5 (C), 152.7 (C-2/ C-8) and 155.4 (C); ***m/z*** (CI) 270.0745 (M^+ +H. $C_{10}H_{13}N_5O_2Cl$ requires 270.0758).

5.1.20 *9-(2,3-Anhydro-β-D-ribofuranosyl)adenine* **110**[225]

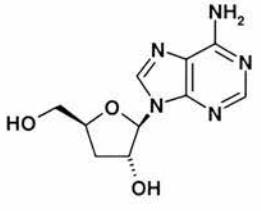


α-Acetoxyisobutyryl bromide (α-AIBBr, 9.0 cm^3, 60 mmol) followed immediately by MeCN / H_2O (2.7 cm^3 / 0.3 cm^3) were added to a suspension of adenosine **166** (4.01 g, 15 mmol) in anhydrous MeCN (300 cm^3) at R.T. The reaction solution was left to stir at R.T for 1 h. Saturated sodium bicarbonate solution (300 cm^3) was then added slowly and the resulting solution was extracted into EtOAc (2 x 300 cm^3). The combined organic layers were washed with brine (100 cm^3) dried and filtered. The solvent was removed under reduced pressure to afford a white foam. TLC (silica, DCM:MeOH (80%: 20%)) analysis

revealed a mixture of 2',3' bromo acetates **188a**, **188b** **189a** and **189b** (Rf. 0.76 and 0.58).[225]

70 cm^3 of Dowex (OH^-) resin (previously washed with dry MeOH) was added to a solution of the crude product mixture of 2',3' bromo acetates in dry MeOH (120 cm^3) and the slurry was stirred at R.T for 1.5 h. The resin was removed by filtration and was washed well with methanol several times. The filtrate and the MeOH washings were combined and concentrated under vacuum to a white foam that was recrystallised (EtOH) to give **110** (3.03 g, 81%) was a white powder; mp 179-180 °C (lit.,[294] 180 °C); δ_H(300 MHz; d_6-DMSO) 3.50 (2 H, m, 2 x 5'-H), 4.18 (1 H, t, *J* 5.0, 4'-H), 4.22 (1 H, d, *J* 1.8, 2'-H / 3'-H), 4.52 (1 H, d, *J* 1.8, 2'-H / 3'-H), 5.08 (1 H, s, 5'-OH, D_2O exchangeable), 6.21 (1H, s, 1'-H), 7.34 (2 H, brs, NH_2, D_2O exchangeable), 8.17 (1 H. s, 2-H / 8-H) and 8.34 (1 H, s, 2-H / 8-H); δ_C(75 MHz; d_6-DMSO) 57.7 (C-2' / C-3'), 58.7 (C-2' / C-3'), 60.8 (CH_2, C-5'), 81.1 (C-4'), 82.0 (C-1'), 118.6 (C), 139.5 (CH, C-2 / C-8), 149.1 (C), 152.6 (CH, C-2 / C-8) and 156.0 (C); ***m/z*** (CI) 250.0944 (M^+ +H. $C_{10}H_{12}N_5O_3$ requires 250.0940).

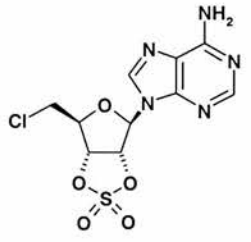
5.1.21 *3'-Deoxy-adenosine* **190**[225]



Superhydride (1M, 50 cm^3, 50 mmol) was added slowly to a solution of 9-(2,3-anhydro-β-D-ribofuranosyl)adenine **110** (1.00 g, 4.01 mmol) in anhydrous 1,4-dioxane (25 cm^3) at 0 °C. The solution was then left to stir for 1 h at 0 °C and for a further 16 h at R.T. The reaction mixture was cautiously acidified (5% HOAc / H_2O, 100 cm^3), and purged with N_2 for 1 h (fume hood) to remove *pyrophoric* triethylborane. Removal of the solvent under reduced pressure gave a yellow residue that was purified using Dowex (OH^-) resin (H_2O

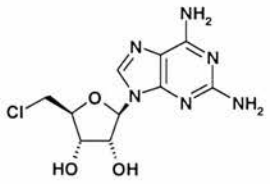
and 30% MeOH / H_2O) to furnish **190** (0.90 g, 89%) as a white powder; mp 225 °C (lit.,[225] 225 °C); δ_H(300 MHz; DMSO) 1.92 (1 H, m, 3'-H), 2.25 (1 H, m, 3'-H), 3.55 (1 H, dd, $^2J_{H,H}$ 12.1 and *J* 3.8, 5'-H), 3.75 (1 H, dd, $^2J_{H,H}$ 12.1 and *J* 3.2, 5'-H), 4.35 (1 H, m, 4'-H) 4.56 (1 H, m, 2'-H), 5.87 (1 H, d, *J* 2.3, 1'-H), 7.32 (2 H, brs, NH_2), 8.14 (1 H, s, 2-H, 8-H) and 8.37 (1 H, s, 2-H / 8-H); δ_C(75 MHz; DMSO) 34.4 (CH_2), 62.9 (CH_2), 75.0 (CH), 81.1 (CH), 91.1 (CH), 119.0 (C), 139.5 (C-2 / C-8), 149.2 (C), 152.8 (C-2 / C-8) and 156.4 (C); ***m/z*** (ES) 274.0924 (M^+ +Na. $C_{10}H_{13}N_5O_3Na$ requires 274.0916).

5.1.22 *5'-Chloro-5'-deoxy-2',3'-O-sulfonyl-adenosine* **193**[226]



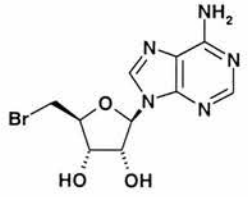
RuCl (tip of small spatula), $NaIO_4$ (1.80 g, 8.49 mmol) and H_2O (15 cm^3) were added sequentially to a suspension of 5'-chloro-5'-deoxy-2',3'-*O*-sulfinyl-adenosine **167a** and **167b** (1.00 g, 3.02 mmol) in MeCN (20 cm^3). The reaction was then left to stir at R.T for 1.5 h. EtOAc (30 cm^3) was added the reaction mixture separated into two layers. The organic layer was collected and concentrated under vacuum to give a grey powder that was recrystallised (ethanol) to afford **193** (0.89 g, 85%) as light grey powder; mp 225-226 °C (lit.,[295] 227-228 °C); δ_H(300 MHz; $CDCl_3$) 3.97 (2 H, m, 2 x 5'-H) 4.73 (1 H, m, 4'-H), 6.06 (1 H, dd, *J* 7.0 and 4.0, 3'-H), 6.51 (1 H, dd, *J* 7.0 and 2.0, 2'-H), 6.67 (1 H, d, *J* 2.6, 1'-H), 7.54 (2 H, brs, NH_2), 8.20 (1 H, s, 2-H / 8-H) and 8.36 (1 H, s, 2-H / 8-H); δ_C(75 MHz; $CDCl_3$); 42.8 (CH_2), 83.2 (CH), 84.3 (CH), 85.3 (CH), 86.8 (CH), 119.1 (C), 139.8 (C-2 / C-8), 148.4 (C), 152.7 (C-2 / C-8) and 156.0 (C); ***m/z*** (ES) 348.0178 (M^+ +H. $C_{10}H_{11}N_5O_5SCl$ requires 348.0169).

5.1.23 *2-Amino-5'-chloro-5'-deoxy-adenosine* **195**



$P(Ph)_3$ (0.250 g, 0.95 mmol) and CCl_4 (1 cm^3, 12 mmol) were added to a suspension of 2-amino-adenosine **194** (250 mg, 0.89 mmol) in anhydrous pyridine (15 cm^3) at R.T. The reaction mixture was then left to stir at R.T for 16 h. MeOH (10 cm^3) was added and the solvent removed under vacuum. Purification by preparative TLC (silica, EtOAc: EtOH: H_2O (45:5:3)) furnished **195** (112 mg, 42%) as an off-white powder; mp 120-122 °C dec.; δ_H(300 MHz; CD_3CN); 3.90 (2 H, m, 2 x 5'-H), 4.20 (1 H, m, 3'-H), 4.36 (1 H, dd, *J* 5.3 and 4.2, 2'-H), 5.38 (1 H, t, *J* 4.2, 4'-H), 5. 75 (1 H, d, *J* 5.3, 1'-H) and 7.68 (1 H, s, 8-H); δ_C(75 MHz; CD_3CN)* 44.3 (CH_2), 71.3 (CH), 72.9 (CH), 83.5 (CH), 88.0 (CH), 112.6 (C), 136.3 (C-8) and 152.3 (C); ***m/z*** (ES) 303 (Cl^{37} M^+ +H, 45%) and 301 (Cl^{35} M^+ +H, 100). For HPLC (UV) analysis see Chapter 3, Figure 3.10. (*missing 2 x quaternary carbons).

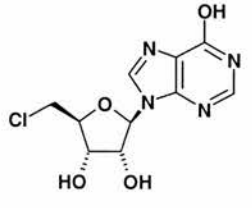
5.1.24 *5'-Bromo-5'-deoxy-adenosine* **201**[230,231]



$P(Ph)_3$ (2.10 g, 7.99 mmol) and CBr_4 (2.50 g, 7.53 mmol) were added to a suspension of adenosine **166** (1.00 g, 3.74 mmol) in anhydrous pyridine (50 cm^3) at R.T and the reaction was left to stir. After 2 h additional CBr_4 (2.50 g, 7.53 mmol) was added and the reaction was then left to stir for a further 12 h. MeOH (20 cm^3) was added and the solvent was

removed under reduced pressure. Purification over silica eluting with EtOAc:EtOH:H_2O (45:5:3) afforded **201** (0.42 g, 34%) as a white amorphous powder mp 199-200 °C (lit.,[230] 200 °C); δ_H(300 MHz; d_6-DMSO) 3.71 (1 H, dd, $^2J_{H,H}$ 10.8 and *J* 6.7, 5'-H), 3.83 (1 H, dd, $^2J_{H,H}$ 10.8 and *J* 5.1, 5'-H), 3.96 (1 H, t, *J* 5.4, 4'-H), 4.21 (1 H, m, 3'-H), 4.77 (1 H, dd, *J* 11.2 and 5.8, 2'-H), 5.92 (1 H, d, *J* 5.6, 1'-H), 5.59 (1 H, d, *J* 6.1, C3'-O*H*), 5.73 (1 H, d, *J* 6.1, C2'-O*H*), 6.41 (2 H, brs, NH_2), 8.14 (1 H, s, 2-H / 8-H) and 8.34 (1 H, s, 2-H / 8-H); δ_C(75 MHz; d_6-DMSO) 33.8 (CH_2), 72.1 (CH), 72.7 (CH, 83.5 (CH), 87.4 (CH), 119.1 (C), 139.8 (C-2 / C-8), 149.4 (C), 152.7 (C-2 / C-8), and 156.1 (C); ***m/z*** (ES) 332 (Br^{81} M^+ +H, 100%), 330 (Br^{79} M^+ +H, 100) and 250 (M^+ -Br, 61).

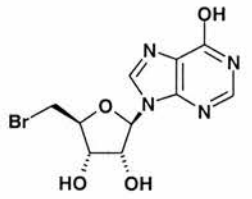
5.1.25 *5'-Chloro-5'-deoxy-inosine (5'-ClDI)* **203**



5'-Chloro-5'-deoxy-adenosine **165** (100 mg, 0.35 mmol) was suspended in purified water (10 cm^3) at R.T and 5'-adenylic acid deaminase (AMPDA, EC 3.5.4.6 from *Aspergillus species*, Sigma Biochemical, A-907, 20 mg) was added and the mixture was stirred for 12 h. The reaction solution was heated (100 °C / 3 min) and centrifugation (14,000 rpm / 15 min) was used to remove the denatured enzyme. Lypohilisation of the supernatant afforded **203** (100 mg, 100 %) as an off white amorphous powder; mp 159-160°C; δ_H(300 MHz; d_6-DMSO) 3.83 (1 H, dd, $^2J_{H,H}$ 11.5 and *J* 6.3, 5'-H), 3.93 (1 H, dd, $^2J_{H,H}$ 11.5 and *J* 4.9, 5'-H), 4.10 (1 H, m, 4'-H), 4.17 (1 H, m, 3'-H), 4.64 (1 H, t, *J* 5.4, 2'-H), 5.91 (1 H, d, *J* 5.4, 1'-H), 8.10 (1 H, s, 2-H / 8-H) and 8.31 (1 H, s, 2-H / 8-H); δ_C(75 MHz; d_6-DMSO) 44.7 (CH_2), 71.1 (C-3'), 73.1 (C-2'), 83.8 (C-4'), 87.3 (C-1'), 124.4 (C), 138.9 (C-2 / C-8),

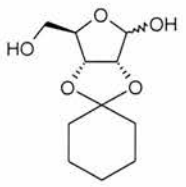
146.0 (C-2 / C-8), 148.3 (C) and 156.5 (C); ***m/z*** (ES) 309.0374 (M^+ +Na. $C_{10}H_{11}N_4O_4ClNa$ requires 309.0367, 100%).

5.1.26 *5'-Bromo-5'-deoxy-inosine* **207**



5'-Bromo-5'-deoxy-adenosine **201** (0.200 g, mmol) was suspended in ultra-pure water (15 cm^3). AMPDA (EC 3.5.4.6, from *Aspergillus species*, Sigma Biochemicals, A-907, 30 mg) was added and the solution was left to stir at 25 °C for 12 h. The sample was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). Lyophilisation of the solution furnished **207** (196 mg, 98%) as an off-white powder; mp 115-120 °C softens, 190-191 °C dec.; δ_H(300 MHz; d_6-DMSO) 3.70 (1 H, dd, $^2J_{H,H}$ 10.7 and *J* 6.4, 5'-H), 3.82 (1 H, dd, $^2J_{H,H}$ 10.7 and *J* 5.2, 5'-H), 4.08-4.13 (1 H, m, 4'-H), 4.17 (1 H, m, 3'-H), 4.67 (1 H, t, *J* 5.7, 2'-H), 5.91 (1 H, d, *J* 5.7, 1'-H), 8.10 (1 H, s, 2-H / 8-H) and 8.33 (1 H, s, 2-H / 8-H); δ_C(75 MHz; d_6-DMSO) 33.7 (CH_2), 71.9 (CH), 73.1 (CH), 83.6 (CH), 87.3 (CH), 124.4 (C), 138.9 (C-2 / C-8), 146.0 (C-2 / C-8) and 148.3 (C) and 156.5 (C); ***m/z*** (ES) 355 (Br^{81} M^+ + Na, 68%) 353 (Br^{79} M^+ + Na, 68) and 250 (M^+ -Br, 100) and ***m/z*** (ES) 352.9844 (M^+ +Na. $C_{10}H_{11}N_4O_4BrNa$ requires 352.9861).

5.1.27 *2,3-O-Cyclohexlidene-D-ribose* **220**[245,246]



Procedure A[245]

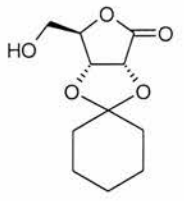
Concentrated H_2SO_4 (10 drops) was added to a solution of D-ribose **218** (15.0 g, 0.1 mol) in cyclohexanone (50 cm^3). The resulting mixture was stirred at R.T for 2 h before solid Na_2CO_3 (0.5g) was added and the solvent removed under reduced pressure. The residue was then dissolved in DCM (100 cm^3) and washed well with H_2O. The product was purified over silica eluting with DCM:MeOH (95%: 5%) to give **220** (8.05 g, 35%) as a viscous yellow oil; ν_{max}(PTFE)/cm^{-1} 3366, 2935, 2869, 1449, 1369, 1163, 1103, 1006 and 941; δ_H(300 MHz; $CDCl_3$) 1.40-1.86 (10 H, m, cyclohexane), 3.70 (2 H, m, 2 x 5'-H), 4.39 (1 H, m, 4'-H), 4.55 (1 H, d, *J* 5.9, 2'-H), 4.79 (1 H, d, *J* 5.9, 3'-H), 5.39 (1 H, brs, 1'-H); δ_C(75 MHz; $CDCl_3$) 23.6 (CH_2), 23.9 (CH_2), 25.0 (CH_2), 34.2 (CH_2), 36.0 (CH_2), 56.5 (C-5'), 81.2 (C-4'), 86.3 (C-2'), 87.8 (C-3'), 102.4 (C-1') and 112.8 (C); ***m/z*** (ES) 253.1043 (M^+ + Na. $C_{11}H_{18}O_5Na$ requires 253.1052)

Procedure B[246]

*p*TSA (0.7g, 3.7 mmol) was added to a solution of D-Ribose **218** (30.0 g, 0.2 mmol), in cyclohexanone (200 cm^3) at R.T. The reaction mixture was then stirred for 12 h. EtOAC (500 cm^3) was then added and the reaction solution was washed with saturated $NaHCO_3$ solution (300 cm^3) and then H_2O (300 cm^3). The organic layer was extracted, dried and filtered and the solvent was removed under reduced pressure (high vacuum). Purification over silica eluting with DCM:MeOH (95%: 5%) furnished **220** (40.1 g, 87%) as a viscous

yellow oil. Spectral and physical properties were identical to those previously reported for **220**.

5.1.28 *2,3-O-Cyclohexlidene-D-ribonolactone* **219**[244,245]



Procedure A[245]

PCC (50.0 g, 0.23 mol) was added to a solution of 2,3-*O*-cyclohexlidene-D-ribose **220** (25.8 g, 0.11 mol) in DCM (330 cm^3) with vigorous stirring. The resulting reaction solution was stirred at R.T for 16 h. Et_2O (1000 cm^3) was added and the resulting mixture was filtered through celite and evaporation of the filtrate gave a yellow oil. Purification over silica (EtOAc:hexanes 2:1) afforded **219** (9.7 g, 38%) as a clear oil; ν_{max} (PTFE)/cm^{-1} 3482, 2922, 2361, 1762, 1448, 1192, 150 and 1115; δ_H(300 MHz; $CDCl_3$) 1.36-1.70 (10 H, m, cyclohexane), 2.85 (1 H, brs, 5'-OH), 3.79 (1 H, dd, *J* 12.4 and 1.7, H, 2'-H / 3'-H), 3.98 (1 H, dd, *J* 12.3 and 2.3, 2'-H / 3'-H), 4.64 (1 H, t, *J* 1.92, 4'-H), 4.75 (1 H, d, *J* 5.6, 5'-H) and 4.84 (1 H, d, *J* 5.6, 5'-H); δ_C(75 MHz; $CDCl_3$) 23.6 (CH_2), 23.7 (CH_2), 24.6 (CH_2), 34.7 (CH_2), 36.2 (CH_2), 61.6 (C-5'), 75.1 (CH), 76.6 (CH), 77.8 (CH), 83.2 (CH), 113.7 (C) and 175.6 (CO); ***m/z*** (ES) 251.0889 (M^+ + Na. $C_{11}H_{16}O_5Na$ requires 251.0895).

Procedure B[245]

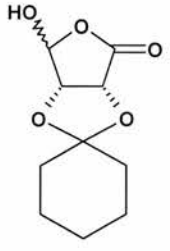
PDC (50.0 g, 132.3 mmol) was added with vigorous stirring to a solution of 2,3-*O*-cyclohexlidene-D-ribose **220** (8.7 g, 37.7 mmol) in DCM (150 cm^3). The reaction solution was then stirred at R.T for 16 h. Et_2O (400 cm^3) was added and the resulting mixture was filtered through celite. Evaporation of the filtrate gave a yellow oil that was purified over

silica (EtOAc / Hexanes 2:1) to give **219** (2.8 g, 33%) as a clear oil. Spectral and physical properties were identical to those previously reported for **219**.

