SYNTHESIS AND EVALUATION OF CARBOCYCLIC NICOTINAMIDE COFACTOR ANALOGUES

Thesis submitted for the Degree of

Doctor of Philosophy

by

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TITLE: **Synthesis and Evaluation of Carbocyclic Nicotinamide Cofactor Analogues**

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ABSTRACT

The history of the discovery and structure elucidation of many important nucleotides and coenzymes is reviewed in chapter 1. Some recent developments in nucleotide chemistry are also discussed including the discovery and biological importance of cyclic ADP ribose and carbocyclic nucleosides such as Aristeromycin and Neplanocin A.

In chapter 2, the preparation of a carbocyclic analogue of NAD^+ (figure1) is reported. This analogue is synthesised both as a mixture of diastereoisomers which are separated by HPLC and as a single diastereoisomer via the homochiral synthesis.

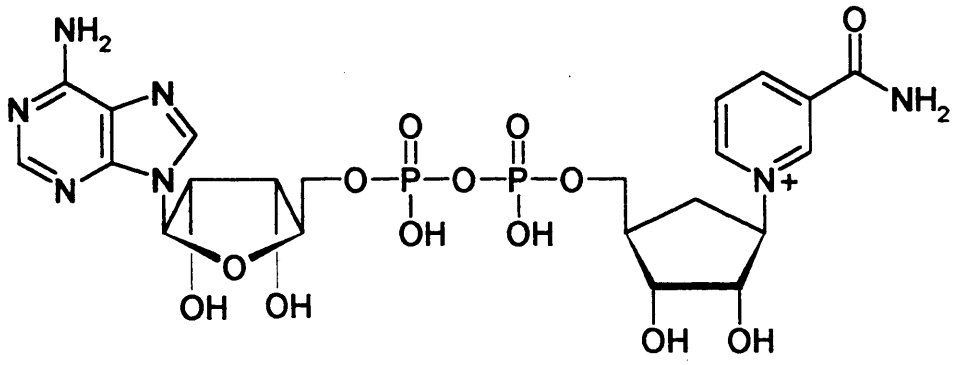

Figure 1

In chapter 3, the kinetic parameters of carbocyclic NAD^+ with yeast alcohol dehydrogenase and glycerol-3-phosphate dehydrogenase are established. Carbocyclic NAD^+ is observed to be a good substrate for both of these enzymes. NMR and molecular modelling studies provide evidence that carbocyclic NAD^+ can adopt the same conformation as NAD^+ without causing any unfavourable interactions. The kinetic isotope effects of carbocyclic NAD^+ and NAD^+ with CH_3CD_2OH and yeast alcohol dehydrogenase were measured and compared. They both were found to have a primary isotope effect of about 2 which is consistent with a C-H bond being broken in the rate limiting step. Carbocyclic NAD^+ was used to probe the stereoselectivity of dehydrogenases, the class A (yeast alcohol dehydrogenase) and class B (glycerol-3-phosphate dehydrogenase) enzymes exhibited the same stereoselectivty with carbocyclic NAD^+ as with NAD^+. The mechanistic consequences of these observations are discussed.

The design, synthesis and characterisation of the first compound on the proposed route to a carbocyclic analogue of cyclic ADP ribose is reported in chapter 4. The importance of cyclic ADP ribose and its analogues are discussed.

STATEMENT

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled "Synthesis and Evaluation of Carbocyclic Nicotinamide Cofactor Analogues" is based on work in the Department of Chemistry at the University of Leicester during the period between October 1993 and September 1996.

The work has not been, and is not concurrently being presented for any other degree.

Signed: A. McGarrigle Date: 22 Sep 97

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ABBREVIATIONS AND SYMBOLS

General and Physical:

br	-	Broad
cm^{-1}	-	Wavenumber
CI	-	Chemical ionisation
δ	-	Chemical shift
d	-	Doublet
EI	-	Electron ionisation
Enz/E	-	Enzyme
FAB	-	Fast atom bombardment
IR	-	Infrared
J	-	Coupling constant
k_{cat}	-	Catalytic constant
K_{eq}	-	Equilibrium constant
K_M	-	Michaelis constant
nmr	-	Nuclear magnetic resonance
ppm	-	Parts per million
q	-	Quartet
s	-	Singlet
S	-	Substrate
t	-	Triplet
tlc	-	Thin layer chromatography
UV	-	Ultraviolet
V_{max}	-	Maximum rate of catalysis

ABBREVIATIONS AND SYMBOLS CONTINUED

Chemical:

Ac	-	Acetate
AMP	-	Adenosine 5'- monophosphate
ADP	-	Adenosine 5'- diphosphate
ATP	-	Adenosine 5'- triphosphate
ADPR	-	Adenosine 5'- diphosphate ribose
cADPR	-	Cyclic adenosine 5'- diphosphate ribose
carba NAD^+	-	Carbocyclic nicotinamide adenine dinucleotide
carba NADH	-	Carbocyclic nicotinamide adenine dinucleotide (reduced form)
DCC	-	Dicyclohexyl carbodiimide
EDTA	-	Ethylene diamine tetracetic acid
Et	-	Ethyl
GPDH	-	Glycerol-3-phosphate dehydrogenase
LADH	-	Liver alcohol dehydrogenase
Me	-	Methyl
NAD^+	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Nicotinamide adenine dinucleotide phosphate (reduced form)
NMO	-	N-methyl morpholine N-oxide
PP_i	-	Inorganic pyrophosphate
TEAB	-	Triethylammonium bicarbonate
THF	-	Tetrahydrofuran
TMS	-	Trimethylsilane
TRIS	-	Tris (hydroxymethyl) amino methane
TSP	-	3- (trimethylsilyl)- propane sulphonic acid sodium salt
YADH	-	Yeast alcohol dehydrogenase

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CHAPTER 1

GENERAL INTRODUCTION

Chapter 1

General Introduction

1.0 Introduction

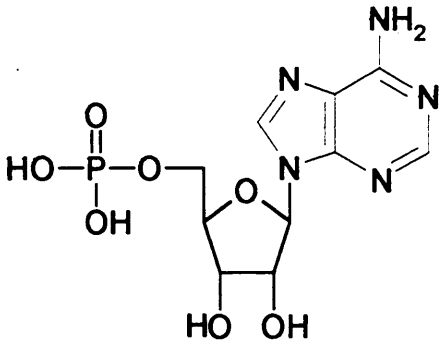
Nucleosides and nucleotides play vital roles in a wide range of metabolic processes in all biological systems. They are the building blocks of both RNA and DNA, as well as featuring predominantly in many coenzymes. Nucleosides are comprised of a heterocyclic base (purine or pyrimidine) linked to the glycosidic position of a sugar (usually D-ribose or 2-deoxy-D-ribose). Nucleotides are the corresponding phosphate esters formed with the sugar hydroxyl groups. These structural units are found in many cofactors e.g. coenzyme A (20), NAD^+ (9), $NADP^+$ (10), FAD (18), ADP (15), ATP (16) and cyclic ADP ribose (23). Cofactors facilitate a wide range of metabolic reactions including group transfer reactions, redox reactions and cyclisation reactions. Nucleotides have also been implicated in signalling as chemical messengers e.g. cAMP (7) and the recently described cyclic ADP ribose (23) which is a second messenger of calcium mobilisation.

1.1 History of Nucleotides and Coenzymes

1.1.1 Historical Discovery of Nucleotides

The term nucleotide was introduced in 1908 by Levene and Mandel[1] to denote the products isolated from acidic digests of thymus nucleic acid. When nucleotides were first discovered the source material was often quoted in the name e.g. "muscle adenylic acid" and "yeast adenylic acid" to describe two isomers of the nucleotide. These names are seldom used now, the nucleotides are now named systematically by quoting the position of the phosphate residue e.g. adenosine-5' phosphate (AMP, 1).

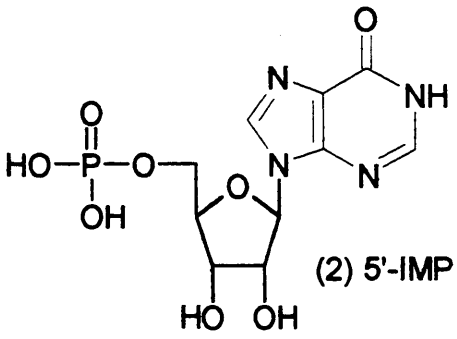
Inosinic acid (IMP, 2) was the first mononucleotide to be discovered, it was obtained from beef muscle extracts by Liebig in 1847.[2] This was almost a quarter of a century before Miescher isolated nucleic acids.[3] Liebig did not detect the presence of a phosphorus in inosinic acid, and the correct structure was not discovered until 1911 when Levene and Jacobs proved that it was the 5' phosphate ester of hypoxanthine ribose.[4]



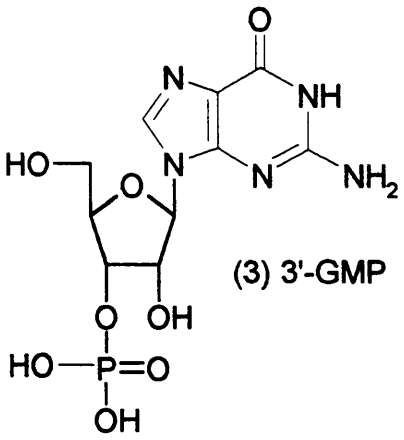
(1)

Inosinic acid is not a common nucleotide and it was formed during Liebigs isolation by the deamination of adenosine 5' monophosphate (AMP, 1). However, the isolation of AMP (1) itself was not achieved until 1927 when it was isolated from muscle. Since then all of the common nucleotides of adenine, cytosine, guanine, thymine and uracil (figure1.1) have been isolated mainly by direct extraction of tissue or organism.[5,6,7,8,9,10] Other minor nucleotides such as methylated adenosines or guanosines have also been isolated. However, the main source of nucleotides are the nucleic acids, which can be degraded to nucleosides and nucleotides by either chemical[11,12] or enzymic methods.[13] A lot of work was done during the first half of this century by Levene and others[14] both to establish the relationship between nucleotides and nucleic acids and to establish the structures of the nucleotides. Hydrolysis of the nucleotides gave the free heterocyclic base and sugar components, these were quickly recognised and the only remaining problem with the structure elucidation was the location of the phosphoryl residue on the sugar.[15] This was determined relatively easily for the 5'-nucleotides and the deoxynucleoside 3'-phosphates.

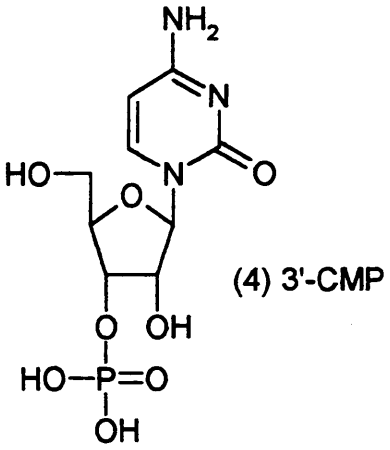
General methods used for the identification of 5'-phosphates included the use of nucleotidases that are specific for nucleoside 5'-phosphates,[16] the formation of boric acid complexes (ribose series only),[17] comparison with synthetic nucleotides and the presence of an unsubstituted 2',3'-*cis* glycol structure by periodate oxidation.[18,19] The behaviour of deoxynucleoside-5' phosphates on ion exchange chromatography compared with the ribonucleotides was also of value.[20] The position of the ester linkage in all of the major nucleoside-5' phosphates and many minor nucleotides was established by the application of the above techniques. X-ray structures have been provided for calcium thymidine-5' phosphate[21] and adenosine-5' phosphate.[22]