Procedure C[244]

D-Ribonolactone **217** (5.00g, 33.8 mmol) was dissolved in cyclohexanone (100 cm^3) containing orange drying perils (enough to cover base of 250 cm^3 rb-flask). Anhydrous $FeCl_3$ (150 mg) was added and the reaction was heated at 50 °C for 3 h. The cooled reaction mixture was stirred with Na_2CO_3 (0.50 g) and charcoal (1.00g) for 15 minutes. The solution was filtered through celite and the solvent removed under vacuum. The resulting residue was evaporated with H_2O (x3) and toluene (x3) to give a pale purple solid. Recrystallisation from DCM and hexane furnished **219** (6.70 g, 87%) as an off-white powder; mp 128 °C (lit.,[244] 128-129 °C). Spectral properties were identical to those previously reported for **220**.

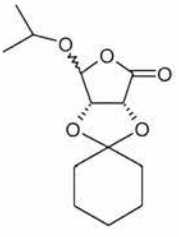
5.1.29 *2,3-O-Cyclohexylidene-L-erthruronolactone* **221**[245]



NaOH (1.73 g, 44 mmol) in H_2O (50 cm^3) and $NaIO_4$ (9.33 g, 44 mmol) in H_2O (50 cm^3) were added to a solution of 2,3-*O*-cyclohexlidene-D-ribonolactone **219** (10.00 g, 44 mmol) in 1,4-dioxane (50 cm^3) at 0 °C with vigorous stirring. The resulting white suspension was stirred at 0 °C for 10 min. A solution of $BaCl_2$ (5.0 g) in H_2O (15 cm^3) was then added and the white ppt. that had formed was filtered off. The filtrate was evaporated and the resulting viscous oil was dissolved in H_2O (45 cm^3). The solution was acidified to ~ pH 3

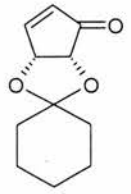
by addition of 10% HCl at 0 °C and extracted with EtOAc (90 cm^3). The aqueous phase was acidified again to ~ pH 3 with 10% HCl and extracted with EtOAc (45 cm^3). The combined organic extracts were dried, filtered and evaporated to dryness to give a solid. Recrystallization (DCM / Hexane) furnished **221** (8.01 g, 85%) as a white crystals; mp 107-108 °C (lit.,[245] 106-108 °C); ν_{max} (PTFE)/cm^{-1} 3345, 1749 (CO), 1364, 1150 and 907; δ_H(300 MHz; $CDCl_3$) 1.55-1.70 (10 H, m, cyclohexane ring), 4.58 (1 H, d, *J* 5.4, 3-H), 4.89 (1H, d, *J* 5.4, 2-H) and 5.81 (1 H, brs, OH); δ_C(75 MHz; $CDCl_3$) 23.6 (CH_2), 23.8 (CH_2), 24.6 (CH_2), 35.0 (CH_2), 36.2 (CH_2), 74.4 (CH), 79.5 (CH), 99.5 (CH), 115.3 (C) and 175.5 (CO); ***m/z*** (ES) 213 (M –H, 100%).

5.1.30 *2,3-(Cyclohexylidenedioxy)-4-hydroxy-4-(2-propyloxy)-butyrolactone* **222**[248]



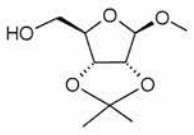
L-Erythruronolactone **221** (7.58 g, 35.5 mmol) and a catalytic amount of pyridinium *p*-toluenesulfonate (0.12 g, 4.7 mmol) were dissolved in dry *iso*-propanol (350 cm^3) and stirred at 60 °C for 12 h. The solution was concentrated under vacuum to afford a viscous oil. Purification over silica afforded **222** (4.35 g, 48%) as a colourless oil; ν_{max} (PTFE)/cm^{-1} 2933, 1790 (CO), 1448, 1369, 1188, 1107, 1043 and 902; δ_H(300 MHz; $CDCl_3$) 1.20 (3 H, d, *J* 4.6, C*H*$_3$), 1.48 (3 H, d, *J* 4.6, C*H*$_3$), 1.30-1.70 (10 H, m, cyclohexane ring), 4.00 (1 H, h, *J* 4.6, C*H*$(CH_3)_2$), 4.50 (1 H, d, *J* 5.4, 3'-H), 4.81 (1 H, d, *J* 5.4, 2'-H) and 5.53 (1 H, s, 1'-H); δ_C(75 MHz; $CDCl_3$) 21.3 (CH_3), 23.1 (CH_3), 23.6 (CH_2), 23.7 (CH_2), 24.7 (CH_2), 35.1 (CH_2), 36.3 (CH_2) 72.4 (CH), 74.3 (CH), 79.3 (CH), 103.0 (CH) and 115.4 (C); ***m/z*** (GC-MS) 213 (M^+ -$(CH_3)_2$, 50%), 169 (45), 140 (44) and 99 (100).

5.1.31 *(2R,3R)-2,3-(Cyclohexylidenedioxy)-4-cyclopentenone* **216**[248]



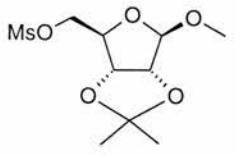
Dimethyl methyl phosphonate (1.50 g, mmol) was dissolved in dry THF (cm^3) at R.T and the solution was cooled to -78 °C. *n*-Butyl lithium (1.6 M in hexane, 7.4 cm^3, 11.8 mmol) was added drop-wise over a 10 min period and the solution was allowed to stir for 15 min. 2,3-(Cyclohexylidenedioxy)-4-hydroxy-4-(2-propyloxy)-butyrolactone **222** (3.04 g, 11.9 mmol) in THF (cm^3) was added rapidly for a dropping funnel and the reaction mixture was stirred at -78 °C for 2.5 h. The solution was allowed to warm to R.T (~ 30 min) and was then poured into Et_2O (200 cm^3) / H_2O (50 cm^3) and shaken well. The organic layer was separated and the aqueous layer was extracted with a further volume of Et_2O (50 cm^3). The combined organic layers were washed with brine, dried, filtered and concentrated (< 50 °C) to give an oil. Purification over silica with Et_2O as the eluent (hexane:Et_2O (2/8)) gave **216** (1.36 g, 59%) as a pale yellow oil; δ_H(300 MHz; $CDCl_3$) 1.32–1.76 (10, H, m, cyclohexane ring), 4.45 (1 H, m, 2-H), 5.26 (1 H, ddd, *J* 5.4, 2.3 and $^4J_{H,H}$ 0.7, 3-H), 6.23 (1 H, d, *J* 6.2, 5-H), 7.60 (1 H, ddd, *J* 6.4, 2.3 and $^4J_{H,H}$ 0.4, 4-H); δ_C(75 MHz; $CDCl_3$) 23.7 (CH_2), 23.5 (CH_2), 24.8 (CH_2), 35.5 (CH_2), 37.1 (CH_2), 76.1 (C-2), 78.2 (C-3), 116.3 (C), 134.2 (C-5) 159.8 (C-4) and 202.3 (CO); ***m/z*** (ES) 217 (M^+ + Na, 80%) and 195 (M^+ +H, 78).

5.1.32 *Methyl-2',3'-O-isopropylidene-β-D-ribofuranoside* **117**[262]



2,2-Dimethoxypropane (50 cm^3, 0.41 mol) followed by BF_3 in diethyl ether (4 cm^3) were added to a solution of dry D(-)ribose **218** (16.59 g, 0.11 mol) in a mixture of anhydrous methanol (60 cm^3) and anhydrous acetone (80 cm^3) at RT. The resulting yellow solution was then allowed to stir for 24 h at R.T. $NaHCO_3$ was added until a pH 7 was achieved. The reaction mixture was then filtered and concentrated under reduced pressure to afford a thick brown oil. Vacuum distillation furnished **117** (13.43 g, 60%) as a colourless oil; bp 89 °C, 3 mm Hg. (lit.,[260] bp 110 °C, 10 mm Hg); δ_H(300 MHz; $CDCl_3$) 1.25 (3 H, s, CH_3), 1.42 (3 H, s, CH_3), 3.37 (3 H, s, OCH_3), 3.58 (1 H, dd, $^2J_{H,H}$ 12.5 and *J* 2.6, 5'-H), 3.66 (1 H, dd, $^2J_{H,H}$ 12.5 and *J* 3.5, 5'-H), 4.36 (1 H, t, *J* 2.7, 4'-H), 4.52 (1 H, d, *J* 5.9, 2'-H), 4.77 (1 H, d, *J* 5.9, 3'-H) and 4.91 (1 H, s, 1'-H); δ_C(75 MHz; $CDCl_3$) 25.0 (CH_3), 26.7 (CH_3), 55.9 (OCH_3), 64.4 (C-5'), 81.8 (C-3'), 86.2 (C-2'), 88.7 (C-4'), 110.3 (C-1') and 112.5 (C); ***m/z*** (CI) 205 (M^+ + H, 12%) 189 (M^+ -CH_4, 5), 173 (M^+ -$HOCH_3$, 100) and 57 (24).

5.1.33 *Methyl-2',3'-O-isopropylidene-5'-O-mesyl-β-D-ribofuranoside* **118**[262,263]



Method A[262]

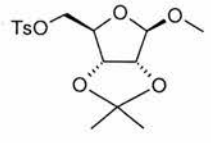
Methanesulfonyl chloride (4.8 cm^3, 61.90 mmol) was added drop-wise to a solution of methyl-2',3'-*O*-isopropylidene-β-D-ribofuranoside **117** (8.00 g, 39.17 mmol) in anhydrous pyridine (20 cm^3) at 0-5 °C. The resulting yellow reaction mixture was left to stir at 0-5 °C overnight before being quenched into ice/water (100 cm^3). The product was extracted with Et_2O (2 x 50 cm^3) and washed with H_2O (2 x 25 cm^3) before being dried and the solvent removed to afford a pale yellow semi-solid. Recrystallisation from EtOAc / hexane afforded **118** (6.78 g, 61%) as a white amorphous, mp. 70-71 °C (lit.,[262] mp. 66-73 °C);

δ_H(300 MHz; $CDCl_3$) 1.32 (3 H, s, CH_3), 1.48 (3 H, s, CH_3), 3.07 (3 H, s, SO_2-CH_3), 3.34 (3 H, s, OCH_3), 4.20 (2 H, d, *J* 7.1, 2 x 5'-H), 4.40 (1 H, t, *J* 7.1, 4'-H), 4.60 (1 H, d, *J* 5.9, 2'-H), 4.70 (1 H, d, *J* 5.9, 3'-H) and 4.99 (1H, s, 1'-H); δ_C(75 MHz, $CDCl_3$); 26.3 (CH_3), 26.8 (CH_3), 38.1 (SO_2-CH_3), 55.7 (OCH_3), 68.8 (CH_2), 79.2 (CH), 81.7 (CH), 84.6 (CH), 85.3 (CH), 109.9 (CH), 113.2 (*C*-(CH_3)$_2$); ***m/z*** (GC-MS) 282 (M^+, 1%), 207 (M^+ -$C_3H_7O_2$, 33), 111 (M^+ -CSO_3H, 20), 79 (60) and 68 (100).

Method B[263]

Methanesulfonyl chloride (1.75 cm^3, 22.6 mmol) was added over a 5 min period to a solution of methyl-2',3'-*O*-isopropylidene-β-D-ribofuranoside **117** (1.85 g, 9.06mmol) in anhydrous DCM (20 cm^3), containing NEt_3 (3 cm^3) at 0°C. The solution was then left to stir at O °C for 5 hours. The reaction was quenched with saturated sodium bicarbonate solution (2 x 14 cm^3) and H_2O (2 x 14 cm^3). The aqueous layers were extracted with DCM (30 cm^3) and the combined organic layers were dried and filtered. The solvent was removed to afford a pale yellow solid. Recrystallisation from EtOH yielded the pure product **118** (2.06 g, 81%) as a white amorphous powder. Spectral and physical properties were identical to those previously reported for **118**.

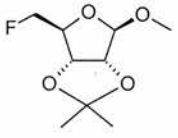
5.1.34 *Methyl-2',3'-O-isopropylidene-5'-O-tosyl- β-D-ribofuranoside* **229**[264]



Purified *p*-toluene sulfonyl chloride (13.80 g, 72.4 mmol) was added portion wise to a solution of methyl-2,3'-*O*-isopropylidene-β-D-ribofuranoside **117** (10.00 g, 49.0 mmol) in anhydrous pyridine (25 cm^3) at 0 °C. The reaction was then left to stir at R.T for 3 h after which time it was poured onto ice-water (50 cm^3) and the resulting white precipitate was

filtered and washed with ice water (3 x 25 cm^3). The white precipitate collected was dissolved in Et_2O (25 cm^3), dried, filtered and the solvent removed under reduced pressure. Subsequent drying under high vacuum afforded **229** (13.82 g, 79%) as a white solid; mp 79-80 °C (lit.,[264] mp 79-80 °C); δ_H(300 MHz; $CDCl_3$) 1.29 (3 H, s, CH_3), 1.45 (3 H, s, CH_3), 2.46 (3 H, s, $ArCH_3$), 3.24 (3 H, s, OCH_3), 4.01 (2 H, dd, *J* 7.2 and 1.5, 2 x 5'-H), 4.31 (1 H, dt, *J* 7.2 and 0.8, 4'-H), 4.54 (1 H, d, *J* 6.0, 2'-H), 4.59 (1 H, dd, *J* 6.0 and 0.8, 3'-H), 4.93 (1 H, s, 1'-H), 7.36 (2 H, d, *J* 8.2, Ar-H) and 7.81 (2 H, d, *J* 8.2, Ar-H); δ_C(75 MHz; $CDCl_3$,) 22.1 ($Ar-CH_3$), 25.2 (CH_3), 26.7 (CH_3), 55.4 (OCH_3), 69.6 (CH_2), 81.7 (CH), 83.9 (CH), 85.2 (CH), 109.8 (CH), 113.1 (C), 128.4 (ArC), 130.3 (ArC), 136.8 (C) and 145 (C); ***m/z*** (ES) 358 (M^+ + Na 100 %).

5.1.35 *Methyl-2', 3'-O-isopropylidene-5'-fluoro-5'-deoxy- β-D-ribofuranoside* **119**[134]



Procedure A

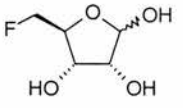
A solution of $TBAF.3H_2O$ in anhydrous MeCN (6.70 g, 21.23 mmol in 10 cm^3) was added to a stirred solution of methyl-2',3'-*O*-isopropylidene-5-*O*-mesyl-β-D-ribofuranoside **119** (2.41 g, 8,52 mmol) in anhydrous MeCN (20 cm^3) at R.T. The reaction was then heated under reflux for 24 h. The reaction mixture was cooled and concentrated under vacuum to give an oil that was partitioned between $CHCl_3$ (100 cm^3) and H_2O (50 cm^3). The organic layer was washed with water (2 x 25 cm^3) dried and filtered. The solvent was removed under vacuum and purification over silica eluting with Et_2O:pet.ether (6:1) afforded **120** (1.09 g, 62%) as a clear oil; δ_H(300 MHz; $CDCl_3$) 1.32 (3 H, s, CH_3), 1.49 (3 H, s, CH_3), 3.33 (3 H, s, OCH_3), 4.28-4.48 (3 H, m, 2 x H-5' and 4'-H), 4.58 (1 H, d, *J* 6.0, 2'-H), 4.68 (1 H, d, *J* 6.0, 3'-H), 4.99 (1 H, d, *J* 2.4, 1'-H); δ_C(75 MHz $CDCl_3$); 25.3 (CH_3), 26.8

(CH_3), 55.3 (OCH_3), 81.4 (d, $^3J_{CF}$ 4.2 , C-3), 82.2-84.5 (d, $^1J_{CF}$ 172.5, C-5'), 84.7-85.0 (d, $^2J_{C,F}$ 22.3, C-4'), 85.4 (C-2), 109.6 (C-1), 113.0 (**C**-(CH_3)$_2$); δ_F(282 MHz, $CDCl_3$) -225.29 (dt, $^2J_{F,H}$ 48.3 and $^3J_{F,H}$ 21.4); ***m/z*** (GC-MS, EI) 191 (M^+ -CH_3, 71%), 175 (-OCH_3, 11), 131 (71), 68 (67), 59 (100) and 43 (100).

Procedure B

TBAF.3H_2O (10.00 g, 31.69 mmol) in anhydrous MeCN (25 cm^3) was added to a solution of methyl-2',3'-*O*-isopropylidene-5'-*O*-tosyl-β-D-ribofuranoside **231** (9.13 g, 25.47 mmol) in anhydrous MeCN (100 cm^3) at R.T. The reaction was then heated under reflux for 24 h. Upon cooling the solution was concentrated under reduced pressure to give a brown oil that was dissolved in EtOAc (50 cm^3) and washed with H_2O (2 x 25 cm^3). The organic layer was dried, filtered and concentrated under vacuum to give a yellow oil. Purification over silica eluting with Et_2O:pet.ether (6:1) gave **120** (3.78 g, 72%) as a clear oil. Spectral and physical properties were identical to those already reported for **120**.

5.1.36 *5-Fluoro-5-deoxy-D-ribose* **120**[133]



Procedure A

Dilute sulfuric acid (0.02 M, 8 cm^3) was added to methyl-2',3'-*O*-isopropylidene-5'-fluoro-5'-deoxy-β-D-ribofuranoside **119** (0.97 g, 4.7 mmol) with stirring and the solution was heated to 100 °C. The reaction was monitored by ^{19}F NMR spectroscopy and found to be complete within 4 h. Calcium carbonate was added to neutralise the excess acid and the product was freed from calcium sulfate by centrifugation. The supernatant was filtered through celite and lyophilised to yield **120** (anomeric mixture, 0.72 g, 89%) pale yellow

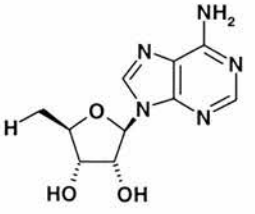
syrup; δ_F(282 MHz, D_2O) -228.95 (β, dt, and $^2J_{F,H}$ 47.5 and $^3J_{F,H}$ 24.2), -231.28 (α, dt, $^2J_{F,H}$ 47.5 and $^3J_{F,H}$ 24.2); δ_C(75 MHz; D_2O) 69.5 (d, $^4J_{C,F}$ 6.6, α / β, C-3), 69.7 (d, $^3J_{C,F}$ 7.2, α / β, C-3), 70. 9, 75.3 (α / β, C-2), 80.84 (d, $^3J_{C,F}$ 22.5, α / β, C-4), 81.4 (d, $^3J_{C,F}$ 17.8, α / β, C-4), 83.2 (d, $^1J_{C,F}$ 167.6, α / β, C-5), 83.6 (d, $^1J_{C,F}$ 168.4, α / β, C-5), 96.7 (α / β, C-1), 101.4 (α / β, C-1); ***m/z*** (GC-MS, CI, after MSTFA derivatisation), 369 (M^+ +H, 7%), 353 (M^+ - CH_4, 40), 279 (100), 263 (71), 217 (36) 189 (73).

The ^{1}H NMR spectrum recorded for **120** was too complex to interpret conclusively.

Procedure B

Methyl-2',3'-*O*-isopropylidene-5'-fluoro-5'-deoxy-β-D-ribofuranoside (1.15 g, 5.56 mmol) was heated to reflux in a suspension of water (25 cm^3) containing amberlite 120 [H^+] resin (0.70 g). The reaction was found by ^{19}F NMR spectroscopy to be complete within 4.5 h. The [H^+] resin was then filtered and the resulting solution was lyophilised to yield **120** (anomeric mixture, 0.64 g, 75%) as a pale yellow oil. Spectral and physical properties were identical to those already reported for **120**.

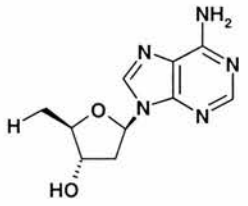
5.1.37 *5'-Deoxy-adenosine* **164**[221,267]



Super hydride (1M in THF, 20 cm^3, 20 mmol) was added slowly over a period of 10 min to a solution of 5'-chloro-5'-deoxy-adenosine **165** (360 mg, 1.26 mmol) in 1,4-dioxane (35 cm^3) at 0 °C. The reaction was left to stir at 0 °C for 1 h and then for 12 h at R.T. The reaction mixture was cautiously acidified (5% HOAc/H_2O, 40 cm^3), and purged with N_2 for 1 h (fume hood) to remove *pyrophoric* triethylborane**.** The solvent was removed under vacuum and chromatography over silica using EtOAc:EtOH:H_2O (45:5:3) as the eluent

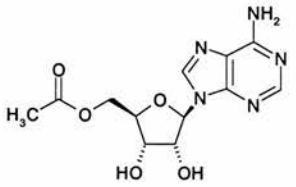
afforded **164** (155 mg, 55%) as a white powder; mp 209 °C (lit.,[267] 211°C); δ_H(300 MHz; d_6-DMSO) 1.30 (1 H, d, *J* 6.1, C*H*$_3$), 3.92-4.00 (2 H, m, 3'-H and 4'-H), 4.67 (1 H, m, 2'-H), 5.16 (1 H, d, *J* 5.9, O*H*), 5.43 (1 H, d, *J* 5.4, O*H*), 5.84 (1 H, d, *J* 4.9, 1'-H), 7.28 (2 H, brs, NH_2), 8.15 (1 H, s, 2-H / 8-H) and 8.32 (1 H, brs, 2-H / 8-H); δ_C(75 MHz; d_6-DMSO) 18.9 (CH_3), 73.0 (CH), 74.6 (CH), 79.7 (CH), 87.8 (CH), 119.1 (C), 139.8 (C-H), 149.4 (C), 152.6 (CH) and 156.0 (C); ***m/z*** (ES) 252.1090 (M^+ +H, $C_{10}H_{14}N_5O_3$ requires 252.1097, 100%).

5.1.38 *2',5'-Dideoxy-adenosine* **232**[268,296]



Superhydride (1M in THF, 10 cm^3, 10 mmol) was added slowly over a period of 10 min to a solution of 5'-*O*-tosyl-2'-deoxy-adenosine **187** (200 mg, 0.49 mmol) in 1,4-dioxane (10 cm^3) at 0 °C. The reaction was then left to stir at 0 °C for 1 h and then for 12 h at R.T. The reaction mixture was cautiously acidified (5% HOAc/H_2O, 20 cm^3) and purged with N_2 for 1 h (fume hood) to remove *pyrophoric* triethylborane. Purification by preparative TLC (silica, EtOAc:EtOH:H_2O (45:5:3)) afforded **232** (110 mg, 67%) as a white powder; mp 186-187 °C (lit.,[296] 185-188 °C); δ_H(300 MHz; CD_3OH); 1.26 (3 H, d, *J* 6.4, C*H*$_3$), 3.61 (1 H, $^2J_{H,H}$ 13,7, *J* 6.8 and 4.5, 2'-H), 3.76 (1 H, m, 2'-H), 3.99 (1 H, dq *J* 6.2 and 4.5, 4'-H), 4.20 (1 H, dt, *J* 6.2 and 4.5, 3'-H), 6.20 (1 H, t, *J* 6.8, 1'-H), 8.10 (1 H, s, 2-H / 8-H) and 8.15 (1 H, s, 2-H / 8-H); δ_C(75 MHz; CD_3OH); 19.6 (CH_3), 40.6 (CH), 76.7 (CH), 84.5 (CH), 85.3 (CH), 120.7 (C), 141.0 (C-2 / C-8), 150.7 (C), 154.2 (C-2 / C-8) and 157.5 and (C); ***m/z*** (ES) 258 (M^+ + Na, 32%), 236 (M^+ +H, 100).

5.1.39 *5'-O-Acetyl-adenosine* **133**[269]



Adenosine **166** (100 mg, 0.37 mmol), vinyl acetate (0.83 mmol) and *Candida antarctica* (200 mg), were suspended in THF (10 cm^3) containing molecular sieves (powder). The reaction was heated at 60 °C for 4 h. The reaction solution was cooled, the enzyme filtered off and washed with MeOH (10 cm^3). Solvents were removed under vacuum and the resulting white solid was recrystallised from MeOH to give **233** (74 mg, 64%) as colourless crystals; mp 132-133 °C (lit.,[269] 132-133 °C); δ_H(300 MHz; CD_3OH) 2.05 (3 H, s, CH_3), 4.25-4.40 (4 H, m, 2 x 5'-H, 3'-H and 4'-H), 4.74 (1 H, t, *J* 4.5, 2'-H), 6.02 (1 H, d, *J* 4.5, 1'-H), 8.21 (1 H, s, 2-H / 8-H) and 8.27 (1 H, s, 2-H / 8-H); δ_C(75 MHz; CD_3OH); 19.3 (CH_3), 63.5 (CH_2), 70.4 (CH), 73.7 (CH), 82.0 (CH), 88.9 (CH), 119.9 (C), 139 (CH), 149.8 (C), 152.5 (CH), 154.1 (C) and 171.1 (CO); ***m/z*** (ES) 332 (M^+ +Na, 100%).

5.2 Biochemical experimental

5.2.1 General methods

All reagents and reference compounds were purchased from either Sigma, Fluka or Berry and Associates (USA).

All of the commercial enzymes used were purchased from Sigma Biochemicals. The details for the enzymes are as follows; 5'-Adenylic acid deminase (EC 3.5.4.6, from *Aspergillus species*, A 1907, 0.11 units / mg), purine nucleoside phosphorylase (PNP, EC 2.4.2.1, unknown bacterial source, N-8264, 15.6 units / mg), xanthine oxidase (EC 1.1.3.22, microbial, X 2252, 7 units / mg), phytase (EC 3.1.3.8, from *Aspergillus ficuum*, P 9792, 3.5 units / mg), L-amino acid oxidase (EC 1.4.3.2, Type VI; crude dried venom from *Crotalus atrox*, A 5147, 0.2 units / mg) FruA aldolase (EC 4.1.2.13, from rabbit muscle, lyophilized powder, A 2714, 10 units / mg) and glucose isomerase (Sweetzyme T, from *Streptomyces murinus*, G-4166). The vector for fuculose-1-phosphate (FucA) aldolase (ATCC number 86984) was purchased from Promochem. The enzyme was over expressed in *Ecoli* by Dr. Hai Deng, University of St. Andrews. The fluorinase used for all of the investigations detailed was over expressed in *E. coli*.

High pressure liquid chromatography (HPLC) analyses were carried out using a Varian Prostar HPLC machine fitted with an analytical C_{18} column (method used Appendix II). For microcentrifugation (50-1500 μl), an Eppendorf 5415C centrifuge was used. ^{19}F NMR analyses were performed on a Bruker avance 500 MHz (operating at 470 MHz) or a Varian unity 500 MHz (operating at 470 MHz). All ^{19}F NMR spectroscopy was carried out using D_2O (~10%) as an internal reference. Chemical shifts are given in ppm and coupling constants (*J*) are given in Hertz (Hz). Electrospray mass spectrometry (ES-MS) was performed on a Micromass LCT time of flight instrument. Gas chromatography mass

spectrometry (GC-MS) was carried out by Dr. J. T. G. Hamilton or Dr. C. McRoberts, Queens University Belfast.

5.2.2 Preparation of a cell free extract (CFE) of *S. cattleya*[109]

5.2.2.1 Culture medium and conditions

Conical flasks (500 cm^3) containing of a chemically defined medium (90 cm^3) were used to grow seed and batch cultures of *Streptomyces cattleya*. The chemically defined medium was prepared as follows; sterile ultra-pure water (450 cm^3), of ion solution (150 cm^3), carbon solution (75 cm^3), sterile phosphate buffer (75 cm^3, 150 mM, pH 7.0) and sterile potassium fluoride solution (3 cm^3, 0.5 M). Seed cultures were prepared by transferring spores and aerial mycelia from a static culture into the prepared solution (90 cm^3) in conical flasks plugged with cotton wool. After shaking for 4-5 days at 28 °C and 180 rpm, an aliquot (0.3 cm^3) of the vegetative mycelium was used to inoculate the batch cultures (90 cm^3). The cultures were incubated at 28 °C and 180 rpm for 6-8 days. After a growth period of 6-8 days, the cells were harvested by centrifugation (25 min, 14,000 rpm, 25 °C). The resultant pellet was washed (100 cm^3 x 3) with KH_2PO_4 (phosphate) buffer (50 mM, pH 7.0) and the final pellet was stored at –80 °C until required. Typically the pellet would remain active for at least 12 months under these storage conditions.

The frozen pellet was resuspended in an appropriate buffer* (typically 0.2g - 0.4g / cm^3). Ultra-sonication (3 x 60 sec, at 60% duty cycle) was used to disrupt the cells and cell debris was removed by centrifugation (14,000 rpm / 20 min, 1.5 cm^3). The resulting clear supernatant was used as the CFE.

*Generally the standard buffer solution used was either 200 mM phosphate (KH_2PO_4) buffer pH 6.8 or 50 mM Tris-HCl buffer pH 7.8.

Ion solution

The following reagents were added to ultra-pure water (900 cm^3); NH_4Cl (6.75 g), NaCl (2.25 g), $MgSO_4.7H_2O$ (2.25 g), $CaCO_3$ (1.13 g), $FeSO_4.7H_2O$ (0.113 g), $CoCl_2.6H_2O$ (0.045 g) and $ZnSO_4.7H_2O$ (0.045 g). The solution was sterilised by autoclaving prior to its use.

Carbon source solution

Glycerol (45 g), monosodium glutamate (22.5 g), *myo*-inositol (1.8 g), *para*-aminobenzoic acid (450 µl of freshly prepared solution 1 mg/ cm^3) and ultra-pure water (900 cm^3). The solution (75 cm^3) was sterilised by filtration into presterilised Schott bottles.

5.2.2.2 Assays to determine the activity of the CFE of *S. cattleya.*

Assay 1: CFE (500 µl), SAM **85** (50 µl, 20 mM) and KF (10 µl, 0.5 M) was incubated for 16 h at 37 °C. The sample was then heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm, 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy. The production of fluoroacetate (FAc) **15** and / or 4-fluorothreonine (4-FT) **16** confirmed that the CFE was active. The presence of **15** or **16** was determined by comparison against reference samples.

Assay 2: CFE (500 µl), 5'-FDA **83** (100 µl, 37.1 mM,) was incubated for 16 h at 37 °C. The sample was then heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy. The production of FAc **15** and / or 4-FT **16** confirmed that the CFE was active. The presence of FAc **15** or 4-FT **16** was determined by comparison against reference samples.

5.2.3 CFE incubation of 5'-FDA 83

5'-FDA **83** (100 µl, 37.1 mM) was incubated at 37 °C for 16 h with a CFE (500 µl, Tris-HCl buffer, 50 mM, pH 7.8) of *S. cattleya*. The sample was then heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy. δ_F(470 MHz; 10% D_2O); (FAc **15**) –216.45 (t, $^2J_{F,H}$ 47.9) and (5'-FDI **87**) -230.58 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9). HPLC analysis (UV detection) against a synthetic reference compound was also used to confirm the production of 5'-FDI **86**.

Control experiments were carried out with denatured CFE. This indicated that 5'-FDA **83** was inert to chemical degradation in the buffer used over an incubation period of 16 h at 37 °C.

5.2.4 Incubation of 5'-FDI 86 in the CFE

5'-FDI **86** (100 µl, 37 mM) was incubated at 37 °C for 16 h with theCFE (500 µl, tris-HCl, buffer 50 mM, pH 7.8). The sample was then heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy. δ_F(470 MHz; 10% D_2O); (5'-FDI **86**) -230.67 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9). HPLC analysis with a reference sample also confirmed that 5'-FDI **86** was present. 5'-FDA **83** could not be detected by either ^{19}F NMR spectroscopy or HPLC analysis.

5.2.5 Assay for the over-expressed fluorinase

SAM **85** (50 µl, 20 mM) and KF (10 µl, 0.5 M) were incubated with the fluorinase (10 mg / ml, 100 µl) at 37 °C for 16 h. The sample was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). HPLC analysis (UV detection)

with a standard was used to confirm the production of 5'-FDA **83**. The the fluorinase was prepared as a solution in either 200 mM phosphate buffer pH 6.8 or 50 mM Tris-HCl buffer pH 7.8.

5.2.6 Enzymatic preparation of *S*-adenosyl-L-methionine (SAM) 85

5.2.6.1 Enzymatic preparation of SAM **85** from 5'-FDA **83**

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The fluorinase was prepared in Tris-HCl buffer pH 7.8 (50 mM). The protein was removed from the solution using a standard heating / centrifugation protocol, prior to analysis.

	Exp1	**Exp2**	**Exp3**
	Volume added (µl)		
5'-FDA **83** (7.4 mM)	100	100	100
L-methionine **84** (100 mM)	40	40	-
Fluorinase (10 mg / ml)	100	-	100
Tris-HCl pH 7.8 (20 mM)	200	240	300

Exp 1: HPLC analysis against a reference sample confirmed the production of SAM **85**. ES-MS analysis also confirmed the production of SAM **85**; ***m/z*** 399 (M^+, 30%) and 298 (M+H, $-C_4H_8NO_2$, 100). The conversion was incomplete as unreacted 5'-FDA **83** was also evident by HPLC analysis.

Exp 2: Only unreacted 5'-FDA **83** was detected by HPLC analysis.

Exp 3: Only unreacted 5'-FDA **83** was detected by HPLC analysis.

5.2.6.2 Enzymatic preparation of SAM **85** from 5'-ClDA **165**

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The fluorinase was prepared as a solution in tris-HCl buffer pH 7.8 (50 mM).

The protein was removed from the solution after the incubation period using a standard heating / centrifugation protocol.

	Exp 1	Exp 2	Exp 3
	Volume added (µl)		
5'-ClDA **165** (10.5 mM)	50	50	50
L-methionine **84** (100 mM)	40	-	40
Fluorinase (10 mg / ml)	100	100	-
Tris-HCl buffer pH 7.8 (50 mM)	200	240	300

Exp 1: HPLC analysis against a reference sample confirmed the production of SAM **85**. ES-MS analysis also confirmed the production of SAM **85**; ***m/z*** 399 (M^+, 25%) and 298 (M +H, $-C_4H_8NO_2$, 100).

Exp 2: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

Exp 3: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

5.2.7 L-methionine analogues as substrates for the fluorinase

5.2.7.1 L-Seleno-methionine **168** as a substrate for the fluorinase.

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The fluorinase was prepared as a solution in Tris-HCl buffer pH 7.8 (50 mM). The protein was removed using a standard heating / centrifugation protocol prior to analysis.

	Exp 1	Exp 2	Exp 3
	Volume added (µl)		
5'-ClDA **165** (10.5 mM)	50	50	50
L-selenomethionine **168** (100 mM)	40	-	40
Fluorinase (10 mg / ml)	100	100	-
Phosphate buffer pH 7.8 (20 mM)	200	240	300

Exp 1: HPLC analysis confirmed the production of a new compound with a retention time later than that of both the starting material 5'-ClDA **165** and SAM **85**. ES-MS (Chapter 3, Figure 3.4) confirmed that L-selenoSAM **169** had been formed; ***m/z*** 348

(Se^{82}, M+H, $-C_4H_8NO_2$, 20%), 346 (Se^{80}, M+H, $-C_4H_8NO_2$, 100), 344 (Se^{78}, M+H, $-C_4H_8NO_2$, 50), 343 (Se^{77}, M+H, $-C_4H_8NO_2$, 20) and 342 (Se^{76}, M+H, $-C_4H_8NO_2$).

Exp 2: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

Exp 3: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

5.2.7.2 L-methionine analogues **171-173** as substrates for the fluorinase

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The fluorinase was prepared as a solution in Tris-HCl buffer pH 7.8 (50 mM). The protein was removed by a standard heating / centrifugation protocol prior to analysis. Appropriate control experiments confirmed that the coupling of 5'-ClDA **165** with compounds **171-173** would not occur in the absence of the fluorinase.

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
	Volumes added (µl)			
5'-ClDA **165** (3.5 mM)	25	25	25	25
L-methionine **84** (100 mM)	5	-	-	-
α-methyl-D/L-methionine **171** (100 mM)	-	5	-	-
N-acetyl-L-methionine **172** (100 mM)	-	-	5	-
L-methionine methyl ester **173** (100 mM)	-	-	-	5
Fluorinase (2.6 mg / ml)	100	100	100	100
Tris-HCl buffer, pH 7.8 (50 mM)	50	50	50	50

Exp 1: The production of SAM **85** was confirmed by HPLC analysis with a reference sample. Unreacted 5'-ClDA **165** was also detected by HPLC.

Exp 2: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

Exp 3: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

Exp 4: The production of SAM **85** was confirmed by HPLC analysis with a reference and by ES-MS; ***m/z*** 399 (M^+, 24%) and 298 (M + H, $-C_4H_8NO_2$, 100).

5.2.7.3 L-Ethionine **175** and D-methionine **176** and as substrates for the fluorinase

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The fluorinase was prepared as a solution in Tris-HCl buffer pH 7.8 (50 mM).

The protein was removed by a standard heating / centrifugation protocol prior to analysis. Control experiments confirmed that the coupling of 5'-ClDA **165** with compounds **175** and **176** would not occur in the absence of the fluorinase.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (μl)		
5'-ClDA **165** (7.0 mM)	100	100	100
L-methionine **84** (100 mM)	40	-	-
L-ethionine **175** (100 mM)	-	40	-
D-methionine **176** (100 mM)	-	-	40
Fluorinase (10 mg / ml)	100	100	100
Tris-HCl buffer, pH 7.8 (50 mM)	300	300	300

Exp 1: The production of SAM **85** was confirmed by HPLC analysis by co-injection with a reference.

Exp 2: Only unreacted 5'-ClDA **165** was detected by HPLC analysis.

Exp 3: The production of a new compound with an identical retention time to SAM **85** was observed. ES-MS analysis showed that the molecule also had the same mass as *S*-adenosyl-L-methionine (SAM) **85**; ***m/z*** 399 (M^+, 100%).

Based on the evidence it was presumed that the compound produced was *S*-adenosyl-D-methionine **176**.

5.2.8 Enzymatic preparation of 2'-deoxy-*S*-adenosyl-L-methionine (2'-deoxy-SAM)

Incubations were carried out at 37 °C for 16 h. Both experiments were performed in duplicate. The fluorinase was prepared as a solution in Tris-HCl buffer, pH 7.8 (50 mM). The protein was removed by a standard heating / centrifugation protocol prior to analysis. Control experiments confirmed that the production of 2'-deoxy-SAM **177** would not occur in the absence of the fluorinase or L-methionine **84**.

	Exp 1	Exp 2
	Volumes added (µl)	
2'-deoxy-5'-ClDA **182** (20 mM)	50	-
2'-deoxy-5'-FDA **181** (20 mM)	-	50
L-methionine **84** (100 mM)	40	40
Fluorinase (10 mg / ml)	100	100
Tris-HCl buffer, pH 7.8 (50 mM)	200	200

Exp 1: The production of a new compound with a retention time later than the starting material was observed by HPLC. ES-MS analysis confirmed that 2'-deoxy-SAM **177** had been produced; ***m/z*** 383 (M^+, 100%).

Exp 2: The production of a new compound with a retention time later than the starting material was observed by HPLC. ES-MS analysis confirmed that 2'-deoxy-SAM **177** had been produced; ***m/z*** 383 (M^+, 100%).

5.2.9 Enzymatic preparation of 2-amino-SAM 198 from 2-amino-5'-ClDA 195

All incubations were carried out at 37 °C for 16 h. All experiments were performed in duplicate. The protein was removed using a standard heating / centrifugation protocol prior to analysis. The fluorinase was prepared in Tris-HCl buffer, 50 mM, pH 7.8.

	Exp 1	Exp 2	Exp 3
	Volumes added (µl)		
2-amino-5'-ClDA **195** (16.6 mM)	50	50	50
L-methionine **84** (100 mM)	80	-	80
Fluorinase (10 mg / ml)	100	100	-
Tris-HCl, pH 7.8 (50 mM)	200	280	300

Exp 1: HPLC analysis (Chapter 3, Figure 3.10) confirmed the production of a new compound with a retention time later than that of the starting material 2-amino-5'-ClDA **196**. ES-MS confirmed that this was 2-amino-SAM **198**; ***m/z*** 414 (M^+, 100%) and 313 (M +H, $-C_4H_8NO_2$, 35).