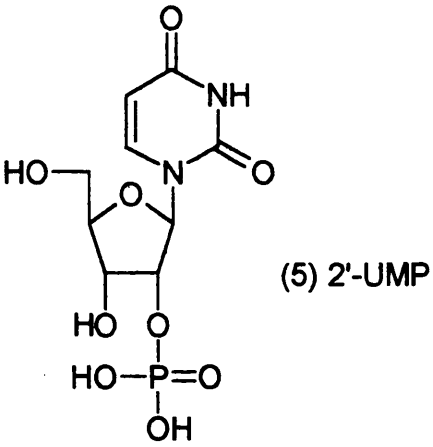
(2) 5'-IMP



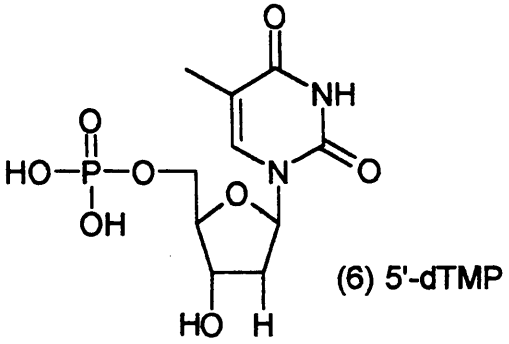
(3) 3'-GMP



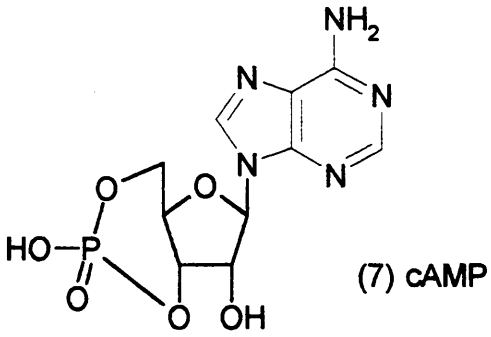
(4) 3'-CMP



(5) 2'-UMP



(6) 5'-dTMP

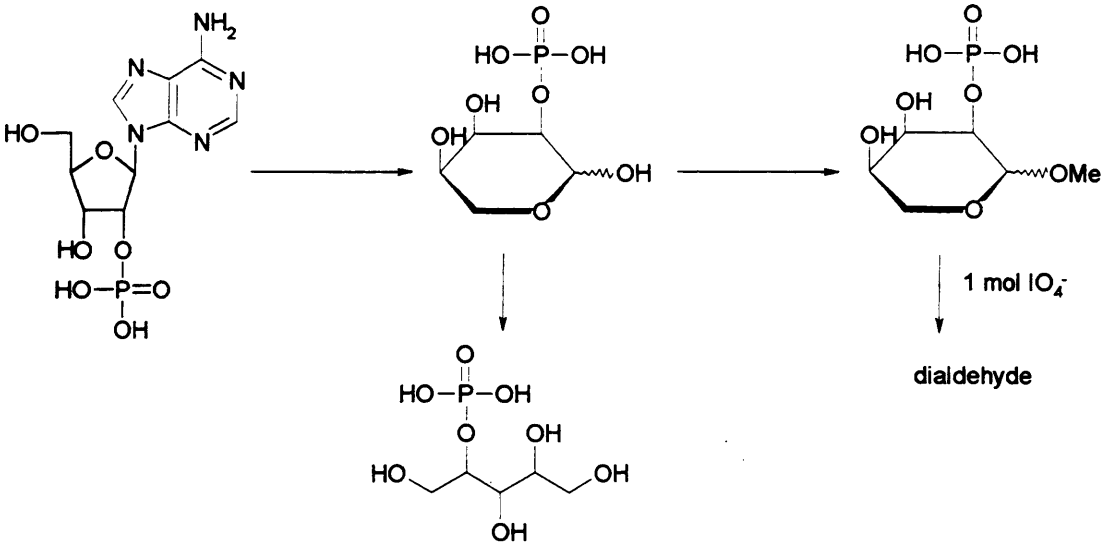


(7) cAMP

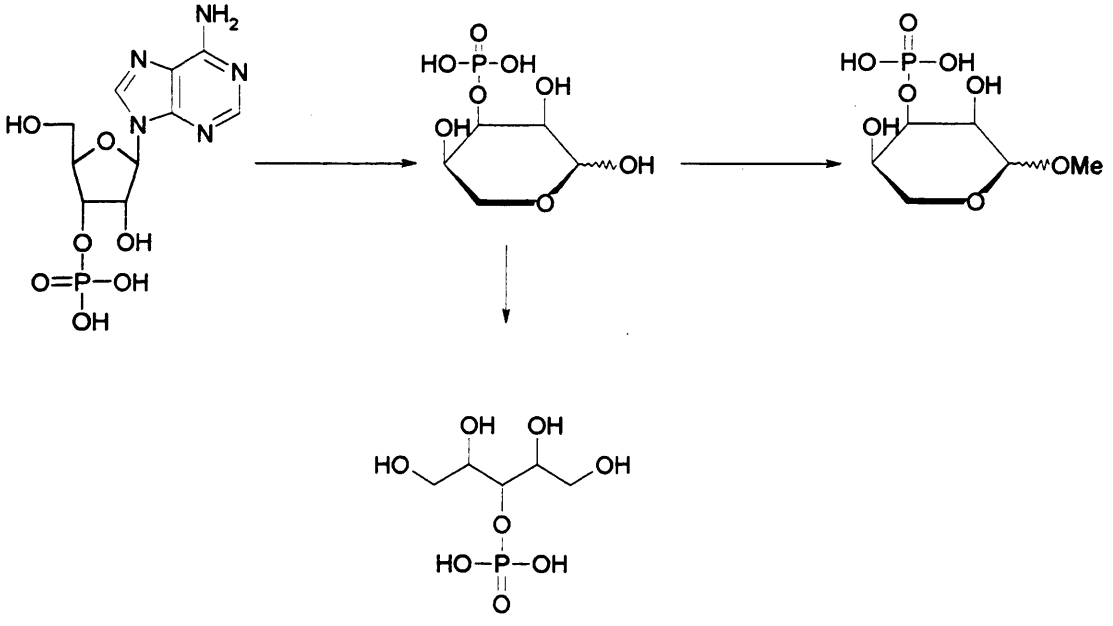
Figure 1.1

The position of the phosphate residue in ribonucleoside 2'- and 3' phosphates were more difficult to determine because of the interconversion between the two positions. Most of the early work by Levene and co-workers is ambiguous, possibly because the nucleotides used were mixtures of the 2' and 3' isomers. In 1949 Cohn isolated two isomeric adenylic acids from the hydrolysate of yeast RNA, by the application of ion exchange chromatography.[23,24] Similar pairs of isomeric nucleotides derived from the other nucleosides were subsequently identified.[25,26,27] The less acidic isomers (with respect to ion exchange chromatography) were

termed "a" and the other "b", the isomers were rapidly interconverted to an equilibrium under acidic conditions,[28,29] but were stable in alkali. The two a and b isomers of adenylic acid were both dephosphorylated to adenosine[30] and both were stable to periodate oxidation,[31] and both isomers gave the same nucleoside cyclic phosphate[32] that on acidic or alkaline hydrolysis gave a mixture of the a and b phosphates.[32,33] Enzymic dephosphorylation of the nucleotides by a specific b nucleotidase suggested that the b isomers were all structurally similar, and this in turn suggested that the a isomers also formed a distinct series.[34] At this point it was clear that the isomers were the 2' and 3' phosphates, although further identification was not yet possible.



Ribitol-2 phosphate (optically active)



Ribitol-3 phosphate (optically inactive)

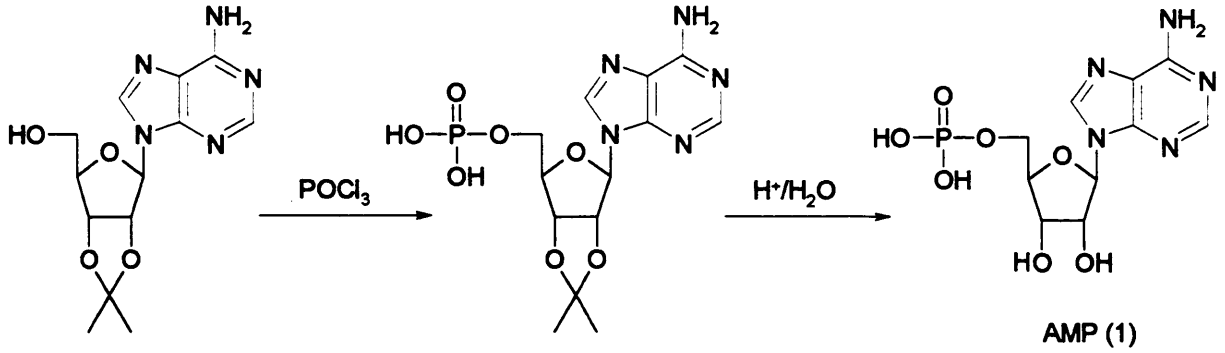
Scheme 1.1

The a isomers of adenylic and guanylic acids gave a greater proportion of ribose-2 phosphate when they were treated with a sulphonic acid resin (acidic form) followed by ion exchange. Similarly the b isomers gave a greater proportion of ribose-3 phosphate. Upon reduction the ribose-3 phosphate gave optically inactive ribitol-3 phosphate whereas ribose-2 phosphate gave optically active ribitol-2 phosphate. The structure of the ribose phosphates was confirmed by periodate oxidation of the derived methyl glycosides (scheme 1.1).[35,36] The assignment of a adenylic acid as adenosine-2' phosphate and b adenylic acid as adenosine-3' phosphate was confirmed by the unambiguous synthesis of adenosine-2' phosphate.[37]

The 2' and 3' phosphate esters of ribonucleosides interconvert via a cyclic 2',3'-phosphate (figure 1.2). These cyclic phosphates were very important in the elucidation of the mechanism of phosphate migration and of the chemical and enzymic degradation of ribonucleic acid.[33] The cyclic phosphates in a five membered ring were shown to be more alkali labile than those in a six or seven membered ring by a factor of 10^6.[38,39] Ring strain is probably the reason for this and the existence of ring strain in cyclic phosphates with five membered rings has been demonstrated thermochemically.[40] Cyclic nucleoside-3',5' phosphates have also been isolated from natural sources even though they have to adopt an unfavourable *trans* configuration.[42,43]

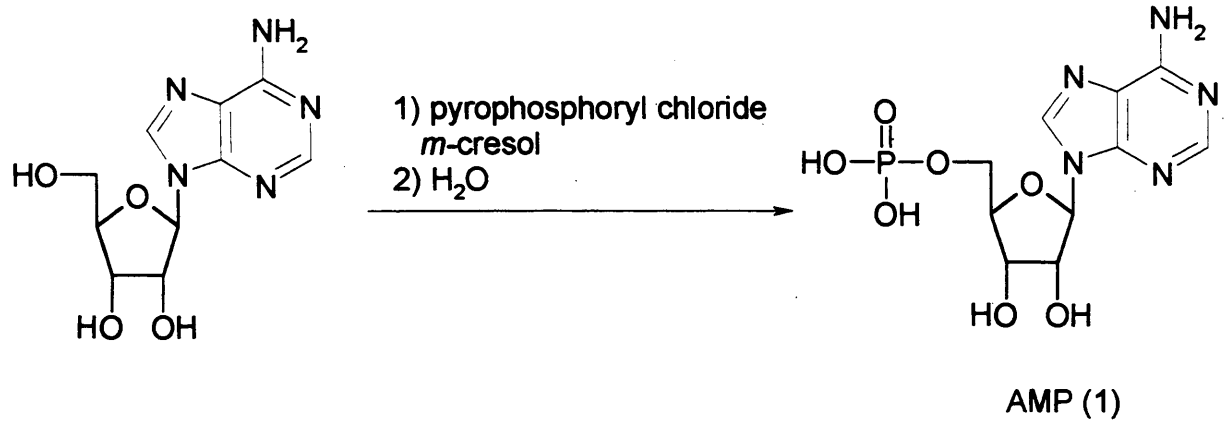
1.1.2 Chemical Synthesis of Nucleotides

Emil Fischer produced the first synthetic nucleotide in 1914 by phosphorylation of glucosyltheophylline with phosphoryl chloride in aqueous barium hydroxide.[45] The nucleotide was obtained as a crystalline solid that on hydrolysis gave a glucose phosphate, the precise location of the phosphate was unknown. Levene and Tipson used the same reagent for the synthesis of nucleotides from naturally occurring nucleosides. Adenosine-5' phosphate (1) was synthesised in low yield from 2',3'-O-isopropylidene adenosine and phosphorous oxychloride (scheme 1.2).[46] The 5' phosphates of inosine[47] and uridine[48] were also prepared in this manner, however all of the yields were low. Phosphoryl chloride is a very reactive phosphorylating agent but as it contains three labile phosphorous-chloride bonds several products are obtained during phosphorylation reactions and hence low yields are obtained. This problem was overcome by the use of dialkyl (or diaryl) phosphorochloridates such as dibenzyl[49] and diphenyl[50] phosphorochloridates. Direct phosphorylation of unprotected nucleosides with phosphoryl chloride in pyridine gave all three monoesters (2',3' or 5'),[51,52,53]



Scheme 1.2

yet again the yields were low. Unprotected ribonucleosides can be phosphorylated directly at the 5' position using several reagents such as phosphoryl chloride in wet trialkyl phosphates,[54] and pyrophosphoryl chloride in *m*-cresol (scheme 1.3).[55] Deoxyribonucleoside-3' phosphates were prepared by the phosphorylation of the nucleoside protected in the 5' position by a trityl or similar group.

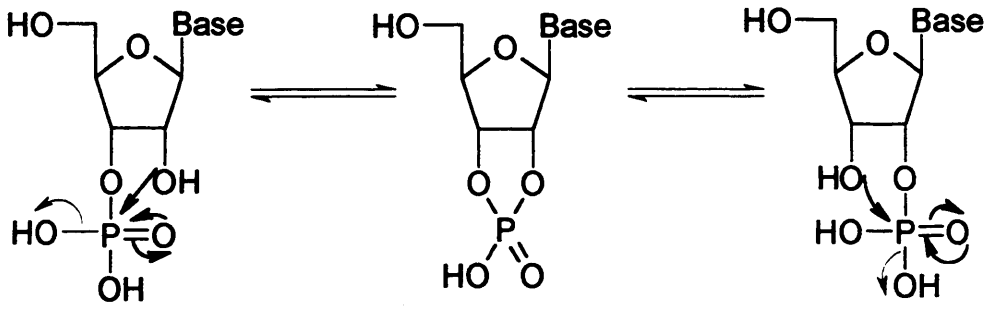


Scheme 1.3

The unambiguous synthesis of ribonucleoside-2' and 3' phosphates was more difficult because of the rapid interconversion of the esters under the usual reaction conditions. A mixture of ribonucleoside-2' and 3' phosphates were prepared by the phosphorylation of 5-trityl adenosine by the phosphorochloridate method, and the isomers were separated by ion exchange chromatography.[33] However, adenosine-2' phosphate was synthesised unambiguously as was uridine-2' phosphate, both routes involved phosphorylation of the 3',5'-di-O-acetyl nucleoside with O-benzyl phosphorous O-diphenyl phosphoric anhydride to

give the phosphite, followed by oxidation and hydrolysis.[37,56] Reaction of dihydropyran with uridine-3',5' cyclic phosphate gave the 2'-O-tetrahydropyranyl derivative of the -3' phosphate.[57]

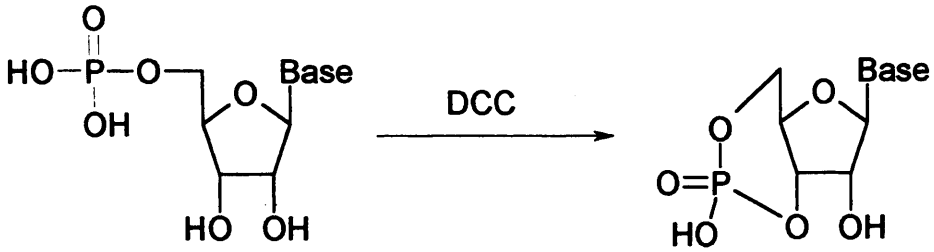
Cyclic ribonucleoside-2',3' phosphates can be prepared in high yield from either the ribonucleoside -2' or -3' phosphates and a dehydrating agent such as DCC[41] or trifluoroacetic anhydride (figure 1.2).[32]



(8) Base = adenine 2',3' cyclic AMP

Figure 1.2

The cyclic-3',5' phosphates of adenosine, uridine, cytidine, guanosine and thymidine have all been prepared by the action of DCC on the nucleoside-5' phosphate (scheme 1.4).[44]