Exp 2: Only unreacted 2-amino-5'-ClDA **195** was detected by HPLC analysis.

Exp 3: Only unreacted 2-amino-5'-ClDA **195** was detected by HPLC analysis.

5.2.10 Transhalogenation reactions mediated by the fluorinase

5.2.10.1 Transhalogenation reaction *via* a SAM **85** intermediate.

All incubations were carried out at 37 °C for 16 h. The fluorinase was prepared as a solution in Tris-HCl buffer, pH 7.8 (50 mM). The protein was removed from the solution using a standard heating / centrifugation procedure prior to analysis.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (μl)		
5'-ClDA **165** (7.0 mM)	100	100	100
L-methionine **84** (100 mM)	40	-	40
KF (0.5 M)	20	20	20
Fluorinase (10 mg / ml)	100	100	-
Tris-HCl buffer pH 7.8 (50 mM)	300	340	400

Exp 1: HPLC analysis with reference samples confirmed the presence of both SAM **85** and 5'-FDA **83** within the sample. The production of 5'-FDA was also confirmed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) -231.09 ppm (dt, $^3J_{F,H}$ 47.0 and $^2J_{F,H}$ 28.7).

Exp 2: Only unreacted 5'-ClDA **165** was identified by HPLC analysis.

Exp 3: Only unreacted 5'-ClDA **165** was identified by HPLC analysis.

5.2.10.2 Transhalogenation reaction *via* a seleno-SAM **169** intermediate.

All incubations were carried out at 37 °C for 16 h. The fluorinase was prepared as a solution in Tris-HCl buffer, pH 7.8 (50 mM). The protein was removed using a standard heating / centrifugation procedure prior to analysis.

	Exp 1	**Exp 2**	**Exp 3**
	Volume added (μl)		
5'-ClDA **165** (7.0 mM)	100	100	100
L-seleno-methionine **168** (100 mM)	40	-	40
KF (0.5 M)	20	20	20
Fluorinase (10 mg / ml)	100	100	-
Tris-HCl buffer pH 7.8 (50 mM)	300	340	400

Exp 1: HPLC analysis with reference compounds confirmed the presence of unreacted 5'-ClDA **165**, seleno-SAM **169** and 5'-FDA **83** within the sample. The production of 5'-FDA **83** was also confirmed by ^{19}F NMR; δ_F(470 MHz, 10% D_2O) -231.19 ppm (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.7).

Exp 2: Only unreacted 5'-ClDA **165** was identified by HPLC.

Exp 3: Only unreacted 5'-ClDA **165** was identified by HPLC.

5.2.10.3 Direct enzymatic fluorination of 5'-BrDA **201**

All incubations were carried out at 37 °C for 16 h. All experiments were carried out in duplicate. The fluorinase was prepared as a solution in Tris-HCl buffer pH 7.8 (50 mM). The protein was removed by heating / centrifugation prior to analysis. Control experiments showed that fluorination of 5'-BrDA **201** did not occur in the absence of the fluorinase at 37 °C.

	Exp1	**Exp2**	**Exp3**
	Volume added (µl)		
5'-BrDA **201** (6.0 mM)	10	10	10
L-methionine **84** (100 mM)	2	-	-
L-selenomethionine **168** (100 mM)	-	2	-
KF (0.5 M)	5	5	5
Fluorinase (10 mg / ml)	100	100	100

Exp 1: HPLC analysis with a reference confirmed the production of 5'-FDA **83**.

Exp 2: HPLC analysis with a reference confirmed the production of 5'-FDA **83**.

Exp 3: HPLC analysis with a reference confirmed the production of 5'-FDA **83**.

5.2.11 Enzymatic preparation of 2'-deoxy-5'-FDA 181

All incubations were carried out at 37 °C for 16 h. The fluorinase was used as a solution in Tris-HCl buffer pH 7.8 (50 mM).The protein was removed from the solution by using a standard heating / centrifugation protocol prior to analysis. All experiments were carried out in duplicate.

	Exp 1	Exp 2	Exp 3
	Volume added (µl)		
2'-deoxy-5'-ClDA **182** (30 mM)	50	50	50
L-methionine (20 mM)	10	-	10
KF (0.5 M)	5	5	5
fluorinase (10 mg / ml)	100	100	-
Tris-HCl buffer pH 7.8 (50 mM)	200	210	300

Exp 1: Co-injection with a synthetic reference confirmed the production of 2'-deoxy-5'-FDA **181**.

Unreacted 2'-deoxy-5'-ClDA **182** and a trace amount of 2'-deoxy-SAM **177** were also detected by HPLC

Exp 2: Only unreacted 2'-deoxy-5'-ClDA **182** was identified by HPLC.

Exp 3: Only unreacted 2'-deoxy-5'-ClDA **182** was identified by HPLC.

	Exp 1	Exp 2	Exp 3
	Volume added (µl)		
2'-deoxy-5'-ClDA **182** (30 mM)	50	50	50
L-seleno-methionine **168** (20 mM)	10	-	10
KF (0.5 M)	5	5	5
Fluorinase (10 mg / ml)	100	100	-
Tris-HCl buffer pH 7.8 (50 mM)	200	200	300

Exp 1: Co-injection with a synthetic reference confirmed the production of 2'-deoxy-5'-FDA **181**. Unreacted 2'-deoxy-5'-ClDA **182** and a small amount of 2'-deoxy-seleno-SAM **196** were also detected by HPLC

Exp 2: Only unreacted 2'-deoxy-5'-ClDA **182** was identified by HPLC.

Exp 3: Only unreacted 2'-deoxy-5'-ClDA **182** was identified by HPLC.

5.2.12 Chloride ion as a substrate for the fluorinase

5.2.12.1 Enzymatic preparation of 5'-ClDA **165**

All incubations were carried out at 37 °C for 16 h. The protein was removed from the solution by heating / centrifugation prior to analysis. The fluorinase was used as a solution in Tris-HCl buffer pH 7.8 (50 mM). L-Amino acid oxidase (EC 1.4.3.2, Type VI; crude dried venom from *Crotalus atrox*, A5147, 0.2 units / mg) was used as a solution (10 mg / ml) in Tris-HCl buffer pH 7.8 (50 mM). The samples were lyophilised to give a white powder. GC-MS analysis was carried out after derivatisation with MSTFA.

	Exp 1	**Exp 2**	**Exp 3**	**Exp 4**
	Volume added (μl)			
SAM **85** (13 mM)	25	25	25	25
KF (0.5 M)	10	10	-	-
KCl (0.5 M)	-	-	10	10
L-amino acid oxidase	200	-	200	-
Fluorinase (2.6 mg / ml)	200	200	200	200
Tris-HCl, pH 7.8, 50 mM	100	300	100	300

Exp 1: The production of 5'-FDA **83** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 470 (M $-CH_3$, 15%), 278 (M $-C_8H_{12}N_5Si$, 50) and 236 (100).

Exp 2: The production of 5'-FDA **83** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 470 (M $-CH_3$, 17%), 278 (M $-C_8H_{12}N_5Si$, 64) and 236 (100).

Exp 3: The production of 5'-ClDA **165** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 486 (M $-CH_3$, 15), 294 (M $-C_8H_{12}N_5Si$, 65) and 236 (100).

Exp 4: No 5'-ClDA **165** was detected by either HPLC or GC-MS.

5.2.12.2 Enzymatic preparation of 5'-ClDI **203**

All incubations were carried out at 37 °C for 16 h. The fluorinase was used as a solution in Tris-HCl buffer pH 7.8 (50 mM). 5'-Adenylic acid deaminase (AMPDA, EC 3.5.4.6, from *Aspergillus species*, A 1907, 0.11 units / mg) was prepared as a solution in ultra-pure water (1 mg / ml). After the incubation period the protein was removed using a standard heating / centrifugation protocol. The samples were lyophilised to give a white powder. GC-MS analysis was carried out after derivatisation with MSTFA.

	Exp 1	Exp 2	Exp 3	Exp 4
	Volume added (µl)			
SAM (20 mM)	100	100	100	100
KF (0.5 M)	40	40	-	-
KCl (0.5 M)	-	-	40	40
APMDA (1 mg / ml)	20	-	20	-
Fluorinase (10 mg / ml)	100	100	100	100
Tris-HCl, pH 7.8, 20 mM	-	20	-	20

Exp 1: The production of 5'-FDI **86** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 471 (M $-CH_3$, 20%), 278 (M $-C_8H_{11}N_4OSi$, 80) and 237 (67).

Exp 2: The production of 5'-FDA **83** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 470 (M $-CH_3$, 13), 278 (M $-C_8H_{12}N_5Si$, 48) and 236 (100).

Exp 3: The production of 5'-ClDI **203** was confirmed by HPLC analysis with a reference and by GC-MS; ***m/z*** 487 (M $-CH_3$, 31%), 294 (M $-C_8H_{11}N_4OSi$, 88) and 230 (230).

Exp 4: Neither 5'-ClDI **203** or 5'-ClDA **165** were detected by either HPLC or GC-MS analyses.

5'-Methylthio-inosine **205** was also detected by HPLC after co-injection with a reference and by GC-MS {***m/z*** 499 (M $-CH_3$ 14%), 281 (30), 237 (56) and 175 (100)} in experiments 1 and 3.

5.2.13 Incubation of 5-fluoro-5-deoxy-D-ribose (5-FDR) 120 in the CFE

Synthetic 5-FDR **120** (100 µl, 50 mM) was incubated at 37 °C for 16 h with CFE (0.2 g / ml, Tris-HCl buffer pH 7.8, 50 mM, 900 µl,). After the incubation period the protein was denatured (100 °C / 3 min) and removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) (5-FDR **120**) -228. 39 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 25.6) and -230.56 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 26.8).

The experiment was repeated several times with various batches of CFE of *S. cattleya*. However in each case only un-metabolised 5-FDR **120** was observed by ^{19}F NMR analysis.

5.2.14 Attempted inhibition of deaminase activity using iodoacetamide

5'-FDA **83** (100 µl, 37.1 mM), iodoacetamide (10 µl, 100 mM) and CFE (0.4g / ml, Tris-HCl buffer pH 7.8, 50 mM, 500 µl) were incubated at 37 °C for different periods of time (Exp 1-3). After the designated incubation time the samples were heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). The products of the incubation were analysed by ^{19}F NMR spectroscopy. The identities of the fluorinated products detected were confirmed by co-injection with reference compounds.

Experiment 1: 2 h incubation.

δ_F(470 MHz; 10% D_2O) (compound A) -230.49 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 28.3), (5'-FDI **86**) -230.14 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9) and (5'-FDA **83**) -231.09 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.7).

Experiment 2: 4 h incubation.

δ_F(470 MHz; 10% D_2O) (compound A) -230.46 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 28.3), and (5'-FDI **86**) -231.15 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9).

Experiment 3: 24 h incubation.

δ_F(470 MHz; 10% D_2O) (5'-FDI **86**) -230.21 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9)

5.2.15 5-FDRP as a biosynthetic intermediate

5.2.15.1 Preparation of 5-FDRP **228**

5'-FDI **86** (10 mg) and PNP (20 mg) were suspended in phosphate buffer (pH 6.8, 200 mM, 2 ml). The sample was left to incubate at 37 °C for 16 h. After incubation the sample was heated (100 °C / 3 min) and the denatured protein was removed from the solution by

centrifugation (14,000 rpm / 15 min). The sample was then analysed by ^{19}F NMR spectroscopy and HPLC (UV detection); δ_F(470 MHz; 10% D_2O) (5-FDRP **228**, 60%) -230.38 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 28.3) and (5'-FDI **86,** 40%) -231.19 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9). HPLC (UV) detection indicated the production of hypoxanthine **230** and the presence of unreacted 5'-FDI **86**. An aliquot (500 µl) was lyophilised and treated with MSTFA prior to GC-MS analysis; ***m/z*** 505 (16%), 382 (11), 353 (14), 300 (24), 299 (100) and 147 (20).

5.2.15.2 CFE incubation of 5-FDRP **228**

The 5-FDRP **228** used in the following experiment was prepared as detailed in section 5.2.15.1 and was found to contain ~50% 5'-FDI **86**. Control experiments carried out with denatured CFE showed that 5-FDRP **228** was chemically stable for of 16 h at 37 °C.

5-FDRP **228** (150 µl) was incubated at 37 °C for 16 h with CFE (0.2 g / ml, Tris-HCl buffer pH 7.8, 50 mM, 500 µl). After the incubation period the protein was denatured (100 °C / 3 min) and removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed ^{19}F NMR spectroscopy. The identities of the new fluorinated products detected were confirmed by co-injection with reference compounds. δ_F(470 MHz, 10 % D_2O) (FAc **15**) -216.78 (t, $^2J_{F,H}$ 48.0) and (5'-FDI **86**, from starting material) -231.10 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 28.9).

5.2.16 CFE preparation of 5-FDR 120

The 5-FDRP **228** used in the following experiments was prepared as detailed in section 5.2.15.1 and was found to contain ~40% 5'-FDI **86**. The phytase enzyme was prepared as a

solution in phosphate buffer (pH 4.6, 200 mM). 5'-FDA **83** was prepared as a solution (10 mg / ml) in ultra-pure water.

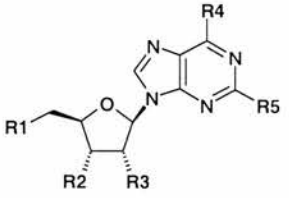
Control 1: 5'-FDA **83** (150 µl, 37.1 mM) was incubated at 37 °C for 16 h with CFE (0.4 g / ml, phosphate buffer pH 4.6, 200 mM, 500 µl). After the incubation period the protein was denatured (100 °C / 3 min) and removed by centrifugation (14,000 rpm /15 min). ^{19}F NMR (10 % D_2O) analysis of the sample showed that FAc **15** was still produced at this lower pH. The production of 5'-FDI **86** was not observed.

Control 2: 5-FDRP **228** (150 µl) was incubated at 37 °C for 16 h with phytase (4 mg /ml, phosphate buffer pH 4.6, 200 mM, 1 ml). After the incubation period the protein was denatured (100 °C / 3 min) and removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy. The ^{19}F NMR analysis with a reference confirmed that all of the starting material 5-FDRP (228) had been converted to 5-FDR **120**. δ_F(470 MHz, 10% D_2O) (5'-FDR **120,** β anomer) -228.41 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 25.6) and (5-FDR **120,** α anomer) -230.60 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 26.8).

Experiment 1: 5'-FDA **83** (150 µl, 37.1 mM) was incubated at 37 °C for 16 h with CFE (0.4 g / ml, phosphate buffer pH 4.6, 200 mM, 500 µl) and phytase (4 mg solid). After the incubation period the protein was denatured (100 °C / 3 min) and removed by centrifugation (14,000 rpm / 15 min). D_2O (100 µl) was added to the supernatant which was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) (5-FDR **120,** β anomer) -228.34 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 25.6) and (5-FDR **120,** α anomer) -230.56 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 26.8).

5.2.17 PNP substrate specificity analysis

General protocol for substrate testing was as follows. The substrate (20 μl, 10 mM) was incubated with partially purified PNP enzyme (150 μl, prepared in phosphate buffer 200 mM, pH 6.8). The protocol used to prepare the partially purified PNP enzyme is detailed in Appendix III, publications. Incubations were carried out at 37 °C for 4 h. A positive (+) sign indicates that the production of a "free" base was confirmed by HPLC analysis (UV detection) confirming enzyme activity. A negative (-) sign indicates that no enzyme activity was observed by HPLC.



	R1	R2	R3	R4	R5	Result
83	F	OH	OH	NH_2	H	+
86	F	OH	OH	OH	H	-
181	F	OH	H	NH_2	H	+
165	Cl	OH	OH	NH_2	H	+
182	Cl	OH	H	NH_2	H	+
203	Cl	OH	OH	OH	H	-
195	Cl	OH	OH	NH_2	NH_2	+
201	Br	OH	OH	NH_2	H	+
190	OH	H	OH	NH_2	H	-
205	SMe	OH	OH	NH_2	H	+
233	OAc	OH	OH	NH_2	H	-
164	H	OH	OH	NH_2	H	+
232	H	OH	H	NH_2	H	+
138	OPO_3	OH	OH	NH_2	H	-
166	OH	OH	OH	NH_2	H	-

5.2.18 Reversibility of the PNP

5.2.18.1 Preparation of 5-ClDRP **236**

5'-ClDI **203** (10 mg) and PNP (20 mg) were suspended in phosphate buffer (pH 6.8, 200 mM, 2 ml). The sample was left to incubate at 37 °C for 16 h. After incubation the sample

was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). The sample was analysed by HPLC which showed the production of hypoxanthine 2 (~60%) and the presence of unreacted 5'-CDI **203** (~40 %).

5.2.18.2 Enzymatic preparation of 2-amino-5'-ClDA **195**

Partially purified PNP from *S. cattleya* (250 µl) was added to an extract of the products from 5.2.18.1 (500 µl). 2-Aminoadenine **242** (1 mg) was added and the sample was incubated at 37 °C for 16 h. After incubation the sample was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). HPLC analysis with a reference confirmed the production of 2-amino-5'-ClDA **195**. ES-MS also confirmed the production of 2-amino-5'-ClDA **195**; ***m/z*** 303 (Cl^{37}, M+H, 30%) 301 (Cl^{35}, M+H, 100%).

5.2.18.3 Enzymatic preparation of 2-amino-SAM **198**

Procedure 5.2.17.2 was repeated but after the PNP enzyme was denatured fluorinase (10 mg / ml, phosphate buffer, pH 6.8, 20 mM, 200 µl) was added an the sample was incubated again at 37 °C for 16 h. After this second incubation the sample was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). HPLC analysis showed the production of a new compound with an identical retention time (by co-injection) to that of 2-amino-SAM **198** produced independently using the protocol detailed in 5.2.9.

5.2.19 CFE preparation of 2-deoxy-5-FDR 250 from 2'-deoxy-5'-FDA 181

Experiment 1: 2'-Deoxy-5'-FDA **181** (150 µl, 30 mM) was incubated with CFE (0.2 g / ml, phosphate buffer pH 6.8, 20 mM, 500 µl) at 37 °C for 16 h. After this second incubation the sample was heated (100 °C / 3 min) and the denatured protein removed by

centrifugation (14,000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) (2-deoxy-5-FDR **250**) -227.67, (2-deoxy-5-FDRP **237**) -230.16, (2'-deoxy-5'-FDI **234**) -230.21. The presence of 2'-deoxy-5'-FDI **234** was also confirmed by HPLC analysis by co-injection with reference sample prepared *via* the treatment of 2'-deoxy-5'-FDA **181** with the commercial AMPDA.

Experiment 2: Phytase (4 mg in 1 ml of phosphate buffer 20 mM, pH 4.6) was added to an aliquot from experiment 1 (250 µl). The sample was then incubated at 37 °C for 16 h. After this second incubation the sample was heated (100 °C / 3 min) and the denatured protein removed by centrifugation (14,000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) (2-deoxy-5-FDR **250**) -227.73 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 23.3) and (2'-deoxy-5'-FDI **234**) -230.21 (dt, $^2J_{F,H}$ 47.6 and $^3J_{F,H}$ 26.0).

5.2.20 Preparation and testing of 5-fluoro-5-deoxy-D-xylulose-1-phosphate 257

FruA aldolase was prepared as a solution in phosphate buffer, pH 6.8, 20 mM (5 mg /ml). DHAP was prepared as a 10 mg / ml aqueous solution. The concentration of the fluoroacetaldehyde **49** was determined by ^{19}F NMR analysis with a reference sample of FAc **15.** Lyophilisation of the samples removed any residual fluoroacetaldehyde **49** or fluoroethanol **149**.

Experiment 1: Preparation of 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**

Ten samples were prepared using the following protocol; fluoroacetaladeyde **49** (30 µl, ~40mM) and DHAP **249** (70 µl) were incubated with FruA aldolase (200 µl) and the total volume of the solution was made up to 1 ml with phosphate buffer (pH 6.8, 20 mM). The samples were then incubated at 37 °C for 12 h. The protein was denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm / 15 min). The supernatants were

combined and lyophilisation gave a white powder that was resuspended in water (5 ml). The resuspended sample was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, 10% D_2O) -228.13 (dt, $^2J_{F,H}$ 46.5 and $^3J_{F,H}$ 15.3)

Experiment 2: Preparation of 5-fluoro-5-deoxy-D-xylulose **258**

Phytase (10 mg) was added to a sample of 5-fluoro-5-deoxy-D-xylulose-1-phosphate 257 (5 ml) prepared using the protocol described in 5.2.20 experiment 1. The pH of the sample was lower to ~ 5 by addition of HCl. The sample was then incubated at 37 °C for 4 h. The protein was denatured by heating (100 °C / 3 min) and removed from the solution by centrifugation (14, 000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, D_2O) -228.54 (dt, $^2J_{F,H}$ 46.8 and $^3J_{F,H}$ 15.7). Note - co-injection with **257** showed a new peak at -228.23 ppm.

Experiment 3: CFE incubation of 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**

5-Fluoro-5-deoxy-D-xylulose-1-phosphate **257** (250 µl) prepared using the protocol described in 5.2.20 experiment 1 was incubated with CFE (0.2 g/ ml, phosphate buffer pH 4.6, 20 mM, 500 µl) at 37 °C for 16 h. The protein was denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy. The identity of the fluorinated compounds were assigned based on co-injection with reference samples; δ_F(470 MHz, D_2O) (FAc **15**) -215.98 (t, $^2J_{F,H}$ 47.9), -228.28 (dt, $^2J_{F,H}$ 46.5 and $^3J_{F,H}$ 15.3) and (fluoroacetaldehyde **49**) -231.20 (dt, $^2J_{F,H}$ 46.5 and $^3J_{F,H}$ 9.8). Co-injection showed peak at -228.28 ppm was unmetabolised **257**.

5.2.21 Preparation and testing of 5-fluoro-5-deoxy-D-ribulose-1-phosphate

The concentration of the fluoroacetaldehyde **49** was determined by ^{19}F NMR analysis with a reference sample of FAc **15**. DHAP **249** was prepared as a 10 mg / ml aqueous solution. Lyophilisation of the samples removed any residual fluoroacetaldehyde **49** or fluoroethanol **149**. FucA aldolase was prepared as a 2 mg / ml solution in phosphate buffer pH 6.8, 200 mM.

Experiment 1: Preparation of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248**

10 samples were prepared using the following protocol; fluoroacetaladeyde **49** (30, ~40 mM) and DHAP **249** (70 µl) were incubated with FucA aldolase (400 µl) and the total volume of the solution was made up to 1 ml by addition of phosphate buffer (pH 6.8, 200 mM). The samples were incubated at 37 °C for 12 h. The protein was denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm / 15 min). The supernatants were combined and freeze dried to give a white powder which was resuspended in ultra-pure water (5 ml). δ_F(470 MHz, 10% D_2O) (5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**, 65%) -228.18 (dt, $^2J_{F,H}$ 46.5 and $^3J_{F,H}$ 15.3) and (5-fluoro-5-deoxy-D-ribulose-1-phosphate **248**, 35%) -231.36 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 20.7).

Experiment 2: Preparation of 5-fluoro-5-deoxy-D-ribulose **259** (Method 1)

Phytase (10 mg) was added to a sample of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248** (5 ml) prepared using the protocol described in 5.2.22 experiment 1. The pH of the sample was lowered to ~ pH 5 by the addition of HCl. The sample was then incubated at 37 °C for 6 h. The protein was denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy; δ_F(470 MHz, D_2O) (5-fluoro-5-deoxy-D-xylulose **258**) -228.50 (dt, $^2J_{F,H}$ 46.8 and $^3J_{F,H}$ 15.8) and (5-fluoro-5-deoxy-D-ribulose **259**) -231.16 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 20.7).

Experiment 3: Preparation of 5-fluoro-5-deoxy-D-ribulose **259** (Method 2)[287]

Glucose isomerase (30 mg) was added to an aqueous solution of 5-FDR **120** (500 µl, 50 mM) containing powdered molecular sieves. The sample was heated at 60 °C for 12h with stirring. Centrifugation (14, 000 rpm / 20 min) was used to remove both the isomerase enzyme and the molecular sieves. ^{19}F NMR analysis was carried out on the supernatant; δ_F(470 MHz, 10% D_2O) (5-FDR **120**, β anomer) -228.50 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 25.6), (5-FDR **120**, α anomer) -230.80 (dt, $^2J_{F,H}$ 47.3 and $^3J_{F,H}$ 26.8) and (5-fluoro-5-deoxy-D-ribulose **259**) -231.19 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 20.7).

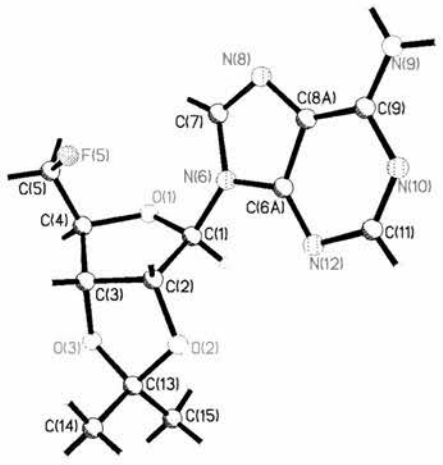
Experiment 4: CFE incubation of 5-fluoro-5-deoxy-D-ribulose-1-phosphate **248**

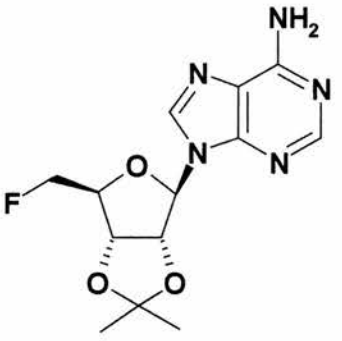
5-Fluoro-5-deoxy-D-ribulose-1-phosphate **248** (250 µl)*, prepared using the protocol described in 5.2.21 experiment 1, was incubated with CFE (0.2 g / ml, phosphate buffer pH 4.6, 20 mM, 500 µl) at 37 °C for 16 h. The protein was denatured by heating (100 °C / 3 min) and removed by centrifugation (14, 000 rpm / 15 min). The supernatant was then analysed by ^{19}F NMR spectroscopy. The identity of the fluorinated compounds were assigned based on co-injection with reference samples; δ_F(470 MHz, D_2O) (FAc **15**) -216.01 (t, $^2J_{F,H}$ 47.8), (5-fluoro-5-deoxy-D-xylulose **258**) -228.57 (dt, $^2J_{F,H}$ 46.8 and $^3J_{F,H}$ 15.8), (fluoroacetaldehyde **49**) -231.20 (dt, $^2J_{F,H}$ 46.5 and $^3J_{F,H}$ 9.8) and (5-fluoro-5-deoxy-D-ribulose **259**) -231.19 (dt, $^2J_{F,H}$ 47.0 and $^3J_{F,H}$ 20.7). The identity of **258** and **259** were confirmed by co-injection with reference samples and indicate that CFE must have some phosphatase activity.

*The sample was known to contain ~ 60% 5-fluoro-5-deoxy-D-xylulose-1-phosphate **257**.

Appendix I

X-ray data for **133**





133

Table 1. Crystal data and structure refinement for **133**.

Identification code	**133**	
Empirical formula	C13 H16 F N5 O3	
Formula weight	309.31	
Temperature	173(2) K	
Wavelength	1.54178 Å	
Crystal system	Monoclinic	
Space group	P2(1)	
Unit cell dimensions	a = 5.4676(16) Å	α= 90°.
	b = 7.754(2) Å	β= 96.531(10)°.
	c = 16.539(5) Å	γ = 90°.
Volume	696.7(3) Å^3	
Z	2	
Density (calculated)	1.474 Mg/m^3	
Absorption coefficient	0.992 mm^{-1}	
F(000)	324	
Crystal size	0.100 x 0.100 x 0.010 mm^3	
Theta range for data collection	2.69 to 49.39°.	
Index ranges	-5<=h<=5, -7<=k<=7, -16<=l<=16	
Reflections collected	5954	
Independent reflections	1259 [R(int) = 0.1682]	
Completeness to theta = 49.39°	91.8 %	
Absorption correction	Multiscan	
Max. and min. transmission	1.0000 and 0.8200	

Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	1259 / 1 / 131
Goodness-of-fit on F^2	1.762
Final R indices [I>2sigma(I)]	R1 = 0.1515, wR2 = 0.3646
R indices (all data)	R1 = 0.1559, wR2 = 0.3682
Absolute structure parameter	0.00
Extinction coefficient	0.020(11)
Largest diff. peak and hole	0.923 and -0.575 e.$Å^{-3}$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for **133**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
O(1)	898(16)	4665(11)	7139(5)	30(3)
C(1)	2310(20)	3214(18)	7212(6)	24(3)
C(2)	3520(30)	3060(20)	8100(7)	32(3)
O(2)	2905(15)	1471(12)	8461(5)	32(3)
C(3)	2230(30)	4520(19)	8517(7)	35(4)
O(3)	345(18)	3547(11)	8849(5)	39(3)
C(4)	1250(30)	5740(20)	7890(8)	52(5)
C(5)	2960(30)	7160(20)	7787(9)	49(4)
F(5)	5372(15)	6592(11)	7783(5)	50(3)
N(6)	4167(19)	3247(12)	6610(5)	25(3)
C(6A)	5690(20)	1860(17)	6466(7)	26(3)
C(7)	4700(30)	4591(19)	6082(7)	28(3)
N(8)	6270(20)	4138(16)	5628(6)	36(3)
C(8A)	7030(30)	2476(19)	5851(7)	28(3)
C(9)	8610(30)	1410(20)	5568(8)	39(4)
N(9)	10180(20)	1697(15)	5018(6)	27(3)
N(10)	8840(20)	-247(15)	5919(7)	41(3)
C(11)	7440(30)	-660(20)	6509(7)	31(3)
N(12)	5717(19)	347(13)	6806(6)	25(3)
C(13)	1120(30)	1790(20)	9041(8)	37(4)
C(14)	2490(30)	1740(30)	9864(9)	50(5)
C(15)	-1010(30)	610(20)	8860(9)	56(5)

Table 3. Bond lengths [Å] and angles [°] for **133**.

O(1)-C(1)	1.364(15)
O(1)-C(4)	1.491(18)
C(1)-N(6)	1.500(17)
C(1)-C(2)	1.544(17)
C(2)-O(2)	1.427(18)
C(2)-C(3)	1.54(2)
O(2)-C(13)	1.464(16)
C(3)-O(3)	1.435(17)
C(3)-C(4)	1.46(2)
O(3)-C(13)	1.449(19)
C(4)-C(5)	1.47(2)
C(5)-F(5)	1.391(18)
N(6)-C(6A)	1.398(17)
N(6)-C(7)	1.411(17)
C(6A)-N(12)	1.300(16)
C(6A)-C(8A)	1.40(2)
C(7)-N(8)	1.255(18)
N(8)-C(8A)	1.390(19)
C(8A)-C(9)	1.32(2)
C(9)-N(9)	1.341(18)
C(9)-N(10)	1.41(2)
N(10)-C(11)	1.35(2)
C(11)-N(12)	1.356(19)
C(13)-C(15)	1.49(2)
C(13)-C(14)	1.48(2)
C(1)-O(1)-C(4)	111.7(9)
O(1)-C(1)-N(6)	110.3(9)
O(1)-C(1)-C(2)	108.9(10)
N(6)-C(1)-C(2)	112.7(10)
O(2)-C(2)-C(1)	111.7(12)
O(2)-C(2)-C(3)	107.8(10)
C(1)-C(2)-C(3)	101.6(11)
C(2)-O(2)-C(13)	109.5(9)
O(3)-C(3)-C(4)	113.1(13)
O(3)-C(3)-C(2)	99.8(11)
C(4)-C(3)-C(2)	107.9(11)

C(3)-O(3)-C(13)	111.8(10)
C(5)-C(4)-C(3)	112.5(13)
C(5)-C(4)-O(1)	110.2(12)
C(3)-C(4)-O(1)	103.4(12)
F(5)-C(5)-C(4)	112.4(13)
C(6A)-N(6)-C(7)	107.1(10)
C(6A)-N(6)-C(1)	124.1(9)
C(7)-N(6)-C(1)	128.8(10)
N(12)-C(6A)-N(6)	126.8(12)
N(12)-C(6A)-C(8A)	129.8(13)
N(6)-C(6A)-C(8A)	103.4(11)
N(8)-C(7)-N(6)	111.5(12)
C(7)-N(8)-C(8A)	107.7(13)
C(9)-C(8A)-N(8)	132.4(14)
C(9)-C(8A)-C(6A)	117.3(14)
N(8)-C(8A)-C(6A)	110.2(13)
N(9)-C(9)-C(8A)	129.2(15)
N(9)-C(9)-N(10)	113.3(13)
C(8A)-C(9)-N(10)	117.3(14)
C(11)-N(10)-C(9)	118.7(13)
N(10)-C(11)-N(12)	127.2(14)
C(6A)-N(12)-C(11)	109.5(12)
O(2)-C(13)-O(3)	102.6(10)
O(2)-C(13)-C(15)	109.1(11)
O(3)-C(13)-C(15)	109.4(12)
O(2)-C(13)-C(14)	107.0(12)
O(3)-C(13)-C(14)	109.5(12)
C(15)-C(13)-C(14)	118.1(13)

Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for **133**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
O(1)	18(5)	18(5)	54(5)	-3(4)	8(4)	17(4)
O(2)	24(5)	38(6)	39(5)	7(4)	25(4)	9(5)
F(5)	32(5)	36(5)	83(6)	14(5)	10(4)	-7(4)
N(6)	33(7)	6(5)	35(5)	1(5)	-3(4)	-9(6)
N(8)	46(9)	27(7)	40(6)	5(5)	22(5)	5(6)
N(9)	30(7)	13(6)	42(5)	-11(5)	24(5)	2(5)
N(10)	49(9)	31(8)	45(6)	-6(5)	12(6)	-26(7)
N(12)	16(6)	14(6)	48(6)	4(5)	17(5)	7(5)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2 x\ 10^3$) for **133**.

	x	y	z	U(eq)
H(1A)	1217	2193	7089	29
H(2A)	5341	3240	8143	38
H(3A)	3362	5093	8953	42
H(4A)	-368	6206	8018	62
H(5A)	2431	7760	7268	58
H(5B)	2891	7999	8234	58
H(7A)	3955	5700	6070	34
H(9A)	10266	2839	4769	32
H(9B)	11256	766	4866	32
H(11A)	7686	-1769	6744	37
H(14A)	1359	1947	10271	75
H(14B)	3772	2632	9909	75
H(14C)	3263	605	9957	75
H(15A)	-1769	797	8301	84
H(15B)	-2226	830	9240	84
H(15C)	-445	-592	8920	84

Table 6. Torsion angles [°] for **133**.

C(4)-O(1)-C(1)-N(6)	-116.1(11)
C(4)-O(1)-C(1)-C(2)	8.0(14)
O(1)-C(1)-C(2)-O(2)	122.5(11)
N(6)-C(1)-C(2)-O(2)	-114.8(12)
O(1)-C(1)-C(2)-C(3)	7.8(14)
N(6)-C(1)-C(2)-C(3)	130.5(12)
C(1)-C(2)-O(2)-C(13)	-106.1(11)
C(3)-C(2)-O(2)-C(13)	4.7(14)
O(2)-C(2)-C(3)-O(3)	-20.2(12)
C(1)-C(2)-C(3)-O(3)	97.3(11)
O(2)-C(2)-C(3)-C(4)	-138.6(13)
C(1)-C(2)-C(3)-C(4)	-21.0(17)
C(4)-C(3)-O(3)-C(13)	144.2(11)
C(2)-C(3)-O(3)-C(13)	29.8(12)
O(3)-C(3)-C(4)-C(5)	157.2(12)
C(2)-C(3)-C(4)-C(5)	-93.3(16)
O(3)-C(3)-C(4)-O(1)	-83.9(14)
C(2)-C(3)-C(4)-O(1)	25.6(17)
C(1)-O(1)-C(4)-C(5)	99.2(13)
C(1)-O(1)-C(4)-C(3)	-21.3(15)
C(3)-C(4)-C(5)-F(5)	43.6(17)
O(1)-C(4)-C(5)-F(5)	-71.2(14)
O(1)-C(1)-N(6)-C(6A)	-172.1(10)
C(2)-C(1)-N(6)-C(6A)	66.0(15)
O(1)-C(1)-N(6)-C(7)	6.5(14)
C(2)-C(1)-N(6)-C(7)	-115.4(13)
C(7)-N(6)-C(6A)-N(12)	-177.3(11)
C(1)-N(6)-C(6A)-N(12)	1.6(18)
C(7)-N(6)-C(6A)-C(8A)	-0.3(12)
C(1)-N(6)-C(6A)-C(8A)	178.5(10)
C(6A)-N(6)-C(7)-N(8)	2.4(15)
C(1)-N(6)-C(7)-N(8)	-176.4(12)
N(6)-C(7)-N(8)-C(8A)	-3.3(15)
C(7)-N(8)-C(8A)-C(9)	179.1(15)
C(7)-N(8)-C(8A)-C(6A)	3.1(15)
N(12)-C(6A)-C(8A)-C(9)	-1(2)

N(6)-C(6A)-C(8A)-C(9)	-178.2(11)
N(12)-C(6A)-C(8A)-N(8)	175.3(13)
N(6)-C(6A)-C(8A)-N(8)	-1.6(13)
N(8)-C(8A)-C(9)-N(9)	8(3)
C(6A)-C(8A)-C(9)-N(9)	-176.2(12)
N(8)-C(8A)-C(9)-N(10)	-177.0(13)
C(6A)-C(8A)-C(9)-N(10)	-1.2(18)
N(9)-C(9)-N(10)-C(11)	176.7(11)
C(8A)-C(9)-N(10)-C(11)	1.0(18)
C(9)-N(10)-C(11)-N(12)	2(2)
N(6)-C(6A)-N(12)-C(11)	179.8(11)
C(8A)-C(6A)-N(12)-C(11)	3.7(19)
N(10)-C(11)-N(12)-C(6A)	-3.9(18)
C(2)-O(2)-C(13)-O(3)	12.8(13)
C(2)-O(2)-C(13)-C(15)	128.7(12)
C(2)-O(2)-C(13)-C(14)	-102.4(13)
C(3)-O(3)-C(13)-O(2)	-27.8(12)
C(3)-O(3)-C(13)-C(15)	-143.5(11)
C(3)-O(3)-C(13)-C(14)	85.6(13)

Symmetry transformations used to generate equivalent atoms:

Table 7. Hydrogen bonds for **133** [Å and °].

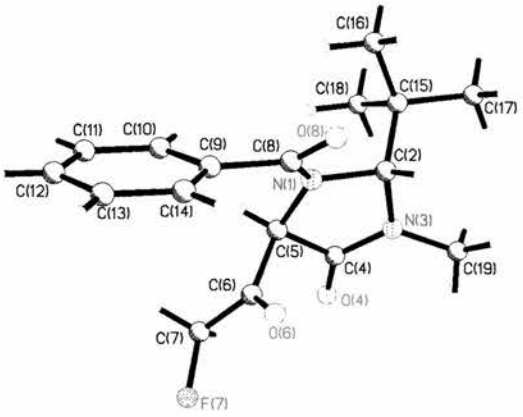
D-H...A	d(D-H)	d(H...A)	d(D...A)	<(DHA)
N(9)-H(9B)...N(8)#1	0.98	2.08	3.050(16)	168.4
N(9)-H(9A)...N(10)#2	0.98	1.96	2.914(16)	162.5

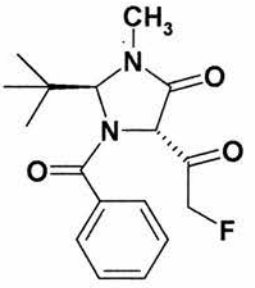
Symmetry transformations used to generate equivalent atoms:

#1 -x+2,y-1/2,-z+1 #2 -x+2,y+1/2,-z+1

Appendix II

X-ray data for (*rac*) **146**





146
(racemic)

Table 1. Crystal data and structure refinement for (*rac*) **146**.