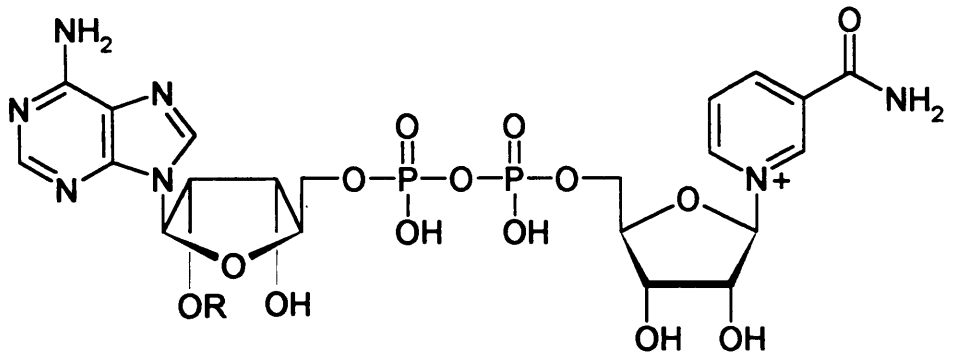
Scheme 1.4

1.2 History of Coenzymes

1.2.1 Historical Discovery and Chemical Synthesis of the Nicotinamide Cofactors NAD^+ (9) and $NADP^+$ (10)

Nicotinamide adenine dinucleotide (NAD^+, 9) was the first nucleotide coenzyme to be discovered by Harden and Young[58] in 1906 as the heat stable cofactor of alcoholic fermentation, but it was not isolated until 1936 when Von Euler[59] and Warburg and Christian[60] both purified the coenzyme. However, the number and diversity of nucleotide coenzymes was not realised until after the development of radioactive tracers and mild isolation techniques

such as paper[61] and ion exchange[29] chromatography. This was mainly due to the extreme lability of the coenzymes under the conditions of "classical" isolation techniques. Warburg and Christian had isolated another coenzyme from yeast in 1931,[62] this heat stable coenzyme displayed properties similar to those of NAD^+ (9), this coenzyme was $NADP^+$ (10). This early work has been reviewed in detail by Singer and Kearney.[63]

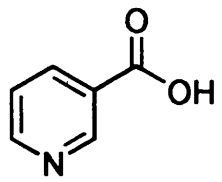


R=H NAD^+ (9)

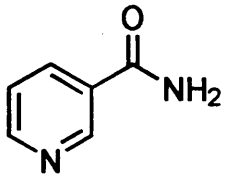
$R=PO_3H_2$ $NADP^+$ (10)

The structure of NAD^+ (9) was established using a combination of degradation and synthesis. Hydrolysis using a mineral acid gave adenine, nicotinamide and two moles of D-ribose 5-phosphate, alkaline hydrolysis gave adenosine diphosphate and the structure of this polyphosphate had already been well established. Enzymatic hydrolysis using a nucleotide pyrophosphatase gave quantitative conversion into adenosine 5'-phosphate and the 5'-phosphate of N-ribosylnicotinamide. The structure of NAD^+ (9) as a P^1,P^2 diester of pyrophosphoric acid was established and Schlenk has summarised the above evidence.[70] The configuration of the glycosidic link in the ribosyl nicotinamide section of NAD^+ (9) was established as β from ^1H nmr studies.[71] The configuration of the other glycosidic linkage followed from the isolation of other adenosine derivatives which were known to be β-ribosides. The structure of $NADP^+$ (10) was elucidated in a similar manner to NAD^+ (9). The position of the third phosphate was established when adenosine 2',5'-diphosphate[72] was isolated by enzymatic hydrolysis of $NADP^+$ (10) using a nucleotide pyrophosphatase. The relationship between NAD^+ (9) and $NADP^+$ (10) and the vitamins nicotinic acid (11) and nicotinamide (12) was clarified by studying the biosynthesis of the coenzymes.[7]

The structures of these coenzymes were confirmed by their synthesis, the synthesis of nucleotide coenzymes involves joining a nucleotide (or a protected nucleotide) to another acid

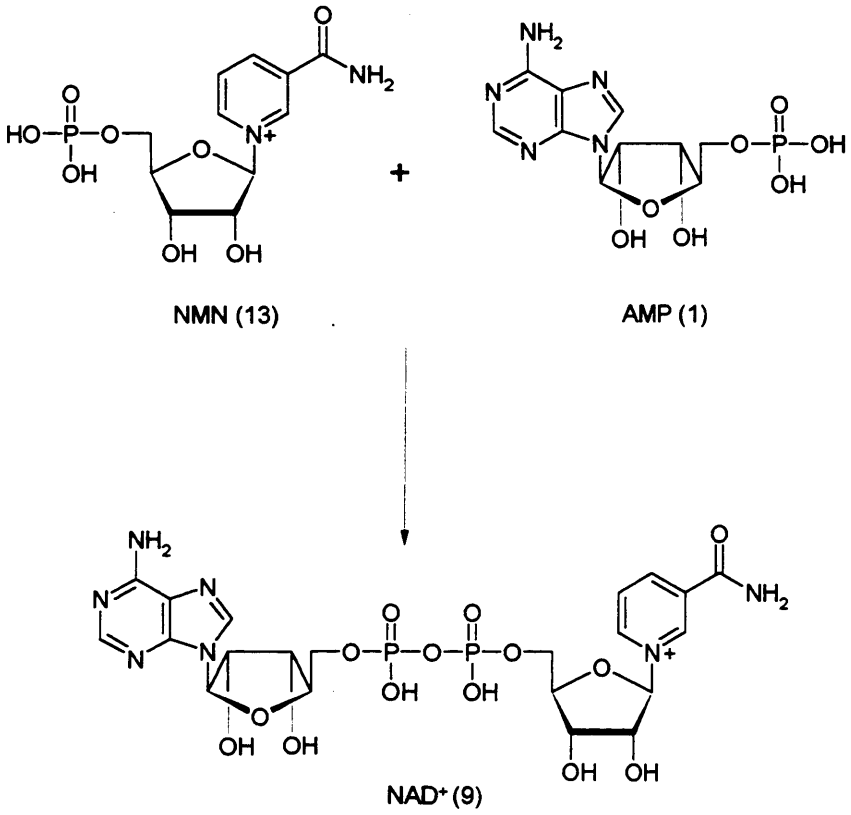


nicotinic acid (11)



nicotinamide (12)

to form an acid anhydride. The classical chemical techniques for the formation of anhydrides have been used in the synthesis of coenzymes, as have other techniques such as the use of carbodiimides and the phosphoramidate method. More recently an effective method has been developed, this involves the controlled nucleophilic displacement of one component of a nucleotide anhydride by a third acid. This procedure is an anion exchange procedure which is analogous to the enzymatic synthesis of nucleotide coenzymes. Other approaches, such as esterification of a preformed pyrophosphate, have occasionally been used, but with a rather low success rate.



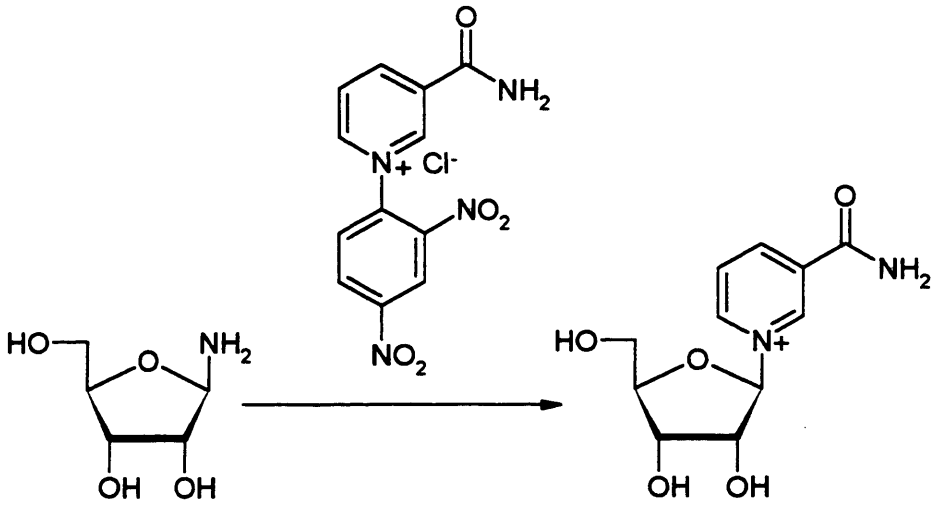
NMN (13) AMP (1)

NAD⁺ (9)

Scheme 1.5

Hughes, Kenner and Todd completed the first synthesis of NAD^+ (9) in 1957[83] by treating a mixture of AMP (1) and nicotinamide mononucleotide (NMN, 13) with DCC (Scheme 1.5). The β anomer predominated in this synthesis. The nicotinamide mononucleotide (NMN, 13) was prepared by phosphorylation of nicotinamide nucleoside (14)

using $POCl_3$.[83] Todd and co-workers prepared the nicotinamide nucleoside (14) in 1957 from triacetyl ribofuranosyl chloride and nicotinamide.[84] Since then other methods have been used to prepare nicotinamide nucleoside (14) (Scheme 1.6).[85] Both of these methods have been used to prepare analogues of NAD^+ (9).[86,87]

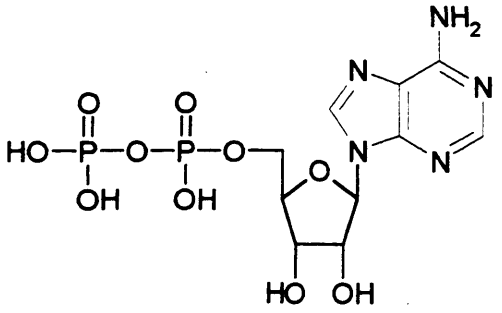


(14)

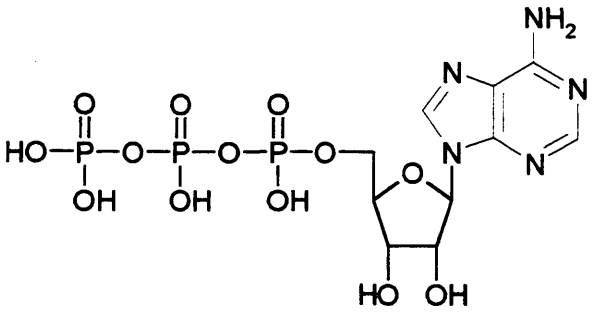
Scheme 1.6

1.2.2 Historical Discovery and Chemical Synthesis of ADP(15) and ATP(16)

Fiske and Subbarow[64] were the first to discover ADP (15) and ATP (16) but it was Lohmann[65] who established the correct formulae. He discovered that ADP (15) had two primary and one



ADP (15)

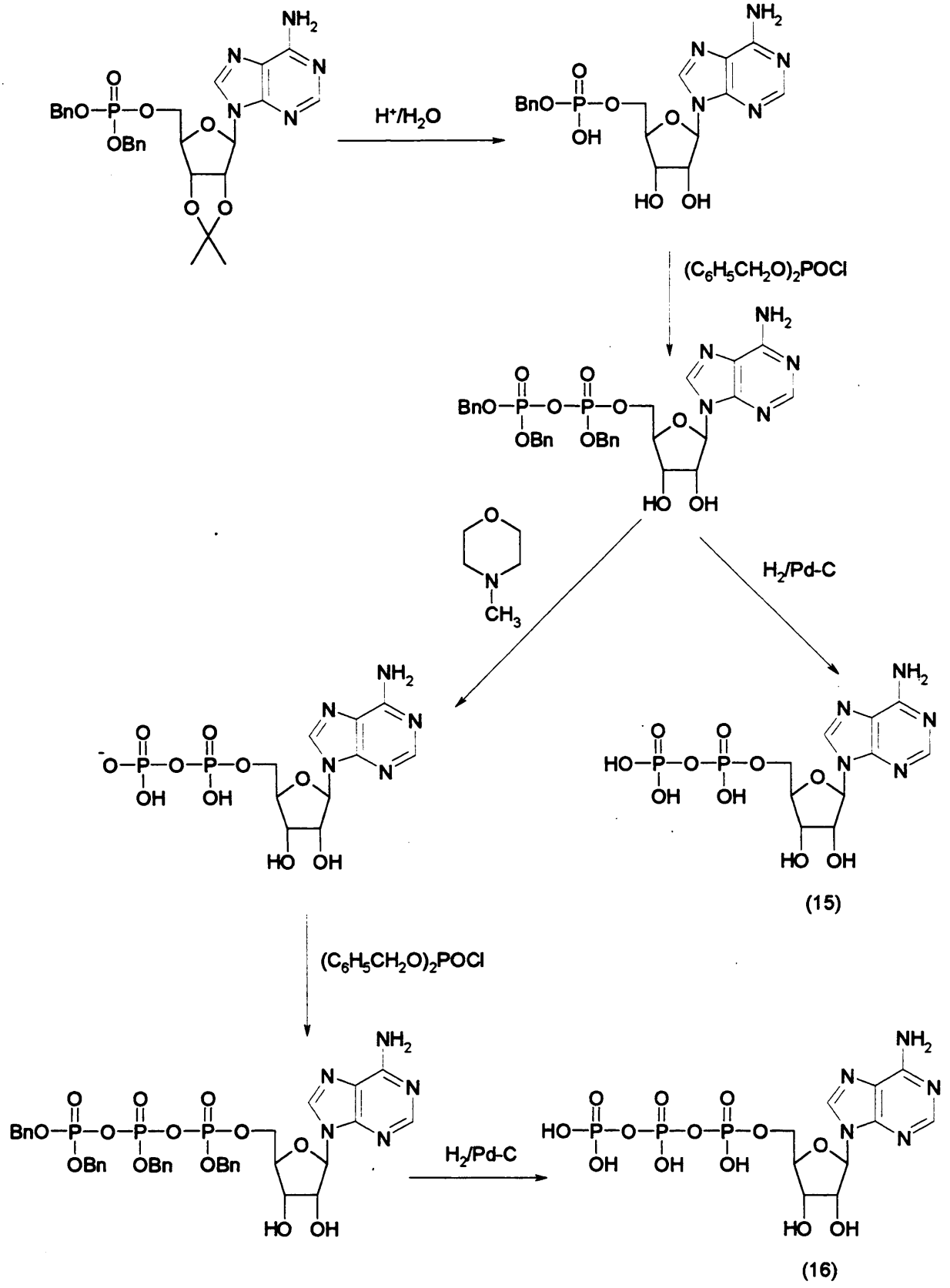


ATP (16)