Empirical formula	C17 H21 F N2 O3	
Formula weight	320.36	
Temperature	93(2) K	
Wavelength	0.71073 Å	
Crystal system	Monoclinic	
Space group	P2(1)/c	
Unit cell dimensions	a = 5.993(2) Å	α= 90°.
	b = 21.450(8) Å	β= 92.515(10)°.
	c = 12.819(5) Å	γ = 90°.
Volume	1646.2(10) $Å^3$	
Z	4	
Density (calculated)	1.293 Mg/m^3	
Absorption coefficient	0.096 mm^{-1}	
F(000)	680	
Crystal size	0.2000 x 0.0500 x 0.0500 mm^3	
Theta range for data collection	2.48 to 25.35°.	
Index ranges	-6<=h<=7, -15<=k<=25, -15<=l<=15	
Reflections collected	9998	
Independent reflections	2929 [R(int) = 0.0674]	
Completeness to theta = 25.35°	97.1 %	
Absorption correction	Multiscan	
Max. and min. transmission	1.0000 and 0.2497	
Refinement method	Full-matrix least-squares on F^2	

Data / restraints / parameters	2929 / 0 / 211
Goodness-of-fit on F^2	1.084
Final R indices [I>2sigma(I)]	R1 = 0.0936, wR2 = 0.2398
R indices (all data)	R1 = 0.1252, wR2 = 0.2615
Extinction coefficient	0.013(5)
Largest diff. peak and hole	0.542 and -0.330 e.$Å^{-3}$

Table 2. Atomic coordinates (x 10^4) and equivalent isotropic displacement parameters ($Å^2$x 10^3) for (*rac*) **146**. U(eq) is defined as one third of the trace of the orthogonalized U^{ij} tensor.

	x	y	z	U(eq)
N(1)	1496(5)	3730(2)	3497(3)	25(1)
C(2)	310(6)	3230(2)	4046(3)	24(1)
N(3)	1524(5)	2678(2)	3692(3)	26(1)
C(4)	3469(6)	2801(2)	3268(3)	26(1)
O(4)	4915(4)	2436(1)	3020(2)	30(1)
C(5)	3533(6)	3505(2)	3043(3)	25(1)
C(6)	3458(7)	3543(2)	1840(4)	30(1)
O(6)	1719(5)	3578(1)	1328(2)	33(1)
C(7)	5704(7)	3516(2)	1393(4)	33(1)
F(7)	5502(4)	3423(1)	323(2)	45(1)
C(8)	266(6)	4237(2)	3121(3)	23(1)
O(8)	-1733(4)	4276(1)	3229(2)	32(1)
C(9)	1549(6)	4753(2)	2632(3)	25(1)
C(10)	3633(6)	4943(2)	3031(4)	28(1)
C(11)	4722(7)	5441(2)	2575(4)	34(1)
C(12)	3710(8)	5747(2)	1731(4)	41(1)
C(13)	1659(8)	5555(2)	1325(4)	39(1)
C(14)	539(7)	5061(2)	1767(3)	31(1)
C(15)	442(6)	3323(2)	5252(3)	25(1)
C(16)	-405(7)	3964(2)	5528(4)	35(1)
C(17)	-1000(7)	2830(2)	5759(4)	32(1)
C(18)	2880(7)	3248(2)	5676(4)	32(1)
C(19)	743(7)	2037(2)	3833(4)	32(1)

Table 3. Bond lengths [Å] and angles [°] for (*rac*) **146**.

N(1)-C(8)	1.388(5)
N(1)-C(5)	1.457(5)
N(1)-C(2)	1.481(5)
C(2)-N(3)	1.472(5)
C(2)-C(15)	1.558(6)
C(2)-H(2A)	1.0000
N(3)-C(4)	1.334(5)
N(3)-C(19)	1.465(5)
C(4)-O(4)	1.221(5)
C(4)-C(5)	1.537(6)
C(5)-C(6)	1.544(6)
C(5)-H(5A)	1.0000
C(6)-O(6)	1.209(5)
C(6)-C(7)	1.486(6)
C(7)-F(7)	1.386(5)
C(7)-H(7A)	0.9900
C(7)-H(7B)	0.9900
C(8)-O(8)	1.215(5)
C(8)-C(9)	1.500(6)
C(9)-C(10)	1.390(6)
C(9)-C(14)	1.405(6)
C(10)-C(11)	1.394(6)
C(10)-H(10A)	0.9500
C(11)-C(12)	1.382(7)
C(11)-H(11A)	0.9500
C(12)-C(13)	1.377(7)
C(12)-H(12A)	0.9500
C(13)-C(14)	1.389(7)
C(13)-H(13A)	0.9500
C(14)-H(14A)	0.9500
C(15)-C(16)	1.513(6)
C(15)-C(17)	1.529(6)
C(15)-C(18)	1.545(6)
C(16)-H(16A)	0.9800
C(16)-H(16B)	0.9800
C(16)-H(16C)	0.9800

C(17)-H(17A)	0.9800
C(17)-H(17B)	0.9800
C(17)-H(17C)	0.9800
C(18)-H(18A)	0.9800
C(18)-H(18B)	0.9800
C(18)-H(18C)	0.9800
C(19)-H(19A)	0.9800
C(19)-H(19B)	0.9800
C(19)-H(19C)	0.9800
C(8)-N(1)-C(5)	124.2(3)
C(8)-N(1)-C(2)	118.3(3)
C(5)-N(1)-C(2)	112.2(3)
N(3)-C(2)-N(1)	100.5(3)
N(3)-C(2)-C(15)	113.9(3)
N(1)-C(2)-C(15)	112.0(3)
N(3)-C(2)-H(2A)	110.0
N(1)-C(2)-H(2A)	110.0
C(15)-C(2)-H(2A)	110.0
C(4)-N(3)-C(19)	121.7(4)
C(4)-N(3)-C(2)	114.7(3)
C(19)-N(3)-C(2)	123.5(3)
O(4)-C(4)-N(3)	128.4(4)
O(4)-C(4)-C(5)	123.9(4)
N(3)-C(4)-C(5)	107.5(3)
N(1)-C(5)-C(4)	102.8(3)
N(1)-C(5)-C(6)	113.2(3)
C(4)-C(5)-C(6)	103.8(3)
N(1)-C(5)-H(5A)	112.1
C(4)-C(5)-H(5A)	112.1
C(6)-C(5)-H(5A)	112.1
O(6)-C(6)-C(7)	124.6(4)
O(6)-C(6)-C(5)	122.1(4)
C(7)-C(6)-C(5)	113.3(3)
F(7)-C(7)-C(6)	110.2(3)
F(7)-C(7)-H(7A)	109.6
C(6)-C(7)-H(7A)	109.6
F(7)-C(7)-H(7B)	109.6
C(6)-C(7)-H(7B)	109.6

H(7A)-C(7)-H(7B)	108.1
O(8)-C(8)-N(1)	121.7(4)
O(8)-C(8)-C(9)	121.5(3)
N(1)-C(8)-C(9)	116.7(3)
C(10)-C(9)-C(14)	120.1(4)
C(10)-C(9)-C(8)	122.1(4)
C(14)-C(9)-C(8)	117.8(3)
C(9)-C(10)-C(11)	120.0(4)
C(9)-C(10)-H(10A)	120.0
C(11)-C(10)-H(10A)	120.0
C(12)-C(11)-C(10)	119.7(4)
C(12)-C(11)-H(11A)	120.2
C(10)-C(11)-H(11A)	120.2
C(13)-C(12)-C(11)	120.5(4)
C(13)-C(12)-H(12A)	119.8
C(11)-C(12)-H(12A)	119.8
C(12)-C(13)-C(14)	120.9(4)
C(12)-C(13)-H(13A)	119.5
C(14)-C(13)-H(13A)	119.5
C(13)-C(14)-C(9)	118.8(4)
C(13)-C(14)-H(14A)	120.6
C(9)-C(14)-H(14A)	120.6
C(16)-C(15)-C(17)	109.1(4)
C(16)-C(15)-C(18)	109.5(3)
C(17)-C(15)-C(18)	108.8(3)
C(16)-C(15)-C(2)	110.2(3)
C(17)-C(15)-C(2)	109.2(3)
C(18)-C(15)-C(2)	110.0(3)
C(15)-C(16)-H(16A)	109.5
C(15)-C(16)-H(16B)	109.5
H(16A)-C(16)-H(16B)	109.5
C(15)-C(16)-H(16C)	109.5
H(16A)-C(16)-H(16C)	109.5
H(16B)-C(16)-H(16C)	109.5
C(15)-C(17)-H(17A)	109.5
C(15)-C(17)-H(17B)	109.5
H(17A)-C(17)-H(17B)	109.5
C(15)-C(17)-H(17C)	109.5

H(17A)-C(17)-H(17C)	109.5
H(17B)-C(17)-H(17C)	109.5
C(15)-C(18)-H(18A)	109.5
C(15)-C(18)-H(18B)	109.5
H(18A)-C(18)-H(18B)	109.5
C(15)-C(18)-H(18C)	109.5
H(18A)-C(18)-H(18C)	109.5
H(18B)-C(18)-H(18C)	109.5
N(3)-C(19)-H(19A)	109.5
N(3)-C(19)-H(19B)	109.5
H(19A)-C(19)-H(19B)	109.5
N(3)-C(19)-H(19C)	109.5
H(19A)-C(19)-H(19C)	109.5
H(19B)-C(19)-H(19C)	109.5

Symmetry transformations used to generate equivalent atoms:

Table 4. Anisotropic displacement parameters ($Å^2 x\ 10^3$) for (*rac*) **146**. The anisotropic displacement factor exponent takes the form: $-2\pi^2[\ h^2\ a^{*2}U^{11} + ... + 2\ h\ k\ a^*\ b^*\ U^{12}\ ]$

	U^{11}	U^{22}	U^{33}	U^{23}	U^{13}	U^{12}
N(1)	23(2)	20(2)	30(2)	2(2)	2(1)	-1(1)
C(2)	23(2)	16(2)	31(2)	1(2)	1(2)	-1(1)
N(3)	28(2)	18(2)	32(2)	-2(2)	-1(1)	-2(1)
C(4)	25(2)	24(2)	27(2)	1(2)	-3(2)	0(2)
O(4)	35(2)	24(2)	32(2)	-1(1)	5(1)	6(1)
C(5)	21(2)	26(2)	28(2)	1(2)	0(2)	2(2)
C(6)	30(2)	19(2)	39(3)	-2(2)	0(2)	-2(2)
O(6)	33(2)	30(2)	34(2)	-3(1)	-8(1)	0(1)
C(7)	38(2)	35(3)	27(2)	2(2)	4(2)	2(2)
F(7)	58(2)	51(2)	26(2)	-1(1)	8(1)	3(1)
C(8)	26(2)	18(2)	25(2)	2(2)	-3(2)	1(2)
O(8)	22(2)	32(2)	43(2)	5(1)	-1(1)	1(1)
C(9)	22(2)	21(2)	33(2)	2(2)	6(2)	3(2)
C(10)	22(2)	23(2)	40(3)	1(2)	4(2)	2(2)
C(11)	32(2)	23(3)	47(3)	-2(2)	8(2)	0(2)
C(12)	42(3)	24(3)	58(3)	5(2)	15(2)	-1(2)

C(13)	51(3)	26(3)	39(3)	8(2)	4(2)	9(2)
C(14)	34(2)	25(2)	34(2)	1(2)	4(2)	6(2)
C(15)	28(2)	20(2)	29(2)	-1(2)	6(2)	-4(2)
C(16)	37(2)	33(3)	34(3)	2(2)	6(2)	3(2)
C(17)	32(2)	29(3)	37(3)	5(2)	10(2)	2(2)
C(18)	34(2)	34(3)	29(2)	-2(2)	0(2)	-1(2)
C(19)	38(2)	23(2)	36(3)	2(2)	1(2)	-5(2)

Table 5. Hydrogen coordinates (x 10^4) and isotropic displacement parameters ($Å^2$x 10^3) for (*rac*) **146**.

	x	y	z	U(eq)
H(2A)	-1286	3211	3786	28
H(5A)	4899	3708	3365	30
H(7A)	6513	3910	1544	40
H(7B)	6577	3171	1722	40
H(10A)	4314	4734	3616	34
H(11A)	6153	5569	2842	41
H(12A)	4435	6093	1429	49
H(13A)	1001	5763	734	46
H(14A)	-884	4933	1489	37
H(16A)	-308	4018	6288	52
H(16B)	-1964	4007	5274	52
H(16C)	507	4282	5200	52
H(17A)	-921	2887	6519	49
H(17B)	-450	2414	5588	49
H(17C)	-2552	2874	5497	49
H(18A)	2950	3309	6434	48
H(18B)	3823	3559	5349	48
H(18C)	3415	2829	5513	48
H(19A)	1266	1775	3268	48
H(19B)	-894	2032	3820	48
H(19C)	1333	1877	4505	48

Table 6. Torsion angles [°] for (rac) **146**.

C(8)-N(1)-C(2)-N(3)	143.8(3)
C(5)-N(1)-C(2)-N(3)	-11.5(4)
C(8)-N(1)-C(2)-C(15)	-94.9(4)
C(5)-N(1)-C(2)-C(15)	109.7(4)
N(1)-C(2)-N(3)-C(4)	15.8(4)
C(15)-C(2)-N(3)-C(4)	-104.1(4)
N(1)-C(2)-N(3)-C(19)	-167.1(3)
C(15)-C(2)-N(3)-C(19)	73.0(4)
C(19)-N(3)-C(4)-O(4)	-6.2(6)
C(2)-N(3)-C(4)-O(4)	171.0(4)
C(19)-N(3)-C(4)-C(5)	169.0(3)
C(2)-N(3)-C(4)-C(5)	-13.8(4)
C(8)-N(1)-C(5)-C(4)	-149.1(4)
C(2)-N(1)-C(5)-C(4)	4.5(4)
C(8)-N(1)-C(5)-C(6)	-37.8(5)
C(2)-N(1)-C(5)-C(6)	115.8(4)
O(4)-C(4)-C(5)-N(1)	-179.2(4)
N(3)-C(4)-C(5)-N(1)	5.4(4)
O(4)-C(4)-C(5)-C(6)	62.7(5)
N(3)-C(4)-C(5)-C(6)	-112.8(3)
N(1)-C(5)-C(6)-O(6)	-20.1(6)
C(4)-C(5)-C(6)-O(6)	90.6(4)
N(1)-C(5)-C(6)-C(7)	161.2(4)
C(4)-C(5)-C(6)-C(7)	-88.1(4)
O(6)-C(6)-C(7)-F(7)	-10.8(6)
C(5)-C(6)-C(7)-F(7)	167.8(3)
C(5)-N(1)-C(8)-O(8)	151.9(4)
C(2)-N(1)-C(8)-O(8)	-0.3(6)
C(5)-N(1)-C(8)-C(9)	-31.7(5)
C(2)-N(1)-C(8)-C(9)	176.1(3)
O(8)-C(8)-C(9)-C(10)	137.7(4)
N(1)-C(8)-C(9)-C(10)	-38.7(6)
O(8)-C(8)-C(9)-C(14)	-39.9(6)
N(1)-C(8)-C(9)-C(14)	143.7(4)
C(14)-C(9)-C(10)-C(11)	0.1(6)
C(8)-C(9)-C(10)-C(11)	-177.4(4)

C(9)-C(10)-C(11)-C(12)	0.6(7)
C(10)-C(11)-C(12)-C(13)	-1.5(7)
C(11)-C(12)-C(13)-C(14)	1.6(8)
C(12)-C(13)-C(14)-C(9)	-0.8(7)
C(10)-C(9)-C(14)-C(13)	0.0(6)
C(8)-C(9)-C(14)-C(13)	177.6(4)
N(3)-C(2)-C(15)-C(16)	167.9(3)
N(1)-C(2)-C(15)-C(16)	54.7(4)
N(3)-C(2)-C(15)-C(17)	-72.3(4)
N(1)-C(2)-C(15)-C(17)	174.5(3)
N(3)-C(2)-C(15)-C(18)	47.0(4)
N(1)-C(2)-C(15)-C(18)	-66.2(4)

Symmetry transformations used to generate equivalent atoms:

Appendix III

The following details the method used for all HPLC analyses carried out.

Analytical C-18 column

Flow rate = 1 ml / minute

UV detection (254 nm)

Buffer A = MeCN 5%: Phosphate buffer 50mM, pH 4.5 95%.

Buffer B = MeCN 20%: Phosphate buffer 50mM, pH 4.5 80%.

Time (minutes)	Buffer A (%)	Buffer B (%)
0	100	0
20	0	100
22	100	0
30	100	0

Appendix IV

List of publications

D. O'Hagan, C. Schaffrath, S. L. Cobb, J. T. G. Hamilton and C. D. Murphy, "Biosynthesis of an organofluorine molecule: A fluorinase enzyme has been discovered that catalyses carbon-fluorine bond formation." *Nature*, 2002, **416**, 279.

C. Schaffrath, S. L. Cobb and D. O'Hagan, "Cell-free biosynthesis of fluoroacetate and 4-fluorothreonine in *Streptomyces cattleya*." (Front cover), *Angew. Chem. Int. Ed.*, 2002, **41**, 3913-3915.

S. L. Cobb, H. Deng, R. McGlinchey, D. O'Hagan, "Identification of 5'-fluoro-5'-deoxy-D-ribose-1-phosphate as an intermediate in fluorometabolite biosynthesis in *Streptomyces cattleya*." *J. Chem. Soc., Chem. Commun.*, 2004, 592-593.

In collaboration with Dr. C. D. Murphy, University College Dublin;

F. G. Boersma, W. C. Roberts, S. L. Cobb and C. D. Murphy, "A F-19 NMR study of fluorobenzoate biodegradation by Sphingomonas sp HB-1." *FEMS. Microbiology. Lett.*, 2004, **237**, 355-361.

Awards

2002. RSC Bio-Organic group prize for best oral presentation at postgraduate symposium, Cambridge University.
2003. Royal Society of Chemistry travel bursary to attend 12th European Carbohydrate Symposium, France.
2004. 2nd Prize for oral presentation, SCI postgraduate meeting, Edinburgh.
2004. 1st Prize for poster presentation, Industry Chemistry Forum, University of St. Andrews.

References

1. R. B. Herbert, "The biosynthesis of secondary metabolites", 2nd Ed. (1988), Chapman and Hal.

2. J. E. Baldwin and E. Ebraham, *Nat. Prod. Rep.*, 1988, **5**, 129.

3. A. M. S. Mayer and M. T. Hamman, *Mar. Biotechnol.,* 2004, **6**, 37.

4. K. C. Nicolaou and R. K Guy, *Angew. Chem. Int. Ed.*, 1995, **34**, 2079.

5. K. C. Nicolaou, W. –M. Dai and R. K Guy, *Angew. Chem. Int. Ed*,1994, **33**, 15.

6. L. Stryer, 'Biochemistry', 3rd Ed. Freeman and Company, New York.

7. J. D. Bul'ock, D. Hamilton, M. A. Hulme, A. J. Powell, H. M. Smalley, D. Shepherd and G. N. Smith, *Can. J. Microbiol.*, 1965, **11**, 765.

8. J. B. Walker, *J. Biol. Chem.*, 1974, **249**, 2397.

9. J. R. Hanson, Natural Products, "The secondary metabolites", 1st Ed. (2003), Royal Society of Chemistry.

10. J. Mann, "Chemical Aspects of Biosynthesis", 1st Ed. (1994), Oxford University Press.

11. J. A. Gundegon, J. S. E. Holker and T. J. Simpson, *J. Chem. Soc., Chem. Commun.*, 1974, 636.

12. R. G. Brereton, M. J. Garson and J. Staunton, *J. Chem. Soc., Perkin I*, 1984, 1027.

13. J. M. Risley and R. L van Etten, *J. Am. Chem. Soc.*, 1980, **102**, 4609.

14. C. Abell and J. Staunton, *J. Chem. Soc., Chem. Commun.*, 1981, 856.

15. L. Sorensen, K. Auclair, J. Kennedy, C. R. Hutchinson and J. C. Vederas, *Org. Biomol. Chem.*, 2003, **1**, 50.

16. G. M. Boersma, T. Y. Dinarieva, W. J. Middelhoven, W. J. H. van Berkel, J. Doran, J. Vervoort and I. M. C. M. Riejtjens, *Appl. Environ. Microbiol.*, 1998, **64**, 1256.