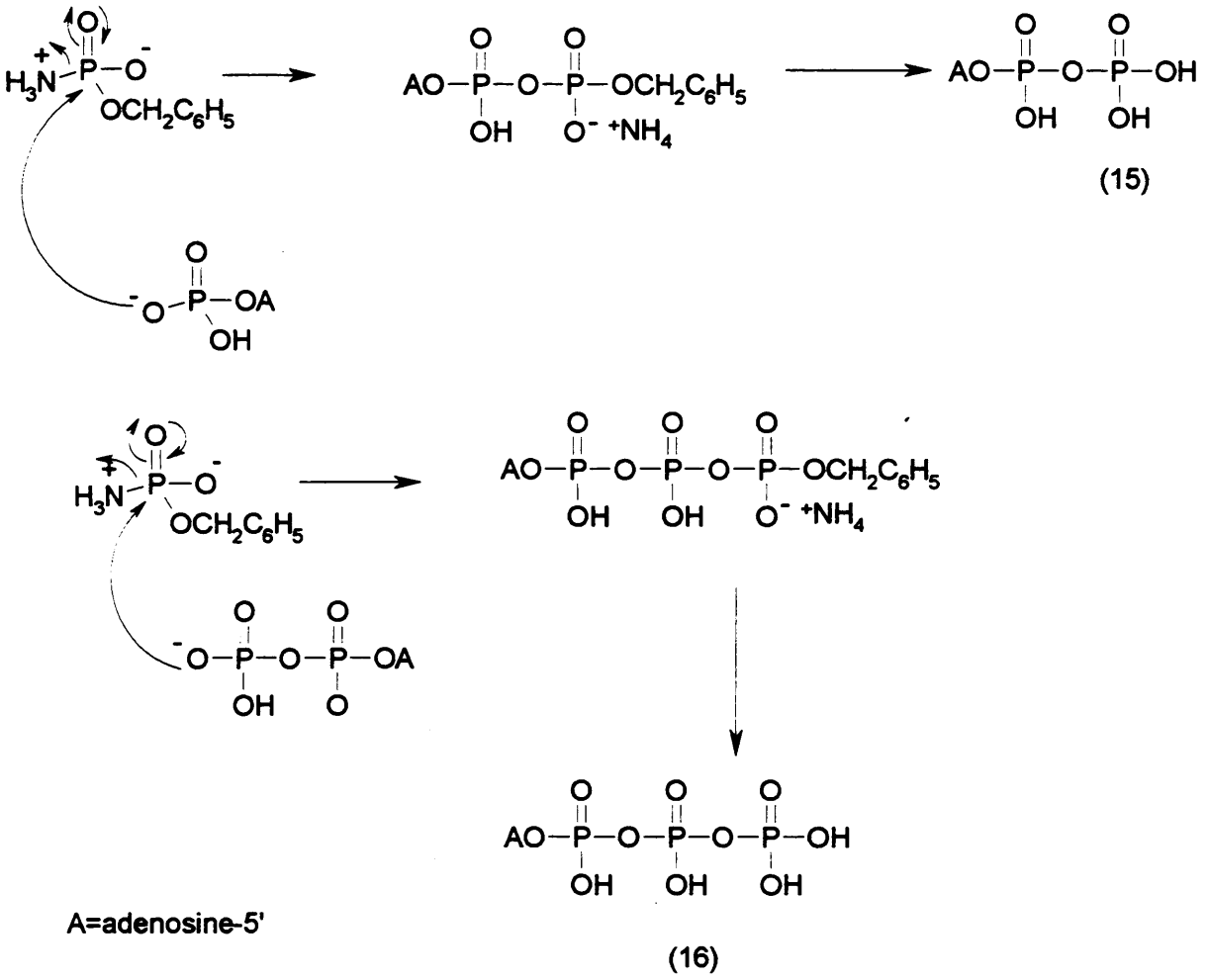
secondary acid dissociation and ATP (16) had three primary and one secondary acid dissociation. Lythgoe and Todd[66] proved that the polyphosphate residue was attached to the 5' hydroxyl group by showing that ATP (16) consumed one equivalent of metaperiodate. Other nucleotide 5'-polyphosphates have also been isolated from natural sources, these include the di- and triphosphates of guanosine, uridine, cytidine and thymidine.[67,68,69]

The first synthesis of a coenzyme was done by Baddiley and Todd in 1947.[74] They prepared adenosine-5' pyrophosphate (ADP, 15) and adenosine-5' triphosphate (ATP, 16) (scheme 1.7) using the phosphorochloridate method, however the yields were low because of the instability of the pyro- and triphosphate reaction intermediates. However, since this early work, the introduction of new techniques has led to the development of more efficient syntheses, such as the use of phosphoramidates. The mono esterified phosphoramidates are good phosphorylating agents and have been used in the syntheses of ADP (15) and ATP (16), as these phosphoramidates do not phosphorylate alcohols under normal conditions, no protecting groups are necessary.

Treatment of adenosine-5' phosphate with triethylammonium phosphoramidate gave a mixture of ADP (15), ATP (16) and a mixture of higher adenosine-5' polyphosphates as well as unreacted nucleotide.[75] Benzyl hydrogen phosphoramidate reacts with pyridinium AMP in a more controlled way to give ADP (15) after removal of the benzyl group.[76] ADP (15) reacts with benzyl hydrogen N-cyclohexyl phosphoramidate to give ATP (16) in high yield (Scheme 1.8).[76] The same method has been used in the syntheses of GDP[77] and other coenzymes.[78]

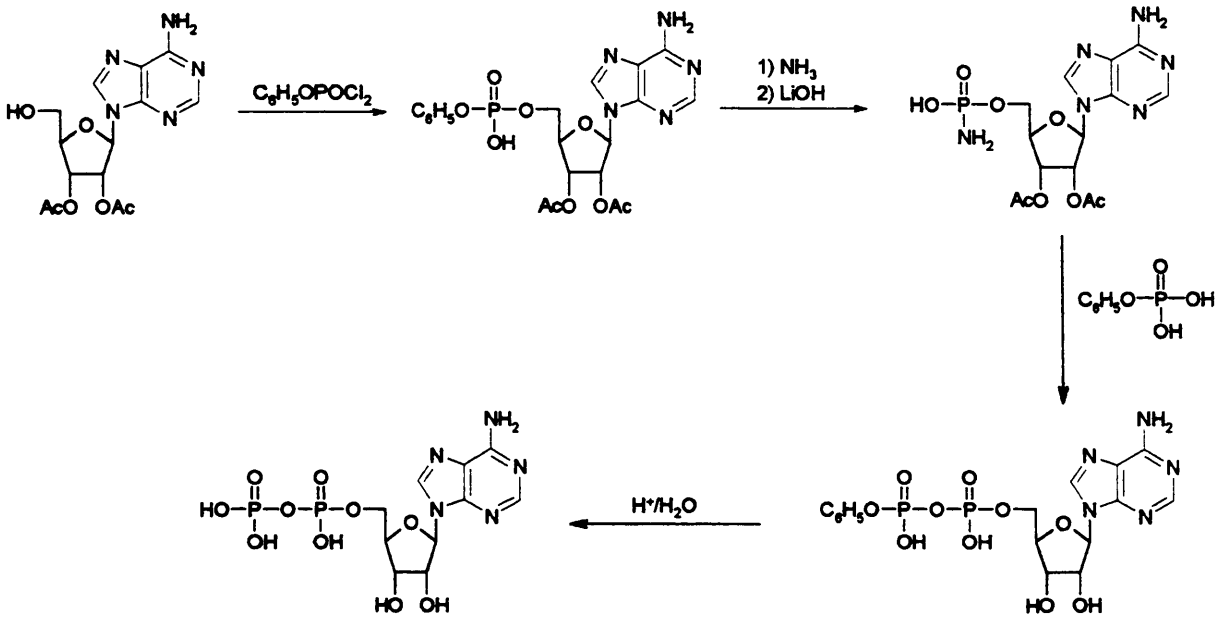


Scheme 1.7



Scheme 1.8

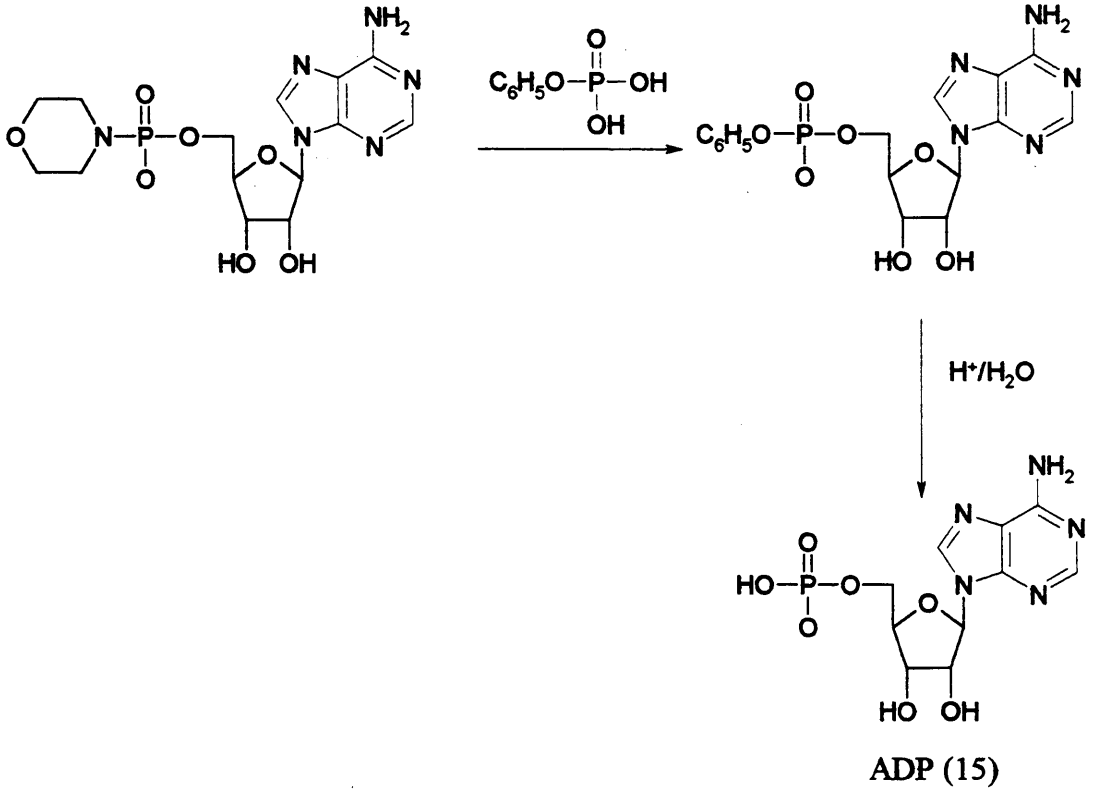
Another approach using the nucleotide-5' phosphoramidates has also been employed, this method is of far greater utility than the previous one, nucleoside-5' phosphoramidates can be obtained directly as either dicyclohexyl guanidinium or ammonium salts from the nucleotide and ammonia in the presence of DCC. The 5'-phosphoramidates of adenosine, cytidine, guanosine and uridine have all been prepared this way. Treatment of a nucleoside-5' phosphoramidate with phenyl phosphoric acid, phosphoric acid[75,79] or dioxan diphosphoric acid[80,81] gives the nucleoside-5' pyrophosphate (Scheme 1.9).



ADP (15)

Scheme 1.9

The yield of adenosine-5' pyrophosphate (15) was improved by the use of adenosine-5' phosphoromorpholidate.[82] This method can be applied in the synthesis of any nucleoside-5' pyrophosphate by using the appropriate nucleoside-5' phosphoromorpholidate. However, the extension of this synthesis to make ATP (16) by the reaction of adenosine-5' phosphoromorpholidate with inorganic pyrophosphate was less successful (Scheme 1.10).[82]

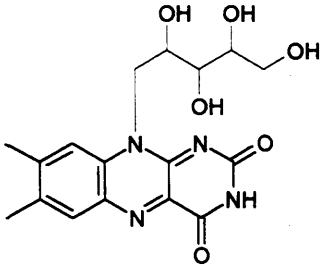


ADP (15)

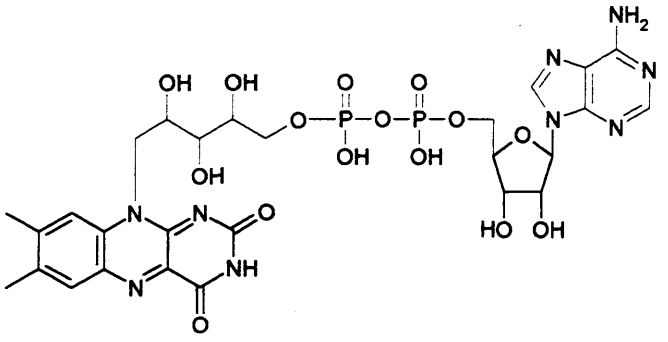
Scheme 1.10

1.2.3 Historical Discovery and Chemical Synthesis of the Flavin Coenzymes FMN (19) and FAD (18)

Riboflavin (17) or Vitamin B2 was one of the earliest vitamins to be discovered, isolated and studied. The flavin coenzymes are phosphate esters of riboflavin. Flavin adenine dinucleotide (FAD, 18) is a pyrophosphate ester of riboflavin. Riboflavin-5' phosphate (19) is not a derivative of D-ribose and is therefore not a nucleotide, however it has acquired the trivial name flavin mononucleotide (FMN). Karrer attempted to isolate FMN (19) from liver preparations,[88] but the coenzyme was contaminated with an adenine nucleotide. This nucleotide must have been released from a more complex form of FMN (19) during the isolation procedure. This more complex form of FMN (19) is now known to be FAD (18). The structure of FAD (18) was determined by a combination of hydrolysis and synthesis. FAD (18) liberated one equivalent of adenine after acid hydrolysis and it was already known to contain three equivalents of phosphate. Photolysis of the coenzyme in alkaline solution



(17)