17. F. X. Prenafeta-Boldú, D. M. A. M. Luykx, J. Vervoort and J. A. M. De Bont, *Appl. Environ. Microbiol.*, 2001, **67**, 1030.

18. M. Sanada, T. Miyano, S. Iwadare, J. M. Williamson, B. H. Arison, J. L. Smith, A. W. Douglas, J. M. Leisch and E. Inamine, *J. Antibiot.*, 1986, **39**, 259.

19. M. R. Amin, "Biosynthesis of Fluoroacetate and 4-Fluorothreonine in Streptomyces cattleya", PhD Thesis, University of Durham, 1996.

20. L. Fowden, *Proc. Roy. Soc.*, 1968, **5**, 171.

21. J. F. Siuda and J. F. DeBernardis, *Lloydia*, 1973, **36**, 107.

22. G. W. Gribble, *Chemosphere*, 2003, **52**, 289.

23. J. D. Marwick, P. C. Wright and J. G. Burgess, *Mar. Biotechnol.*, 1999, **1**, 495.

24. P. R Jensen and W. Fenical, *Annu. Rev. Microbiol*, 1994, **48**, 559.

25. A. Qureshi and D. J. Faulkner, *Nat. Prod. Lett.*, 1999, **13**, 59.

26. K. Ballschmiter, *Chemoshpere*, 2003, **52**, 313.

27. K. C. Nicolaou, H. J. Mitchell, N. F. Jain, N. Winssinger, R. Hughes and T. Bando, *Angew. Chem. Int. Ed.*, 1999, **38**, 240.

28. K. C. Nicolaou, N. C. C. Boddy, S. Brase, N. Winssinger, *Angew. Chem. Int. Ed.*, 1999, **38**, 2097.

29. S. Nakanishi, K. Ando, I. Kawamoto, T. Yasuzawa, H. Sano, H. Kase, *J. Antibiot.*, 1989, **42**, 1775.

30. Y. Kan, B. Sakamoto, T. Fujita and H. Nagai, *J. Nat. Prod.*, 2000, **63**, 1599.

31. N. Sitachitta, B. L. Marques, R. T. Williamson, J. Rossi, M. A. Roberts, W. H. Gerwick, V. A. Nguyen and C. L. Wills, *Tetrahedron*, 2000, **56**, 9103.

32. N. Itoh, M. Tsujita, T. Ando, G. Hisatomi and T. Higashi, *Phytochemistry*, 1997, **45**, 67.

33. R. Kazlauskas, P. T. Murphy, R. J. Wells, J. A. Baird-Lmabert and D.D. Jamieson, *Aust. J. Chem*, 1983, **36**, 165.

34. A. Durgrillion and R. Gaetner, *Eur. J. Endocrinol.*, 1995, **132**, 735.

35. P. G. Williams, W. Y. Yoshida, R. E. Moore and V. J. Paul, *Org. Lett.*, 2003, **5**, 4167.

36. F. Borrelli, C. Campagnuolo, R. Capasso, E. Fattorusso and O. Taglialatela-Scafati, *Eur. J. Org. Chem.*, 2004, 3227.

37. K.-H. van Pée, and S. Unversucht, *Chemosphere*, 2003, **52**, 299.

38. P. D. Shaw and L. P. Hager, *J. Am. Chem. Soc.*, 1959, **81**, 6257.

39. L. P. Hager, D. R. Morris, F. S. Brown and H. Eberwein, *J. Biol. Chem.*, 1966, **241**, 2147.

40. S. L. Neidleman and J. Geigert, "Biohalogenation: Principles, Basic Roles and Applications", (1986), Ellis Horwood, Chichester.

41. M. Almeida, S. Filipe, M. Humanes, M. F. Maia, R. Melo, N. Severino, J. A. L. de Silva, J. J. R. F. de Silva and R. Wever, *Phytochemistry*, 2001, 58, 633.

42. A. Butler, *Coord. Chem. Rev.*, 1999, **187**, 17.

43. P. Messerschmidt, L. Prade and R. Wever, *Biol. Chem.*, 1997, **378**, 309.

44. H.-A. Wagneknecht and W.-D. Woggon, *Chem. Biol.*, 1997, **4**, 367.

45. K.–H. van Pée, *Ann. Rev. Microbiol.*, 1996, **50**, 375.

46. H. J. Hecht, H. Sobek, T. Haag, O. Pfeifer and K.-H. van Pée, *Nature Struct. Biol.*, 1994, **1**, 532.

47. M. Picard, J. Gross, E. Lubbert, S. Tolzer, S. Krauss, K. –H. van Pée and A. Berkessel, *Angew. Chem. Int. Ed.*, 1997, **36**, 1196.

48. B. Hoffman, S. Tolzer, I. Pelletier, J. Altenbuchner, K. –H. van Pée and H. J. Hecht, *J. Mol. Biol.*, 1998, **279**, 889.

49. P. E. Hammer, D. S. Hill, S. T. Lam, K. –H. van Pee and M. J. Ligon, *Appl. Eniviron. Microbiol.*, 1997, **63**, 2147.

50. S. Kirner, P. E. Hammer, D. S. Hill, A. Altman I. Fischer, L. J. Weislo, M. Lanahan, K.-H. van Pee and J. M. Ligon, *J. Bacteriol.*, 1998, **180**, 1939.

51. C. Dong, A. Kotzsch, M. Dorward, K. –H. van Pée and J. H. Naismith, *Acta Cryst.*, 2004, D60, 1438.

52. J. Hatung, *Angew. Chem. Int. Ed.*, 1999, **38**, 1209.

53. D. O'Hagan and D.B. Harper, *J. Fluor. Chem.*, 1999, **100**, 127.

54. F. A. Cotton and G. Wilkinson, "Advanced Inorganic Chemistry," 5th Edition (1988), Wiley Interscience.

55. J. S. C. Marias and J. Onderstepoort, *Vet. Sci. Amin. Ind.*, 1943, **18**, 203.

56. J. S. C. Marias and J. Onderstepoort, *Vet. Sci. Amin. Ind.*, 1944, **20**, 67.

57. L. E. Twigg, D. R. King, L. H Bowen, G. R. Wright and C. T. Eason, *Aus. J. Bot.*, 1996, **44**, 411.

58. T. Vartiannen and J. Gynther, *Fd. Chem. Toxic*, 1984, **22**, 307.

59. M. M De Oliveira, *Experientia*, 1963, **19**, 586.

60. D. O'Hagan, R. Perry, J. M. Lock, J. M. Meyer, L. Dasaradhi, J. T. G. Hamilton and D. B. Harper, *Phytochemistry.*, 1993, **33**, 1043.

61. R. A. Peters, R. W. Wakelin, P. Buffa and L. C. Thomas, *Proc. Roy. Soc. B*, 1953, **10**, 497.

62. C. J. Lovelace, G.W. Miller and G.Wilkie, *Atoms. Environ.*, 1968, **2**, 187.

63. M.H. Yu and G.W Miller, *Environ. Sci. Technol.*, 1970, **4**, 492.

64. H. L. Carrell, J. P. Glusker, J. J. Villiafrance, A. S. Mildvan, R. J. Dummel and E. Kun, *Science*, 1970, **170**, 1412.

65. R. Keck, H. Haas and J. Retey, *FEBS Lett.*, 1980, **203**, 65.

66. H. Luable, M.C Kennedy, M.H Emptage, H. Beinert, C.D. Stout, *Proc. Nat. Acad. Sci.* USA, 1996, **93**, 611.

67. E. Kun, E. Kirsten and M. L. Sharma, *Dev. Bioener. Biomemb.*, 1978, **2**, 285.

68. R. A. Peters and M. Shorthouse, *Nature*, 1967, **216**, 80.

69. R. A. Peters and M. Shorthouse, *Nature*, 1971, **231**, 123.

70. R. A. Peters, P. J. Hall, P. F.V. Ward and N. Sheppard, *J. Biochem.*, 1960, **77**, 17.

71. P. F. V. Ward and P. J. Hall and R. A. Peters, *Nature*, 1964, **201**, 611.

72. J. T. G. Hamilton and D. B. Harper, *Phytochemistry.*, 1997, **44**, 1179.

73. D.B. Harper, D. O'Hagan and C.D. Murphy, pp 141-169, "Fluorinated natural products: occurence and biosynthesis. In: The Handbook of Environmental Chemistry, Vol 3. Naturally production of organohalogen compounds" by G.W. Gribble, (2003) Springer-Verlag Berlin.

74. X. H. Xu, G. Yao, Y. Li, J. Lu, C. Lin, X. Wang and C. Kong, *J. Nat. Prod.*, 2003, **66**, 285.

75. D. Robert, P. Edward and H. Charle, *J. Am. Chem. Soc.*, 1957, **79**, 4559.

76. S. Ozaki, Y. Watanabe, T. Hoshiko, H. Mitzuno, K. Ishirawa and H. Mori, *Chem. Pharm. Bull*, 1984, **32**, 733.

77. R. Ignoffo, *J. Am. Health-Syst. Pharm.*, 1999, **56**, 2417.

78. S. O. Thomas, V. L. Singleton, J. A. Lowery, R. W. Sharpe, L. M. Pruess, J. N. Porter, J. H. Mowat and N. Bohonos, *Antibiotics Annu.*, 1957, 716.

79. R. I. Hewitt, A. R. Grumble, L. H. Tayler and W. S. Wallace, *Antibiotics Ann.*, 1956-1957, 722.

80. G. O. Morton, J. E. Lancaster, G. E. Van Lear, W. Fulmor and W. E. Meyer, *J. Am. Chem. Soc.*, 1969, **91**, 1535.

81. D. A. Shuman, R. K. Robins and M. J. Robins, *J. Am. Chem. Soc.*, 1969, **91**, 3391.

82. I. D. Jenkins, J. P. H. Verheyden and J. G. Moffatt, *J. Am. Chem. Soc.*, 1976, **98**, 3346.

83. A. R. Miguire, W. D. Meng, S. M. Roberts and A. J. Willets, *J. Chem. Soc., Perkin Trans. I*, 1993, 1795.

84. Merck & Co. Inc., U.S. Patent 3,950,357, April, 1976.

85. J. S. Kahan, F. M. Kahan, R. Goegelman, S. A. Currie, M. Jackson, E. O. Stapley, T. W. Miller, A. K. Miller, D. Hendlin, S. Mochales, S. Hernandez, H. B. Woodruff and J. Birnbaum, *J. Antibiot.*, 1979, **32**, 1.

86. J. M. Williamson, E. Inamine, K. E. Wilson, A. W. Douglas J. M. Liesch and G. Albers-Schonberg, *J. Biol. Chem.,* 1985, **260**, 8, 4637.

87. T. Tamura, M. Wada, N. Esaki and K. Soda, *J. Bacteriol.*, 1995, **177**, 2265.

88. K. A. Reid, J. T. G Hamilton, R. D. Bowden, D. O'Hagan, L. Dasaradhi, M. R. Amin and D. B. Harper, *Microbiology*, 1995, **141**, 1385.

89. J. J. M. Meyer and D. O'Hagan, *Phytochemistry*, 1992, **31**, 2699.

90. P. Goldman and G.W. A. Milne, *J. Biol. Chem.*, 1966, **241**, 5557.

91. D. B. Harper and E. R. Blakley, *Can. J. Microbiol.*, 1971, **17**, 645.

92. J. T. G. Hamilton, C. D. Murphy, M. R. Amin, D. O'Hagan and D. B. Harper, *J. Chem. Soc., Perkin Trans. I*, 1998, 759.

93. S. J. Moss, C. D. Murphy, J. T. G. Hamilton, W. C. McRoberts, D. O'Hagan, C. Schaffrath and D.B. Harper, *J. Chem. Soc., Chem. Commun.*, 2000, 2281.

94. C. D. Murphy, S. J. Moss and D. O'Hagan, *Appl. Environ. Micobiol*, 2001, **67**, 4919.

95. J. H. Eckfelt and T. Yonetani, *Methods Enzymol.*, 1982, **89**, 474.

96. J. Van der Ploeg, M. P. Smidt, A. S. Landa and D. B. Janssen, *Appl. Environ. Micobiol.*, 1994, **60**, 1599.

97. C. D. Murphy, D. O'Hagan and C. Schaffrath, *Angew. Chem. Int. Ed.*, 2001, **40**, 4479.

98. H. Ogawa, T. Gomi and M. Fujioka, *J. Biochem. Cell. Biol.*, 2000, **32**, 289.

99. H. Yoshida, N. Arai, M. Sugoh, J. Iwabuchi, K. Shiomi, M. Shinose, Y. Tanake and S. Omura, *J. Antibiot.,* 1994, **47**, 1165.

100. H. Lackner, I. Bahner, N. Shigematsu, L. K Pannell and A. B. Mauger, *J. Nat. Prod.*, 2000, **63**, 352.

101. R. J. Mead and W. Segal, *Aust. J. Bio. Sci.,* 1972, **25**, 327.

102. R. J. Mead and W. Segal, *Phytochemistry*, 1973, **12**, 1977.

103. L. S. Leung and P. A. Frey, *Biochem. Biophys. Res. Commun.*, 1978, **81**, 274.

104. J. J. Meyer and D. O'Hagan, *Phytochemistry*, 1992, **31**, 499.

105. D. L. Zechel, S. P. Reid, O. Nashiru, C. Mayer, D. Stoll, D. L. Jakeman, P. A. J. Warren and S. G. Withers, *J. Am. Chem. Soc.*, 2001, **123**, 4350.

106. R. E. London and S. A. Gabel, *Arch. Biochem. Biophys.*, 1996, **334**, 332.

107. C. Schaffrath, "Biosynthesis and Enzymology of Fluorometabolite Production in Streptomyces Cattleya", PhD Thesis, University of St. Andrews, 2002.

108. C. Schaffrath, C. D. Murphy, J. T. G. Hamilton and D. O'Hagan, *J. Chem. Soc., Perkin Trans. I.*, 2001, 3100.

109. W. Jones, L. C. Kurz and R. Wolfenden, *Biochemistry*, 1989, **28**, 1242.

110. K. W. Pankiewicz, *Carbohydr. Res.*, 2000, **327**, 87.

111. C. H. Tann, P. R. Brodfuehrer, S. P. Brundidge, C. Sapino J.r. and H. G. Howell, *J. Org. Chem.*, 1985, **50**, 3644.

112. A. Graul and J. Silvestre, *Drugs Future*, 1999, **23**, 1176.

113. J. He, I. Mikhailopulo and F. Seela, *J. Org. Chem.*, 2003, **68**, 5519.

114. V. Glacon and F. Seela, *Helv. Chim. Acta.*, 2004, **87**, 1239.

115. M. J. Robins, Y. Fourun and R. Mengel, *J. Org. Chem.*, 1974, **39**, 1564.

116. V. E. Marquez, C. Tseng, K. –H. Mitsuya, S. Aoki, J. A. Kelley, H. Ford J.r., J. S. Roth, S. Broder, D. G. Johns and J. S. Driscoll, *J. Med. Chem.*, 1990, **33**, 978.

117. A. M. Kawasaki, M. D. Casper, S. M. Freier, E. A. Lesnik, M. C. Zounes, L. L. Cummins, C. Gonzalez and P.D. Cook, *J. Med. Chem.*, 1993, **36**, 831.

118. J. A. Wright, N. F. Taylor, *Carbohydr. Res.*, 1968, **6**, 347.

119. J. A. Wright, N. F. Taylor and J. J. Fox, *J. Med. Chem.*, 1974, **39**, 1564.

120. U. Reichman, K. A. Watanabe and J. J. Fox, *Carbohydr. Res.*, 1975, **42**, 238.

121. G. Howell, P. R. Brodfeuhrer, S. P. Brundidge, D. A. Benigni and C. Sapino J.r., *J. Org. Chem.*, 1988, **53**, 85.

122. K. W. Pankiewicz, J. Krzeminski, L. A. Ciszewki, W. Y. Ren and K. A. Watanabe, *J. Org. Chem.*, 1992, **57**, 535.

123. J. Krezminski, B. Nawort, K. W. Pankiewicz and K. A. Watanabe, *Nucleosides & Nucleotides*, 1991, **10**, 781.

124. S. Uesugi, H. Niki, M. Ikehara, H. Iwahashi and Y. Kyogoku, *Tetrahedron Lett.*, 1979, 12, 4073.

125. K. W. Pankiewicz, J. Krzeminski and K. A. Watanabe, *J. Org. Chem.*, 1992, **57**, 7315.

126. K. Miyai, R. K. Robins and R. L. Tolman, *J. Med. Chem.*, 1972, **15**, 1092.

127. Y. Morizawa, T. Nakayma. A. Yasuda and K. Uchinda, *Bull. Chem. Soc. Jpn.*, 1989, **62**, 2119.

128. C. Battistini, A. Giordani, A. Ermoli and G. Franceschi, *Synthesis*, 1990, **1990**, 900.

129. G. R. Owen, J. P. H. Verheyden and J. G. Moffatt, *J. Org. Chem.*, 1976, **41**, 3010.

130. K. Biggadlike and A. D. Brorthwick, *J. Chem. Soc., Chem. Commun.*, 1990, 1380.

131. C. O. –Yang, W. Kurz, E. M. Eugui, M. J. McRoberts, J. P. H. Verheyden, L. J. Kurz and K. A. M . Walker, *Tetrahedron Lett.*, 1992, **33**, 41.

132. S. Ajmera, A. R. Bapat, E. Stephenian and P. V. Danenberg, *J. Med. Chem.*, 1988, **31**, 1094.

133. H. M. Kissman and M. J. Weiss, *J. Am. Chem. Soc.*, 1958, **80**, 5559.

134. M. Sharma, Y. X. Li, M. Ledvina and M Bobek, *Nucleosides & Nucleotides*, 1995, **14**, 1831.

135. G. Resnati, *Tetrahedron*, 1993, **49**, 9385.

136. H. Vorbrüggen and C. Ruh-Pohlenz, "Handbook of nucleoside synthesis", 1st Ed (2001), Wiley Inter-Science.

137. T. W. Greene and P. G. M. Wuts, "Protective groups in organic synthesis", 3rd (1999), Wiley Inter-Science.

138. S. B. Ha and V. Nair, *Tetrahedron Lett.*, 1996, **10**, 1567.

139. C. J. Marasco, Jr, D. L. Kramer, J. Miller, C. W. Porter, C. J. Bacchi, D. Rattendi, L. Kucera, N. Iyer, R. Bernacki, P. Pera and J. R. Sufrin, *J. Med. Chem.*, 2002, **45**, 5112.

140. M. Pankaskie, M. Abdel and M. Mahmoud, *J. Med. Chem.*, 1980, **23**, 121.

141. A. A. Minnick and G. L. Kenyon, *J. Org. Chem.*, 1988, **53**, 4952.

142. W. Jahn, *Chem. Ber.*, 1965, **98**, 1705.

143. J. Uzawa and K. Anzai, *Can. J. Chem.*, 1985, **63**, 3537.

144. K. Anzai and K. Matsui, *Bull. Chem. Soc. Jpn.*, 1973, **46**, 618.

145. G. Ceulemans, F. Vandenriessche, J. Rozenski and P. Herdewijn, *Nucleosides & Nucleotides*, 1995, **14**, 117.

146. M. J. Thompson, A. Mekhalfia, D. P. Hornby and G. M. Blackburn, *J. Org. Chem.*, 1999, **64**, 7467.

147. D. Gani and A. W. Johnson, *J. Chem. Soc., Perkin Trans. I.*, 1982, 1197.

148. R. K. Sharma and J. L. Fry, *J. Org. Chem.*, 1983, **48**, 2112.

149. D. P. Cox, J. Terpinski and W. Lawrynowicz, *J. Org. Chem.*, 1984, **49**, 3216.

150. M. –I. Lim, J. D. Moyer, R. L. Cysyk and V. E. Marquez, *J. Med. Chem.*, 1984, **27**, 1536.

151. C. H. Jurgensen, B. E. Huber, T. P. Zimmerman and G. Wolberg, *J. Immunol.*, 1990, **144**, 653.

152. M. M. Mansuri and J. C. Martin, *Ann. Reports Med. Chem.*, 1988, 161.

153. H. Mitsuya and S. Broder, *Nature*, 1987, **325**, 773.

154. Y. –K. Chow, M. S. Hirsch, D. P. Merril, L. J. Betchel, J. J. Eron, J. C. Kaplan and R. T. D'Aquila, *Nature*, 1993, **361**, 650.