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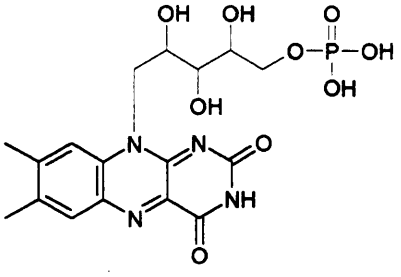
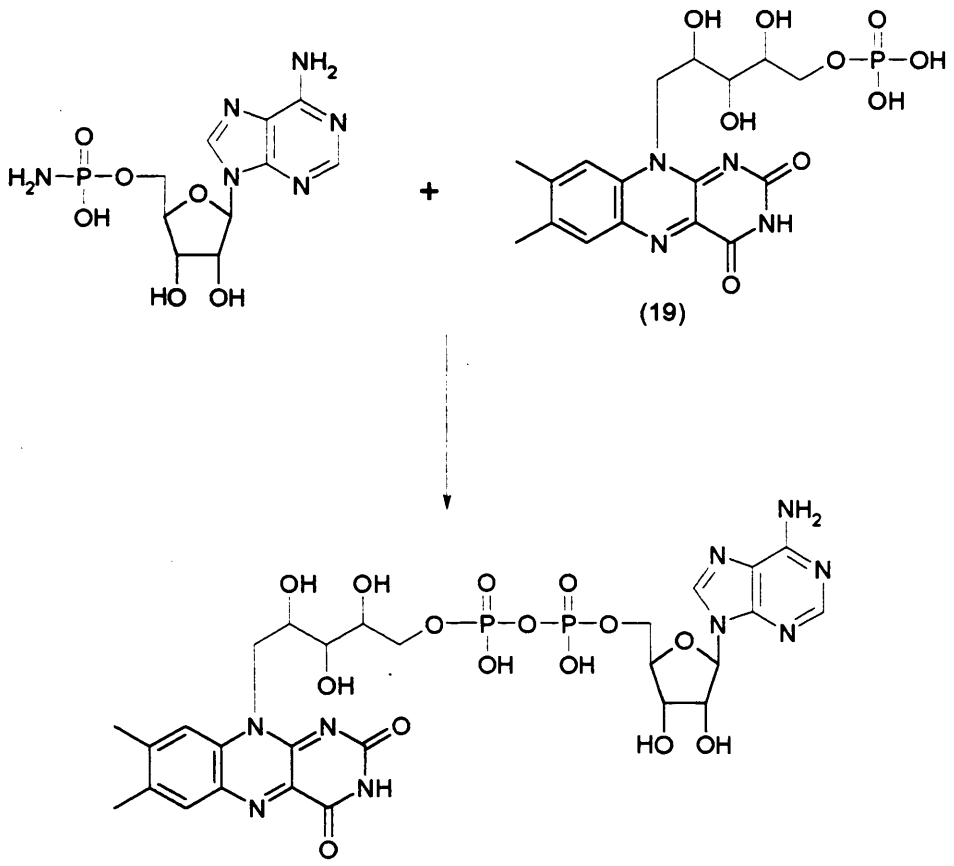


(19)

gave one equivalent of lumiflavin. Careful hydrolysis with dilute acid gave AMP (1) and FMN (19) in equivalent amounts.[89] The structure (19) was proposed and this was confirmed by synthesis. FAD (18) has been synthesised in many ways.[90,91,92,93] Khorana and Moffatt formed the pyrophosphate bond in high yield by the action of adenosine-5' phosphoramidate on FMN (19) (scheme 1.11).[93] The same techniques that were used to form the pyrophosphate bonds in the nucleotide 5' polyphosphates syntheses can be used in the synthesis of flavin coenzymes e.g. phosphoramidates, phosphorochloridates and phosphoromorpholidates.



(19)

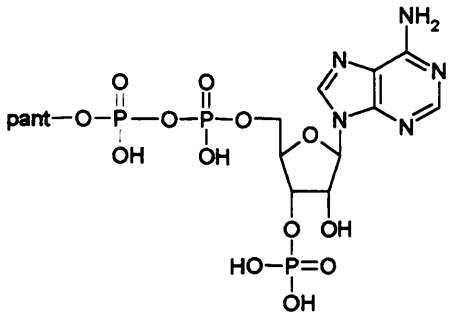
(18)

Scheme 1.11

1.2.4 Historical Discovery and Chemical Synthesis of Coenzyme A (20)

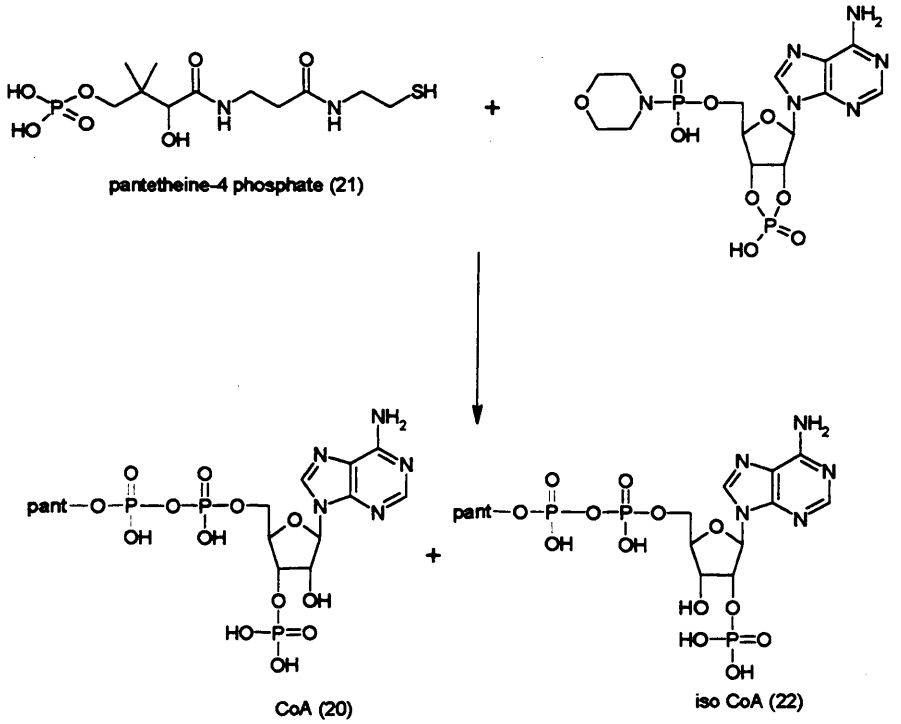
Coenzyme A (CoA, 20) was discovered by Lipmann in 1945,[94] it was originally isolated from fresh liver, but the inconvenience of handling large amounts of fresh liver led to the development of micro-organisms such as yeast as more convenient sources of the coenzyme. The structure was elucidated as a result of the work done by Baddiley,[95] Lipmann[96] and Snell,[97] treatment with an intestinal phosphatase gave adenosine, pantetheine and three moles of phosphate. More specific enzymatic degradation established the position of the phosphate

groups. "Dephosphoro-coenzyme A" plus one mole of orthophosphate was obtained using a nucleotidase which specifically cleaves nucleoside 3'-phosphates. A pyrophosphatase yielded adenosine 3',5'-diphosphate (a known compound) and panthetheine 4'-phosphate (21).
The position of the phosphate in the panthetheine 4'-phosphate (21) was established by comparison with a synthetic specimen of known structure. The structure was confirmed by the total synthesis of CoA (20) by Khorana,[98] Michelson[99] and Lynen.[100] Khorana's synthesis



CoA (20)

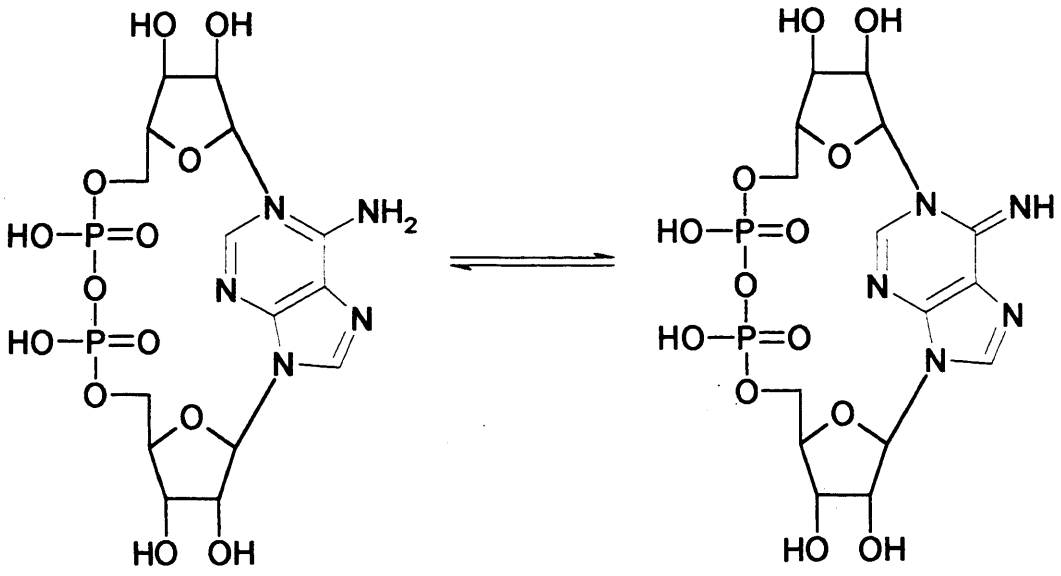
involved coupling pantetheine-4 phosphate (21) and a siutably substituted adenosine-5' phosphoromorpholidate, iso CoA (22) was produced as a byproduct (scheme 1.12).[98] Michelson obtained a better yield of CoA (20) from P^1-adenosine-2',3' cyclic phosphate-5'-P^2-diphenyl pyrophosphate and pantethine-4,4' diphosphate, followed by reduction of the disulphide and opening of the cyclic phosphate with ribo nuclease.[99] Lynen's synthesis of CoA (20) involved using pyrophosphoryl chloride for the formation of the pyrophosphate bond.[100]



Scheme 1.12

1.3 Recent Developments in Nucleotide Chemistry

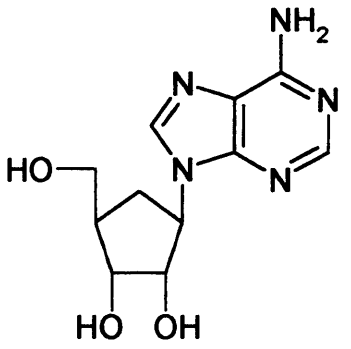
The field of nucleotide chemistry is still ongoing as novel nucleotides are being discovered. Cyclic ADP ribose (23) is an interesting example of a nucleotide which has only recently been discovered. It is a cyclic dinucleotide which has been implicated in the process of calcium release in cells (figure 1.3).



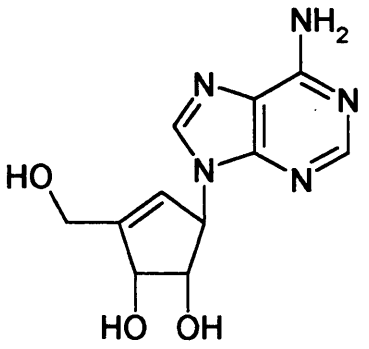
Cyclic ADP Ribose (23)

Figure 1.3

There has also been a lot of interest in the area of carbocyclic nucleosides where the ring oxygen of the ribose has been replaced by a methylene unit. Some of these are naturally occurring materials e.g. aristeromycin (24).

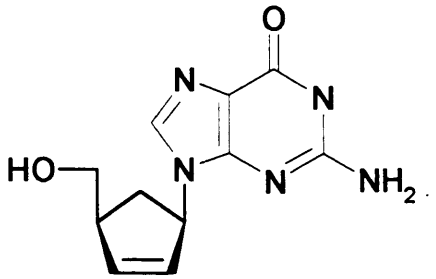


Aristeromycin (24)



Neplanocin A (25)

Some of these carbocyclic nucleotides have generated a lot of interest as potential anti viral agents. Aristeromycin (24) and neplanocin A (25) are both naturally occurring carbocyclic nucleotides and they both demonstrate significant antitumor activity. Aristeromycin is the carbocyclic analogue of adenosine and the racemic version was first synthesised in 1966 by Shealy and Clayton[101] and aristeromycin was isolated as a single enantiomer in 1968.[102] Neplanocin A was not isolated until 1981,[103] and this renewed synthetic interest. However, in the last ten years these compounds have received a lot of attention as potential anti HIV agents, and one of the most interesting is carbovir (26).[108] Tetrahydrofuran rings and cyclopentyl rings show a large amount of conformational and structural similarity and this allows the carbocyclic compounds to display a wide variety of biological effects. Unlike the nucleosides, carbocyclic nucleosides do not have a glycosidic bond and this makes them resistant to the action of some enzymes which can deactivate the parent nucleoside by cleavage of this bond.



(26)

1.4 Summary

Since the initial discovery of nucleotides and nucleotide coenzymes the introduction of modern analytical techniques has led to the separation and identification of many compounds. This has been followed by a desire to synthesise nucleotides and their analogues, and to discover the biological functions of these compounds. The carbocyclic analogues of nucleosides are important due to their anti viral and anti tumor properties, and in particular the carbocyclic analogues of the nicotinamide cofactors are interesting as they can be used as tools to investigate the modes of action of these important cofactors.