155. E. De Clercq, *Anticancer Res*, 1987, 1023.

156. B. Wahren, A. Larsson, U. Ruden, A. Sundqvist and E. Solver, *Antimicrob. Agents Chemother.*, 1987, **31**, 317.

157. V. Nair and T. B. Sells, *Synlett*, 1991, 753.

158. M. R. Almond, J. L. Collins, B. E. Reitter, J. L. Rideout, G. A. Freeman and M. H. St. Clair, *Tetrahedron Lett.*, 1991, **32**, 5745.

159. R. Vince and J. Brownwell, *Biochem. Biophys. Res. Commun.*, 1990, **168**, 912.

160. P. Ciuffreda, S. Casti and E. Santaniello, *Tetrahedron,* 2000, **56**, 3239.

161. A. L. Margolin, D. R. Borcherding, D. W.-K. Dominique and N. Margolin, *J. Org. Chem.*, 1994, **59**, 7214.

162. M. R. Amin, D. B. Harper, J. Moloney, C. D. Murphy, J. A. K. Howard and D. O'Hagan, *J. Chem. Soc. Chem. Commun.*, 1997, 1471.

163. R. von Naef and D. Seebach, *Helv. Chim. Acta*, 1985, **68**, 135.

164. R. Fitzi and D. Seebach, *Tetrahedron*, 1988, **44**, 5277.

165. H. L. Schubert, R. M. Blumenthal and X. D. Cheng, *Trends in Biochemical Sciences*, 2003, **28**, 329.

166. M. Fontecave, M. Atta and E. Mulliez, *Trends in Biochemical Sciences*, 2004, **29**, 243.

167. G. L. Cantoni, *J. Biol. Chem.*, 1953, **204**, 403.

168. G.L. Cantoni, *Annu. Rev. Biochem.*, 1975, **44**, 435.

169. J. Komoto, T. Yamada, Y. Takata, G. D. Marham and F. Takusagawa, *Biochemistry*, 2004, **43**, 1821.

170. R. J. Parry and A. Minta, *J. Am. Chem. Soc.*, 1982, **104**, 871.

171. G. D. Marham, D. W. Parkin, F. Mentch and V. L. Schramm, *J. Biol. Chem.*, 1987, **262**, 5609.

172. A. Jeltsch, *Chem. BioChem.*, 2002, **3**, 274.

173. N. Ohsawa, *Biosci. Biotechnol. Biochem.*, 2001, **65**, 2397.

174. J. M. Attieh, *J. Biol. Chem.*, 1995, **65**, 9250.

175. R. W. Woodward, M. D. Tsai, H. G. Floss, P. A. Crooks, and J. K. Coward, *J. Biol. Chem.*, 1980, **255**, 9124.

176. A. L. Pogolotti, A. Ono, R. Subramaniam and D. V. Santi, *J. Biol. Chem.*, 1988, **263**, 7461

177. D. V. Santi, A. Norment and C. E. Garrett, *Proc. Natl. Acd. Sci.* USA. 1984, **81**, 6993.

178. J. C. Wu and D. V. Santi, *J. Biol. Chem.*, 1987, **262**, 4778.

179. L. Chen, A. MacMillan and G. L. Verdine, *J. Am. Chem. Soc.*, 1993, **115**, 5318.

180. M. O'Gara, S. Klimasauskas, R. J. Roberts and X. Cheng, *J. Mol. Biol.*, 1996, **261**, 634.

181. G. L. Stoner and M. A. Eisenberg, *J. Biol. Chem.*, 1975, **250**, 4029.

182. H. **Kack**, K. J. Gibson, A. A. Gatenby, G. Schneider and Y. Lindovist, *Acta. Crystallogr. D.*, 1999, 55, 610.

183. R. A. John, *Biochem. Biophys. Acta.,* 1995, **1248**, 81.

184. M. Jakubowicz, *Acta. Biochimica. Polonica*, 2002, **49**, 757.

185. H. Dejima, M. Kobayashi, H. Takasaki, N. Takeda, A. Shirahata and K. Samejima, *Biol. Pharm. Bull.*, 2003, **26**, 1005.

186. W. D. Tolbert, J. L. Ekstrom, I. I. Mathews, J. A. Secrist III, P. Kappor, A. E. Pegg and S. E. Ealick, *Biochemistry*, 2001, **40**, 9484.

187. J. L. Ekstrom, W. D. Tolbert, H. Xiong, A. E. Pegg and S. E. Ealick, *Biochemistry*, 2001, **40**, 9495.

188. C. W. Tabor and H. Tabor, *Ann. Rev. Biochem.*, 1976, **45**, 285.

189. K. Samejima and Y. Nakazawa, *Arch. Biochem. Biophys.*, 1980, **210**, 241.

190. G. R. Bjork, J. U. Ericson, C. E. Gustafsson, T. G. Hagervall, Y. H. Jonsson and P. M. Wikstrom, *Annu. Rev. Biochem.*, 1987, **56**, 263.

191. C. Grimm, G. Klebe, R. Ficner and K. Reuter, *Acta. Crystallogr.*, 2000, 56, 484.

192. D. Iwata-Reuyl, *Bioorg. Chem.*, 2003, **31**, 24.

193. S. G. Van Lanen and D. Iwata-Reuyl D, *Biochemistry,* 2003, **42**, 5312.

194. S. G. Van Lanen, S. D. Kinzie, S. Matthieu, T. Link, J. Culp and D. Iwata-Reuyl, *J. Biol. Chem.*, 2003, **278**, 10491.

195. S. Daoud Kinzie, B. Thern and D. Iwata-Reuyl, *Org. Lett.*, 2000, **2**, 1307.

196. F. Berkovitch, Y. Nicolet, J. T. Wan, J. T. Jarrett and C. L. Drennan, *Science*, 2004, **303**, 76.

197. P. A. Frey and S. J. Booker, *Adv. Protein Chem.*, 2001, **58**, 1.

198. J. Cheek and J. B. Broderick, *J. Biol. Inorg. Chem.*, 2001, **6**, 209.

199. G. Layer, D. W. Heinz, D. Jahn and W. –D Schubert, *Curr. Opin. Chem. Biol.*, 2004, **8**, 468.

200. T. Jarrett, *Curr. Opin. Chem. Biol.*, 2003, **7**, 174.

201. B. T. Sum Bui, D. Florentin, F. Fournier, O. Ploux, A. Méjean and A. Marquet, *FEBS Lett.*, 1998, **440**, 226

202. N. B. Ugulava, C. J. Sacanell and J. T. Jarrett, *Biochemistry*, 2001, **40**, 8352.

203. S. Ollagnier de Choudens, E. Mulliez and M. Fontecave, *FEBS Lett.*, 2002, **532**, 465.

204. A. De Meijere, *Chem. Rev.*, 2003, **103**, 931.

205. H. –W. Liu and C. T. Walsh p 959 in "The Chemistry of the Cyclopropyl Group" by Z. Rappoport, (1987), J. Wiley and Sons.

206. C. Guerard, M. Breard, F. Courtois, T. Drujon and O. Ploux, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 1661.

207. F. R. Talyor and J. E. Cronan Jr., *Biochemistry*, 1979, **18**, 3292.

208. E. Q. Lederer, *Rev.*, 1969, **23**, 453.

209. C. C. Huang, C. V. Smith, M. S. Glickman, W. R. Jacobs Jr. and J. C. Sacchettini, *J. Biol. Chem.*, 2002, **277**, 11559.

210. P. H. Buist and D. B. Maclean, *Can. J. Chem.*, 1981, **60**, 371.

211. M. S. Glickman, J. S. Cox and W. R. Jacobs Jr., *Mol. Cell.*, 2000, **5**, 717.

212. C. Schaffrath, H. Deng and D. O'Hagan, *FEBS. Lett.*, 2003, **547**, 111.

213. C. Dong, F. Huang, H. Deng, C. Schaffrath, J. B. Spencer, D. O'Hagan and J. H. Naismith, *Nature*, 2004, **427**, 561.

214. D. A. Dixon and B. E. Smart, *J. Phys. Chem.*, 1991, **95**, 1602.

215. D. O'Hagan and R. J. M. Goss, A. Meddour and J. Courtieu, *J. Am. Chem. Soc.*, 2003, **125**, 379.

216. C.D. Cadicamo, J. Courtieu, H. Deng and A. Meddour and D. O'Hagan, *Chem. Biochem.*, 2004, **5**, 685.

217. D. B. Harper and D. O'Hagan, *Nat. Prod. Rep.*, 1994, **11**, 123.

218. P. Goldman, *J. Biol. Chem.*, 1965, **240**, 3434.

219. P. Goldman and G. W. A. Milne, *J. Biol. Chem.*, 1965, **241**, 5557.

220. K. A. Francesconi, J. S. Ednmonds and R. V. Stick, *J. Chem. Soc., Perkin Trans I*, 1992, 1349.

221. M. J. Robins, F. Hansske, S. F. Wunk and T. Kanai, *Can. J. Chem.*, 1991, **69**, 1468.

222. K. Samejima, Y. Nakazawa and I. Matsunaga, *Chem. Pharm. Bull.*, 1978, **26**, 1480.

223. K. Ramalingam and R. W. Woodward, *J. Org. Chem.*, 1984, **49**, 1291.

224. R. T. Borchardt, J. A. Huber and Y. S. Wu, *J. Org. Chem.*, 1976, **41**, 565.

225. M. J. Robins, J. S. Wilson, D. Madej, N. H. Low, F. Hansske and S. F. Wnuk, *J. Org. Chem.*, 1995, **60**, 7902.

226. M. J. Robins, E. Lewandowska and S. J. Wnuk, *J. Org. Chem.*, 1998, **63**, 7375.

227. J. Fuentes, M. Angulo and M. A. Prasera, *J. Org. Chem.*, 2002, **67**, 2577.

228. J. Plavec, C. Thibaudeau and J. Chattopadhyaya, *J. Am. Chem. Soc.*, 1994, **116**, 6558.

229. I. Luyten, C. Thibaudeau and J. Chattopadhyaya, *J. Org. Chem.*, 1997, **62**, 8800.

230. K. L. Brown and X. Zou, *J. Am. Chem. Soc.*, 1992, **114**, 9643.

231. T. Tsuji and K. Takenaka, *Nucleosides & Nucleotides*, 1987, **6**, 575.

232. S. S. Chen, J. H. Walgate and J. A. Duerre, *Arch. Biochem. Biophys.*, 1971, **146**, 51.

233. F. B. Tutwiler and R. L. McKee, *J. Am. Chem. Soc.*, 1954, **76**, 6342.

234. E. W. Taylor, P. van Roey, R. F. Schinazi and C. K. Chu, *Antiviralchem. Chemother.*, 1990, **1**, 163.

235. C. Thibaudeau, A. Kumar, S. Bekiroglu, A. Matsuda, V. E. Marquez and Chattopadhyaya, *J. Org. Chem.*, 1998, **63**, 5447.

236. T. Kusaka, H. Yamamoto, M. Shibata, M. Muroi, T. Kishi and K. J. Mizuno, *J. Antibiot.*, 1968, **21**, 255.

237. V.P Rajappan and S. W. Schneller, *Bioorg. Med. Chem.*, 2003, **11**, 5199.

238. M. Cowart, M. J. Bennett and J. F. Kerwin Jr., *J. Org. Chem.*, 1999, **64**, 2240.

239. B. M. Trost, R. Madsen, S. D. Guile and B. Brown, *J. Am. Chem. Soc.*, 2000, **122**, 5947.

240. F. Burlina, A. Favre, J. –L. Fourrey and M. Thomas, *Bioorg. Med. Chem. Lett.*, 1997, **7**, 247

241. D. R. Borcherding, S. A. Scholtz and R. T. Borchardt, *J. Org. Chem.*, 1987, **52**, 5457.

242. M. S. Wolfe, B. L. Andreson, D. R. Borherding and R. T. Borchardt, *J. Org. Chem.*, 1990, **55**, 4712.

243. Y. H. Jin, P. Liu, J. Wang, R. Baker, J. Higgins and C. K. Chu, *J. Org. Chem.*, 2003, **68**, 9012.

244. D. Beer, R. Meuwly and A. Vasella, *Helv. Chim. Acta.*, 1982, **65**, 2570

245. D. Lui and C. A. Caperelli, *Synthesis*, 1991, 933.

246. K. Mori and H. Kikuchi, *Liebigs Ann. Chem.*, 1989, 1267.

247. T. Hudlicky, M. G. Natchus, and T. C. Nugent, *Synth. Commun.*, 1992, **22**, 151.

248. C. L. Mariën, E. L. Esmans, F. Lemière and R. A. Dommisse, *Synth. Commun.*, 1997, **27**, 205.

249. M. S. Wolfe, Y. Lee, W. J. Bartlett, D. R. Borcherding and R. T. Borchardt., *J. Med. Chem.*, 1992, **35**, 1782.

250. W. Versees and J. Steyaert, *Curr. Opin. Struc. Bio.*, 2003, **13**, 731.

251. A. Fedorov, W. Shi, G. Kicska, E. Fedorov, P. C. Tyler, R. H. Furneaux, J. C. Hanson, G. Gainsford, J. Z. Larese, V. L. Schramm and S. C. Almo, *Biochemistry*, 2001, **40**, 853.

252. M. D. Erion, K. Takabayashi, H. B. Smith, J. Kessi, S. Wagner, S. Hönger, S. L. Shames and S. E. Ealick, *Biochemistry*, 1997, **36**, 11725.

253. M. J. Pugmire and Steve. E. Ealick, *Biochem. J.*, 2002, **361**, 1.

254. M. D. Erion, J. D. Stoeckler, W. C. Guida, R. L. Walter and S. E. Ealick, *Biochemistry*, 1997, **36**, 11735.

255. H. G. Willams-Ashman, J. Seidenfeld and P. Galletti, *Biochem. Pharmacol.*, 1982, **31**, 277.

256. K. Fabianowska-Majewska, J. Dudley and L. Fairbanks, *Acta. Biochimica. Polonica.*, 1994, **41**, 391.

257. G. B. Evans, R. H. Furneuax, V. L. Schramm, V. Singh and P. C. Tyler, *J. Med. Chem.*, 2004, **47**, 3275.

258. A. K. Sinhababu, R. L. Bartel, N. Pochopin and R. T. Borchardt, *J. Am. Chem. Soc.*, 1985, **107**, 7628.

259. J. A. Duerre, *J. Biol. Chem.*, 1962, **237**, 3737.

260. A. G. Barrett and S. Lebold, *J. Org. Chem.*, 1990, **55**, 3853.

261. N. J. Leonard and K. L. Carraway, J. Heterocyclic chemistry, 1966, **3**, 485.

262. A. P. Rauter, F. Ramoa-Ribeiro, A. C. Fernandes and J. A. Figueiredo, *Tetrahedron*, 1995, **51**, 6529.

263. C. A. Wartchow, P. Wang, M. D. Bednarski and M. R. Callstrom, *J. Org. Chem.*, 1995, **60**, 2216.

264. F. Sarabia-Garcia and F. Jorge Lopez-Herrera, *Tetrahedron*, 1996, **52**, 4757.

265. Q. Zhang and H.-W. Liu, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 145.

266. B. Curti, V. Massey and M. Zmudka, *J. Biol. Chem.,* 1968, **243**, 2306.

267. P. Herdewijn, *Tetrahedron*, 1989, **45**, 6563

268. W. Thomas and F. Seela, *Helv. Chim. Acta.*, 1996, **79**, 169.

269. P. Ciuffreda, S. Casati and E. Santaniello, *Tetrahedron*, 2000, **56**, 3239.

270. R. L. Miller, C. L. Sabourin and T. A. Krenitsky, *Biochem. Pharmacol.* 1987, **36**, 553.

271. D. Toorchen and R. L. Miller, *Biochem. Pharmacol.* 1991, **41**, 2023.

272. L. Y. Ghoda, T. M. Savarese, C. H. Northup, R. E. Parks Jr., J. Garofalo, B. B. Ellenbogen and C. J. Bacchi, *Mol. Biochem. Parasitology.*, 1988, **27**, 109.

273. H. M. Kalckar, *J. Bio. Chem.*, 1947, **167**, 461.

274. W. J. Hennen and C. –H. Wong, *J. Org. Chem.*, 1989, **54**, 4692.

275. H. Komatsu and T. Araki, *Tetrahedron Lett.*, 2003, **44**, 2899.

276. J. A. Montgomery, A. T. Sortnacy-Fowler, S. D. Clayton, J. M. Riordan and J. A. Secrist III, *J. Med. Chem.*, 1992, **35**, 397.

277. A. Giner-Sorolla and J. H. Burchenal, *J. Med. Chem.*, 1971, **14**, 816.

278. Z. Janeba, P. Francom and M. J. Robins, *J. Org. Chem.*, 2003, **68**, 989.

279. A. Bzowska and Z. Kazimierczuk, *Euro. J. Biochem.*, 1995, **233**, 886.

280. E. S. Furfine and R. H. Abeles, *J. Biol. Chem.*, 1987, **263**, 9598.

281. J. W. Wray and R. H. Abeles, *J. Biol. Chem.*, 1994, **270**, 3147.

282. W. –D. Fessener and V. Helaine, *Curr. Opin. Chem. Biol.*, 2001, **12**, 574.

283. R. Schoevaart, F. van Rantwijk and R. A. Sheldon, *J. Chem. Soc., Chem. Commun.*, 1999, 2465.

284. R. Schoevaar, "Applications of aldolases in organic synthesis", PhD Thesis, Technische Unversiteit Delft, 2000.

285. R. Schoevaart, F. van Rantwijk and R. A. Sheldon, *J. Org. Chem.*, 2000, **65**, 6940.

286. M. D. Bednarski, E. S. Simon, N. Bischofberger, W. –D. Fessener, M. –J. Kim, W. Lees, T. Saito, H. Waldmann and G. M. Whitesides, *J. Am. Chem. Soc.*, 1989, **111**, 627.

287. M. Ebner and A. E. Stütz, *Carbohydr. Res.*, 1998, **305**, 331.

288. W. –D. Fessener and G. Sinerius, *Angew. Chem. Int. Ed.*, 1991, **30**, 555.

289. V. M. Clark, A. R. Todd and J. Zussman, *J. Chem. Soc.*, 1951, 2952.

290. R. Fitzi and D. Seebach, *Angew. Chem. Int. Ed.*, 1986, **25**, 345.

291. J. V. Davisson, D. R. Davis, V. M. Dixit and D. C. Poulter, *J. Org. Chem.*, 1987, **9**, 1794.

292. G. Kowollik, K. Gaertner, G. Etzold and P. Langen., *Carbohydr. Res.*, 1970, **12**, 301.

293. L. M. Beachham, *J. Org. Chem.*, 1979, **44**, 3100.

294. H. Schirmeister-Tichy, K. T. Iacono, N. F. Muto, J. W. Homan, R. J. Suhadolnik and W. Pfleiderer, *Helv. Chim. Acta.*, 1999, **82**, 597.

295. K. Kikugawa, *Chem. Pharm. Bull.*, 1977, **25**, 2181.

296. M. Ikehara, *Chem. Pharm. Bull.*, 1979, **27**, 2647.