CHAPTER 2

THE SYNTHESIS OF CARBA NAD^+ (28) and PSEUDO CARBA NAD^+ (29), CARBOCYCLIC ANALOGUES OF NAD^+

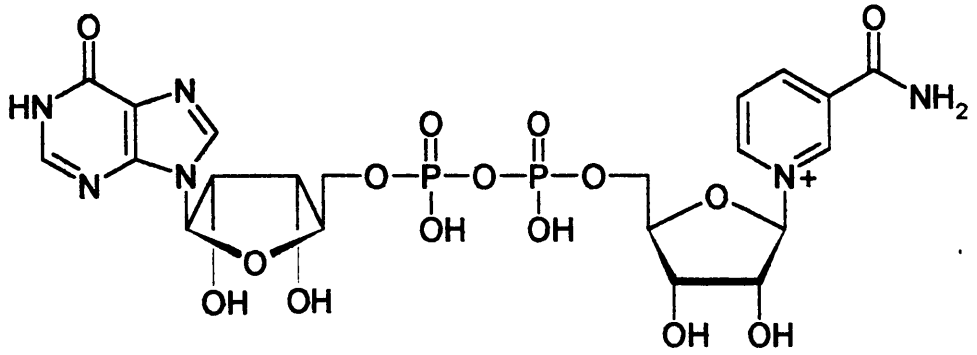
Chapter 2

The Synthesis of Carba NAD^+ (28) and Pseudo Carba NAD^+ (29), Carbocyclic Analogues of NAD^+

2.0 Introduction

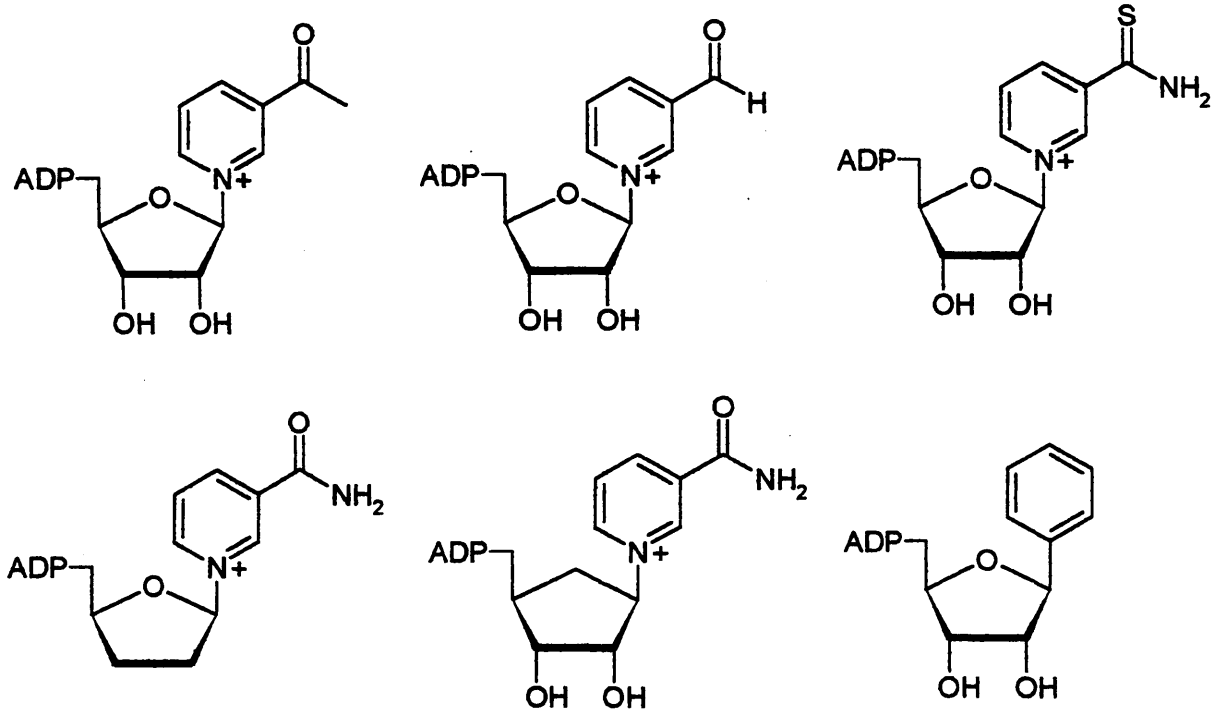
NAD^+ (9) in association with the appropriate protein apoenzymes, is a cofactor for a wide variety of dehydrogenation reactions, it is comprised of two ribose units, a nicotinamide ring, an adenine moiety and a pyrophosphate linkage. The two pyridine nucleotide coenzymes NAD^+ (9) and $NADP^+$ (10) are required for more than 370 different kinds of enzymes, this is 17% of all classified enzymes.[104,105] As these coenzymes are present in most living systems and are involved in more enzymatic reactions than any other coenzyme they are arguably the most important of coenzymes. In most cases these coenzymes are involved in the hydride transfer process of dehydrogenases. The coenzyme is acting as an intracellular electron carrier. One point which differentiates the pyridine nucleotide coenzymes from other coenzymes such as bioitin and thiamine pyrophosphate is that the pyridine nucleotide cofactors do not function catalytically, they do not return to their original form at the end of the reaction. This means that $NAD(P)^+$ (9 and 10) can be considered as a special kind of substrate or cosubstrate. In the redox reactions of NAD^+ (9), it is the 4 position of the nicotinamide ring which is involved in the hydride transfer process. The rest of the molecule can be considered to be involved in the interaction of the coenzyme with the dehydrogenase enzyme to position the coenzyme in relation to the substrate and catalytic groups on the enzyme.

NAD^+ (9) also acts as a substrate in ADP ribosylation reactions and in this role it is the ADPR portion of the molecule which is more important, the nicotinamide ring only acts as a leaving group. In these two widely differing roles of NAD^+ (9) the various parts of the coenzyme must interact in different ways with the enzymes and therefore changing any part of the NAD^+ (9) to make an analogue can have pronounced effects on the activity. These analogues can be used as tools to probe the mode of action of the NAD^+ (9).



(27)

The first NAD^+ analogue to be synthesised was nicotinamide hypoxanithine dinucleotide (27), it was prepared by Schlenk and coworkers in 1938[106] by the treatment of NAD^+ (9) with nitrous acid. Since then many NAD^+ analogues have been made by both enzymatic and chemical methods. These analogues have included alterations to all parts of the NAD^+ and some analogues contain multiple alterations (figure 2.1).[107]

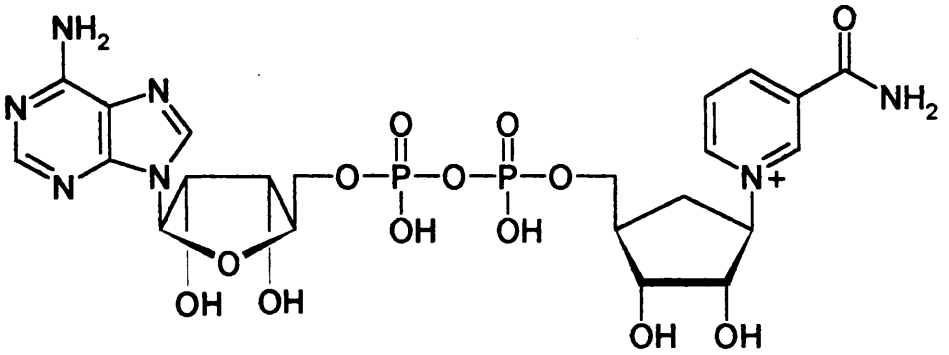


(28)

Some Examples of NAD^+ Analogues

Figure 2.1

NAD^+ (9) contains an unstable glycosidic linkage; however in the carbocyclic analogue (28) the ring oxygen of ribose has been replaced by a carbon thus removing the glycosidic linkage. NAD^+ (9) itself is very labile to hydrolysis, the nicotinamide moiety is a good leaving group



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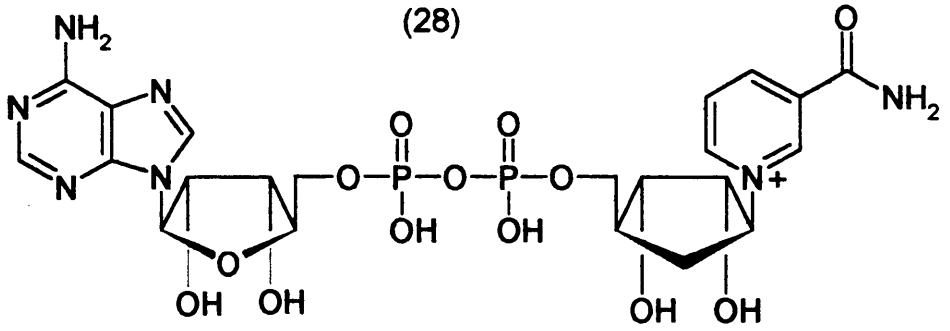
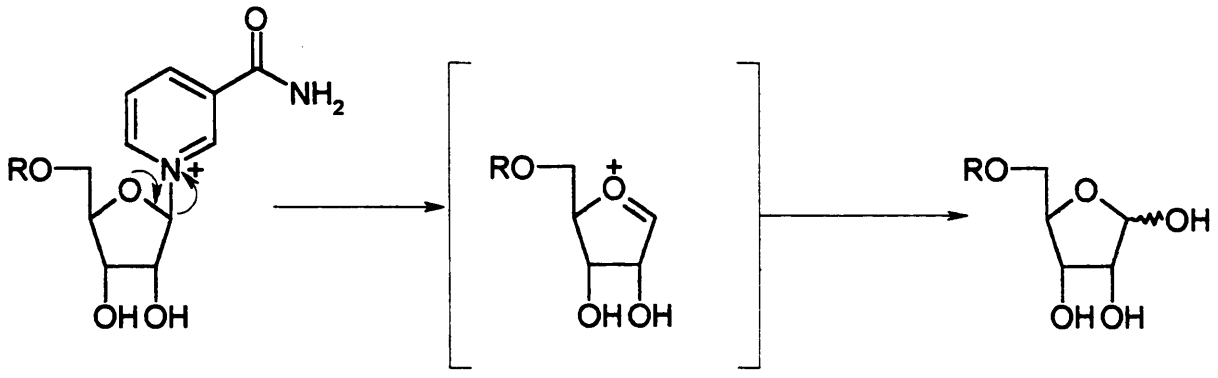


(29)

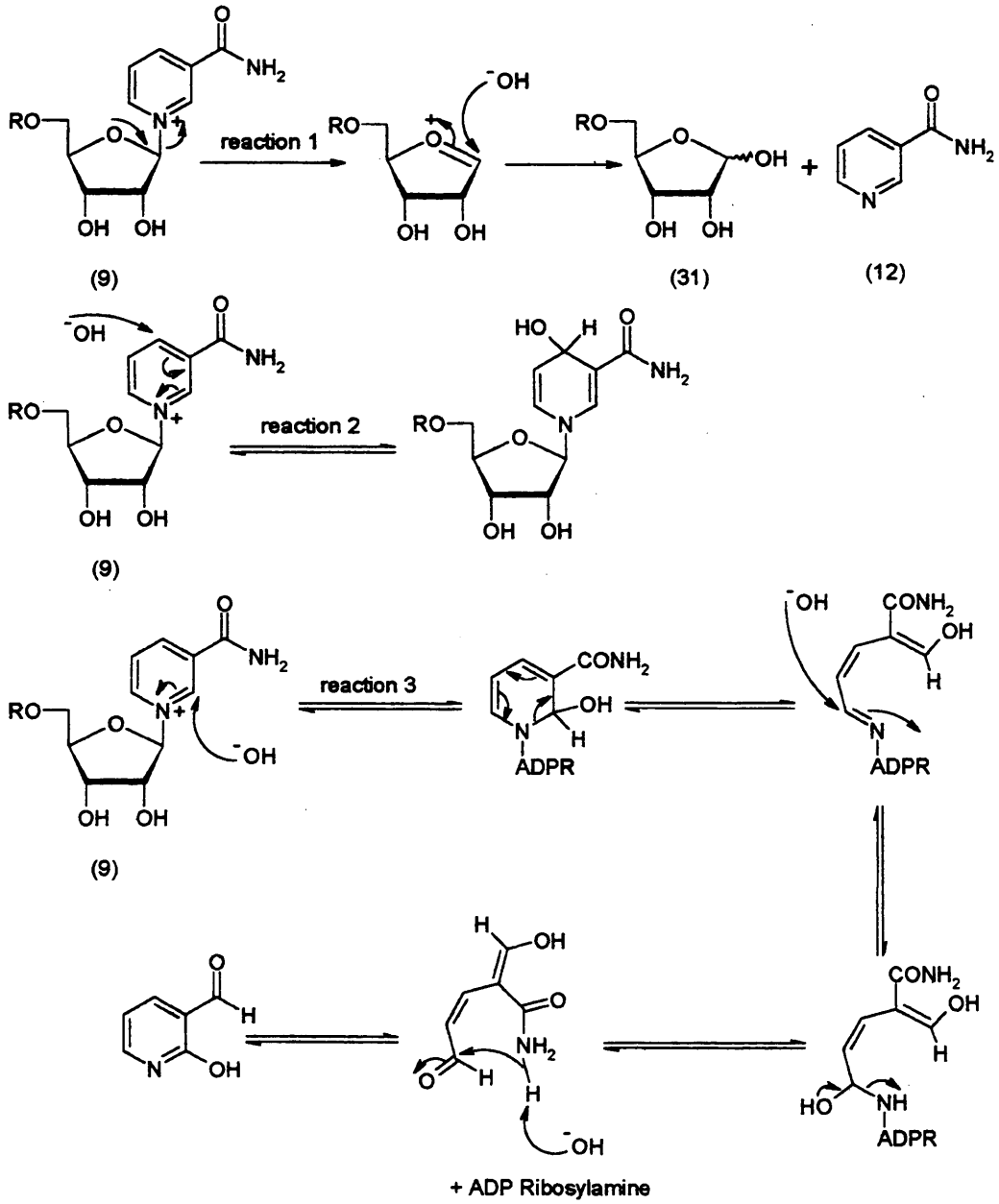
(scheme 2.1), thus by removing the glycosidic linkage, the cation intermediate loses stability and the carbocyclic analogue of NAD^+ (28) should be more stable than NAD^+ (9) itself. Another feature of the carbocyclic analogue is that it cannot undergo epimerization between the α and β forms. In acidic solution the reduced form of the NAD coenzyme undergoes epimerization (figure 2.2) which involves ring opening of the ribose ring, the carbocyclic cyclopentane ring cannot undergo this reversible ring opening. The acid/base stability of NAD^+ (9) and NADH (30) are complimentary, NAD^+ (9) is base labile and acid stable whereas NADH (30) is stable to acid but base labile. NAD^+ (9) can undergo three distinct reactions in base (scheme 2.2), reaction 1 involves the direct nucleophilic replacement of the nicotinamide with a hydroxide ion to give ADP ribose (31), reaction 2 is addition of the hydroxide ion *para* to the nitrogen in the nicotinamide ring, this reaction is reversible, and



(9) (31)

Scheme 2.1

reaction 3 is addition of the hydroxide ion ortho to the nitrogen at the 2 position of the nicotinamide ring.[105] Reaction 3 is analogous to a Zincke reaction (Scheme 2.17).



Scheme 2.2

NADH (30) is stable to base as the 1,4 dihydro nicotinamide ring is a poor leaving group also the ring is electron rich so the nucleophilic addition of hydroxide to the double bonds does not occur readily. The NADH (30) is labile in acidic solution and one of the main reactions involved is epimerization between the α and β forms of the coenzyme (figure 2.2).

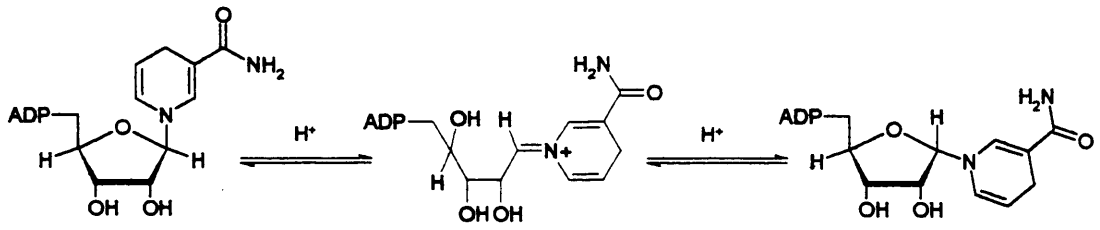
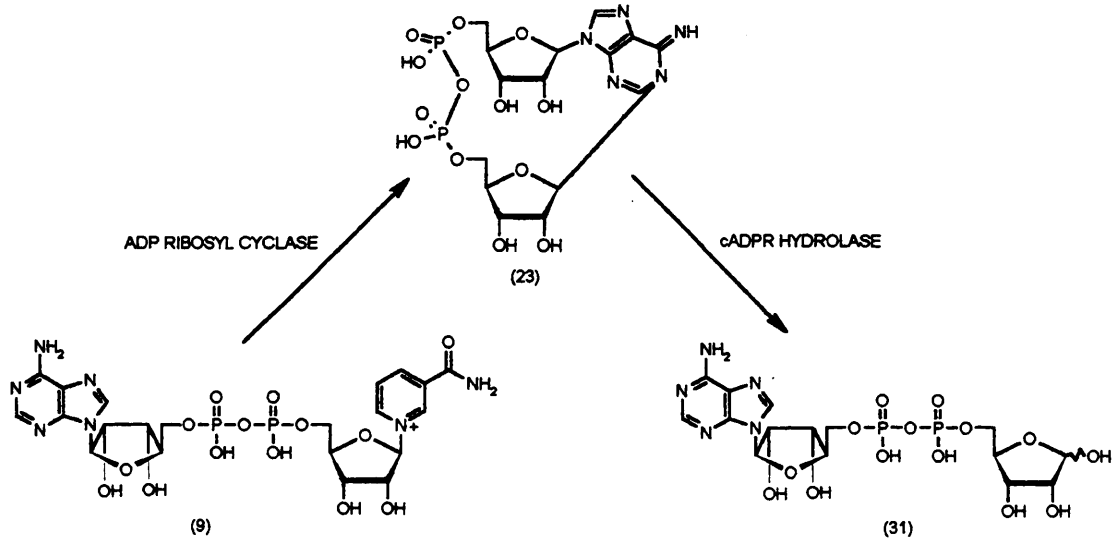


Figure 2.2

Carbocyclic NAD^+ (28) has been shown to be a substrate for both yeast and horse liver alcohol dehydrogenase,[87] as expected it was resistant to cleavage by NAD glycohydrolases, and pseudo carbocyclic NAD^+ (29) has been shown to be a potent competitve inhibitor for a variety of NAD glycohydrolases.[87] The pseudo carbocyclic NAD^+ (29) is not a substrate for YADH and LADH.

The metabolic pathway involving the novel NAD^+ metabolite cyclic ADP ribose (23)[107] is shown in scheme 2.3 cADPR (23) is made from NAD^+ (9) by ADP ribosyl cyclase, and broken down to ADP ribose (31) by cADPR hydrolase. Cyclisation of NAD^+ (9) to give cADPR (23) involves loss of the nicotinamide group. Removal of the glycosidic linkage reduces the ability of the nicotinamide to act as a leaving group and thus carbocyclic NAD^+ (28) could potentially act as an inhibitor of the ADP ribosyl cyclase enzyme.



Scheme 2.3

Recently a lot of work has been done in the area of carbocyclic nucleosides, some of which are naturally occurring materials. Carbocyclic analogues of purine and pyrimidine nucleosides in which the ring oxygen atom of the furanose ring has been replaced by a methylene group have generated a lot of interest as potential anti-viral agents in recent years; carbovir (26) has proven to be a potent and selective inhibitor of HIV-1 *in vitro*,[108] and the naturally occurring compounds aristeromycin (24) and neplanocin A (25) both show good anti tumor activity.[102,103]

Emil Fischer summarised the importance of nucleotide analogues when he made the following statement when he published the synthesis of a phosphorylated theophylline glucoside, the first nucleotide analogue of its kind:

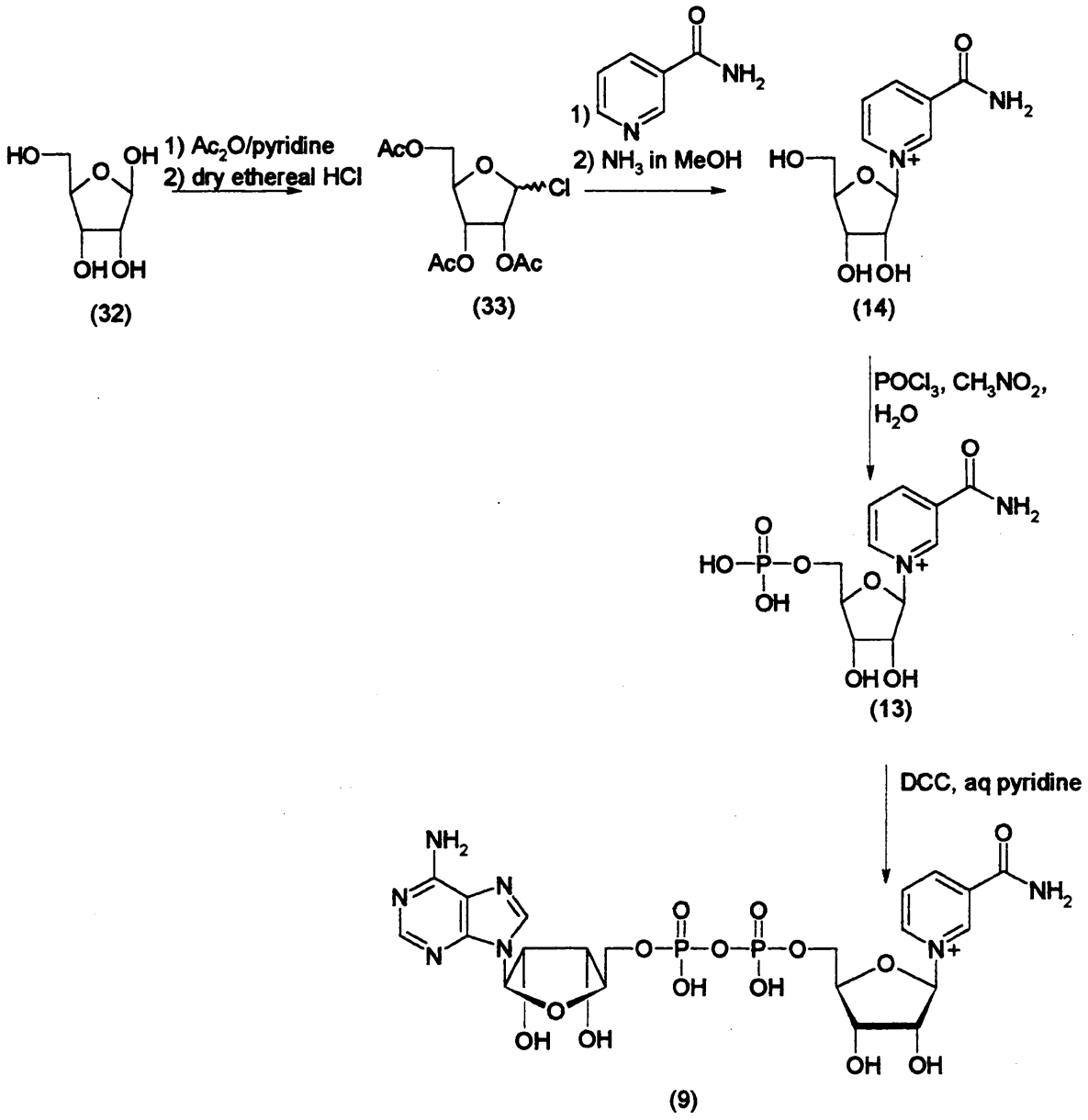
"It seems to me that further advances in understanding the natural nucleotides and nucleic acids can follow from these experiences with theophyllineglucoside phosphoric acid.... The possibility of synthesising this class of compounds allows many substances to be made that are more or less closely related to the natural nucleic acids. How are these substances going to react with various living organisms? Are they going to be rejected, or degraded or are they going to be incorporated into the cell nucleus? I am bold enough to hope that under especially favourable conditions the last case, the assimilation of synthetic nucleic acids without cleavage of the individual molecule can occur. This would however lead to fundamental changes of the organism which perhaps resemble mutations, the permanent changes observed in nature."[109,110]

2.1 Aims

The aim of this project was to synthesise both carbocyclic NAD^+ (28) and pseudo carbocyclic NAD^+ (29) and then use these analogues to study the stereochemical preferences of dehydrogenases, and to investigate the potential of these analogues (28) and (29) as inhibitors of the ADP ribosyl cyclase enzyme.

2.2 Results and Discussion

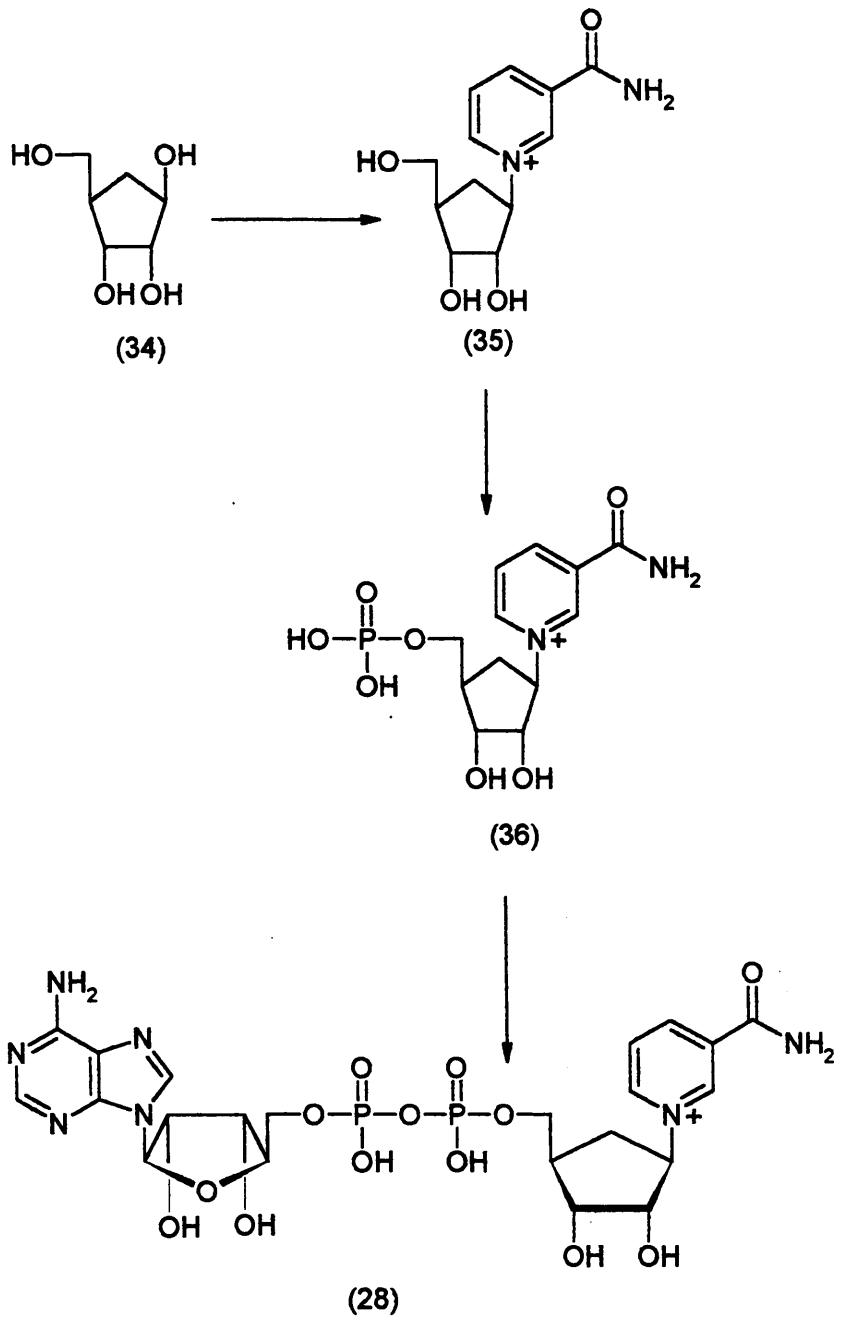
The first synthesis of NAD^+ (9) was completed in 1957[83] (scheme 2.4). This involved the formation of the nicotinamide nucleoside (14) from Tri-O-acetylribofuranosyl chloride (33) and nicotinamide (12), the tri-O-acetylribofuranosyl chloride (33) was obtained from D-ribose (32). The nucleoside was phosphorylated using $POCl_3$ in water and nitromethane. Coupling of the nucleotide to AMP (1) in the presence of DCC gave mainly the β anomer of NAD^+ (9).



Scheme 2.4

2.2.1 The Prins Reaction Approach

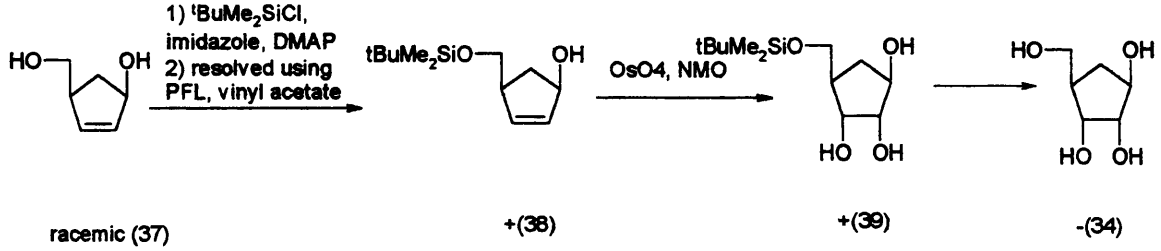
The first approach to the synthesis of carbocyclic NAD^+ (28) involved an attempt to make a carbocyclic analogue of D-ribose (34) (scheme 2.5). It was reported in the literature that the cyclopentane derivative (34) could be produced in a high state of optical purity using an



Proposed route to carba NAD^+ (28)

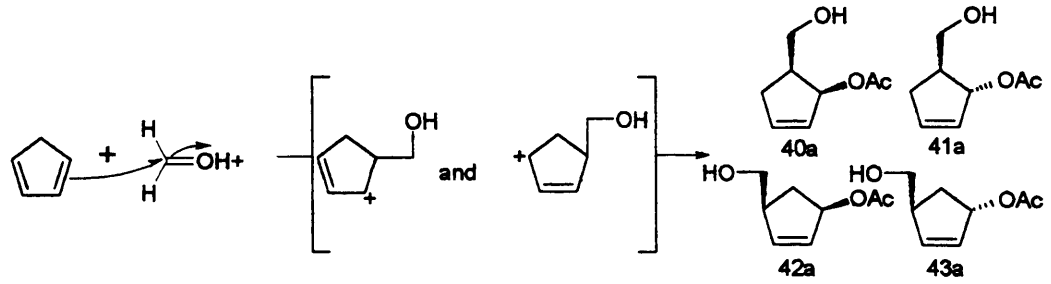
Scheme 2.5

enzyme catalysed esterification reaction (scheme 2.6).[111] The diol (37) is one of the products formed via the Prins reaction of cyclopentadiene with formaldehyde in acetic acid.[112,113,114]



Scheme 2.6

(scheme 2.7). The reaction yields four isomeric products (40a), (41a), (42a) and (43a), (figure 2.3). The literature[112] claims that the acetates are inseparable but that the *cis* diols (40b) and



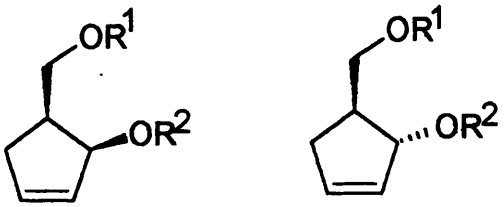
Scheme 2.7

(42b) can be separated from the *trans* diols (41b) and (43b) by "careful chromatography". A separation of the diols was attempted using 9:1 MeOH:CH_2Cl_2 as the elutant on a column of silica gel 60. Two spots were obtained by tlc, the spots were close together and the initial assumption was that the two spots were due to the separation of the *cis* diols from the *trans* diols:

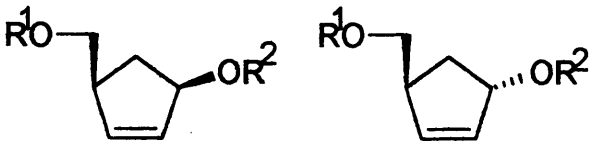
the first spot had Rf = 0.4 (A)

the second spot had Rf = 0.3 (B)

The two mixtures (A) and (B) were produced in the ratio (A):(B) 72:28, the literature[112] gives a ratio of *cis:trans* 82:18. The tentative assumption was made that A was the *cis* diol mixture (40b) and (42b) and B was the *trans* diol mixture (41b) and (43b) (figure 2.3).



40a,b,c 41a,b,c



42a,b,c 43a,b,c

a R^1=H R^2=Ac

b R^1=H R^2=H

c R^1=SiBu^{t}Me$_2$ R^2=H

Figure 2.3

The literature[112] states that monosilylation of the *cis* diols (40b) and (42b) allowed the monosilylates (40c) and (42c) to be separated by "careful chromatography". Both mixtures

(A) and (B) were silylated using $Me_2{}^tBuSiCl$, DMAP and imidazole then separated by column chromatography using petroleum ether:ethyl acetate 9:1 as the elutant on a column of silica gel 60. Both mixtures gave two spots by tlc, (A) gave (C) and (D), (B) gave (E) and (F):

Rf of (C) = 0.08

Rf of (D) = 0.03

Rf of (E) = 0.10

Rf of (F) = 0.04

(A), (B), (C), (D), (E) and (F) were examined by ^1H nmr spectroscopy, (C), (D), (E) and (F) were examined by ^{13}C nmr spectroscopy. From the ^1H nmr data for (A) and (B) it can be seen that both spectra contain a forest of overlapping peaks which implies that both (A) and (B) are still mixtures. The proton nmr spectra of (C) and (D) both show two signals between 4.6-5.9ppm and the ^{13}C nmr spectra show two signals between 132-135ppm, this indicates the presence of a carbon carbon double bond and points to (C) and (D) being cyclopentene derivatives. The proton nmr spectra also show multiplets rather than singlets for the methyl groups 0.05-0.1ppm and the ^{t}butyl groups 0.87-0.9ppm. The ^{13}C nmr spectra also give more than one methyl signal between -0.19 and -5.7ppm and more than one signal for the quaternary carbon of ^{t}butyl. This means that (C) and (D) contain more than one type of $SiMe_2{}^tBu$ group which implies that they are still mixtures (figure 2.4). Both the ^1H and ^{13}C nmr data for (D) correspond well with the literature data[111] for β-(tert-butyldimethylsiloxymethyl) cyclopent-2-en-1β-ol (34c) along with some extra peaks, this too suggests that (D) is a mixture. The ^1H and ^{13}C nmr data for (E) and (F) do not show any signals for sp^2 carbons so there are no cyclopentene derivatives present.

It was concluded that (A) was still a mixture of both *cis* and *trans* diols (40b), (41b), (42b) and (43b) and (B) was a mixture of products from a side reaction. A tlc of (A) in methanol gave two spots that were very close together:

first spot Rf = 0.45

second spot Rf = 0.43

these two spots were assumed to be the *cis* diols and the *trans* diols after separation. However, the separation of the isomers proved to be so difficult that a new chiral starting material sought.

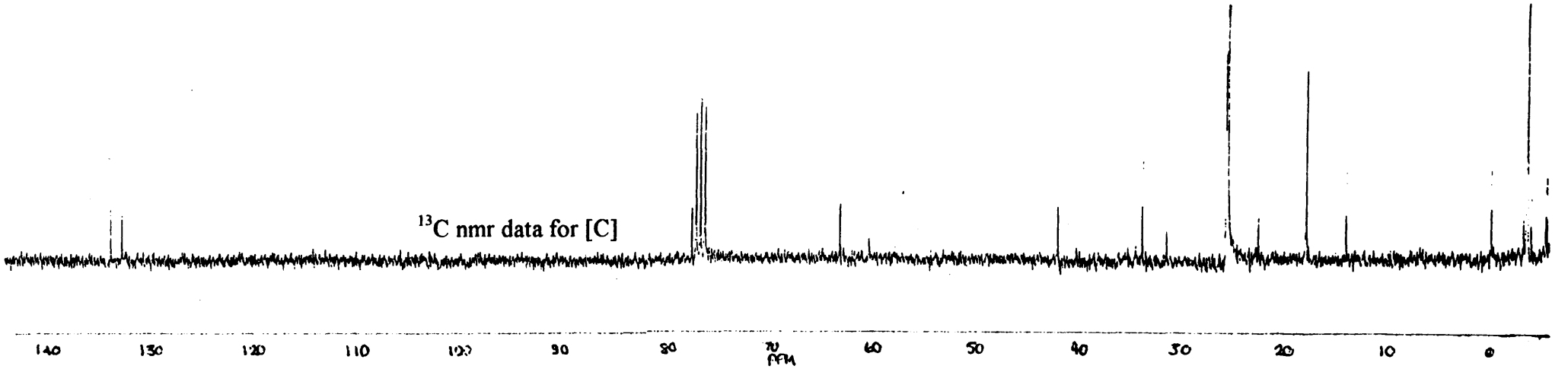
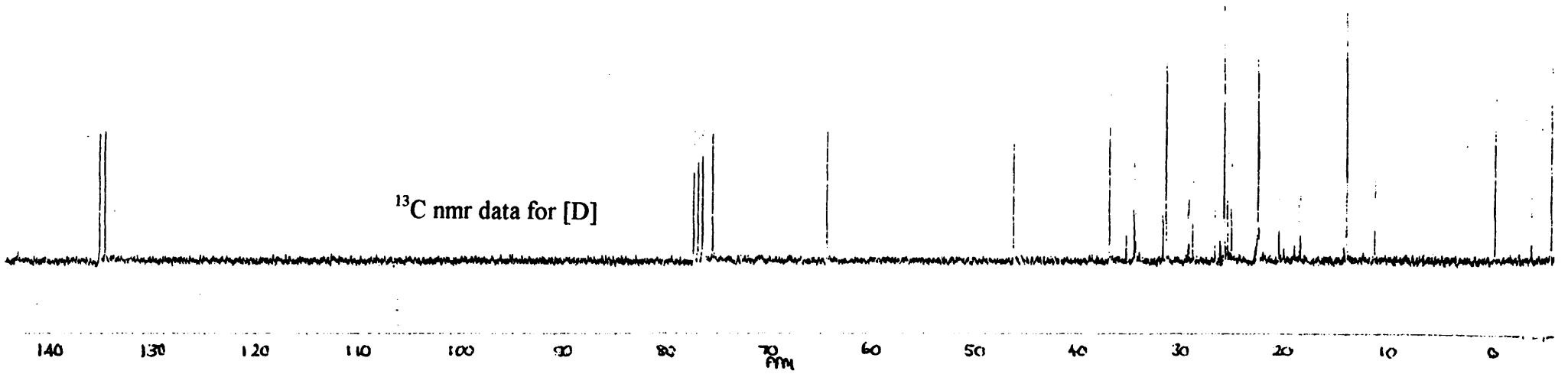
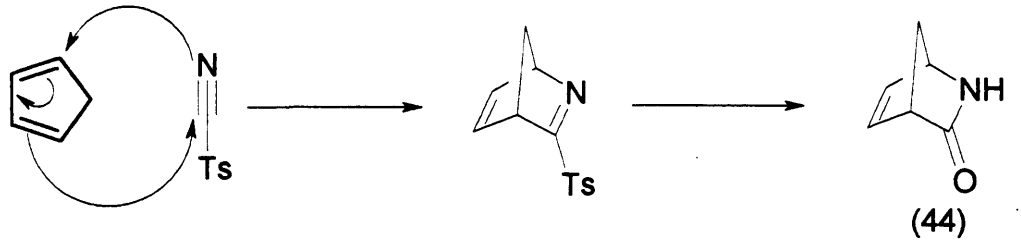

^{13}C nmr data for [C]
140
130
120
110
100
90
80
70
ppm
60
50
40
30
20
10
0

Figure 2.4


^{13}C nmr data for [D]
140
130
120
110
100
90
80
70
ppm
60
50
40
30
20
10
0

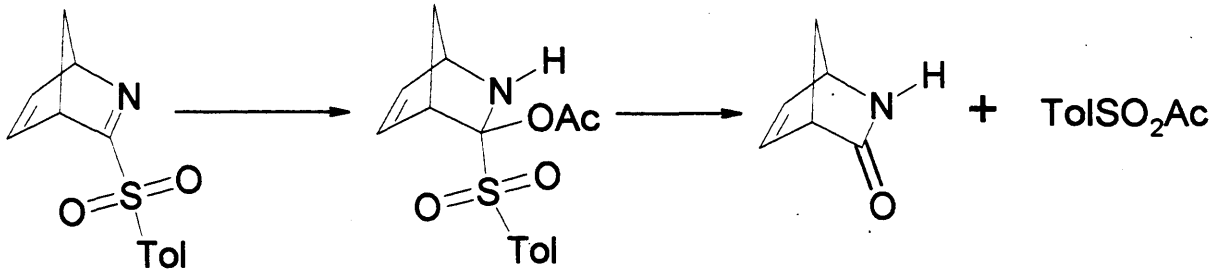
2.2.2 The 2-Azabicyclo[2.2.1]hept-5-en-3-one (44) Approach

The racemic lactam, 2-azabicyclo[2.2.1]hept-5-en-3-one (44) is readily available from a Diels-Alder reaction between cyclopentadiene and tosyl cyanide followed by acidic work up[115,116] (scheme 2.8). The original mechanism proposed for the hydrolysis step involved the addition



Scheme 2.8

of water across the sulphonyl imine group in the presence of acetic acid to generate a tetrahedral intermediate. Collapse of this tetrahedral intermediate would give lactam (44) and tolyl sulphinic acid, the assumption was made that the acetic acid acted only as a catalyst for the addition of water. However, a recent study using both ^{18}O labelled water with acetic acid



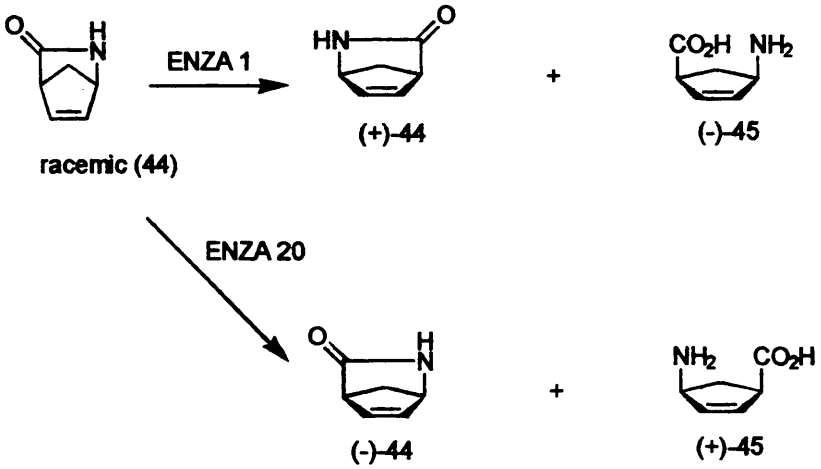
(44)

Scheme 2.9

and ^{18}O labelled acetic acid alone in $CDCl_3$ has shown that the ^{18}O label incorporated into the carbonyl of the lactam (44) comes from the acetic acid and not the water. This study also showed that the insoluble byproduct of this reaction was a mixture of acetyl tolyl sulphinate and an addition product of acetyl tolyl sulphinate with cyclopentadiene[117] and not tosyl tolyl sulphinate as originally reported (scheme 2.9).[115]

The racemic lactam (44) can be kinetically resolved into the two separate enantiomers by the use of two distinct whole cell biocatalysts ENZA-1 (*Rhodococcus* equi NC1B 40213) and ENZA-20 (*Pseudomonas solanacearum* NC1B 40249) (scheme 2.10). Mutant strains of ENZA-1 and ENZA-20 have also been constructed, these mutants hyperexpress the γ-lactamase activity.[118] Use of the mutants as whole cell biocatalysts has enabled extremely rapid rates of catalysis to be carried out in the presence of very high substrate concentrations,

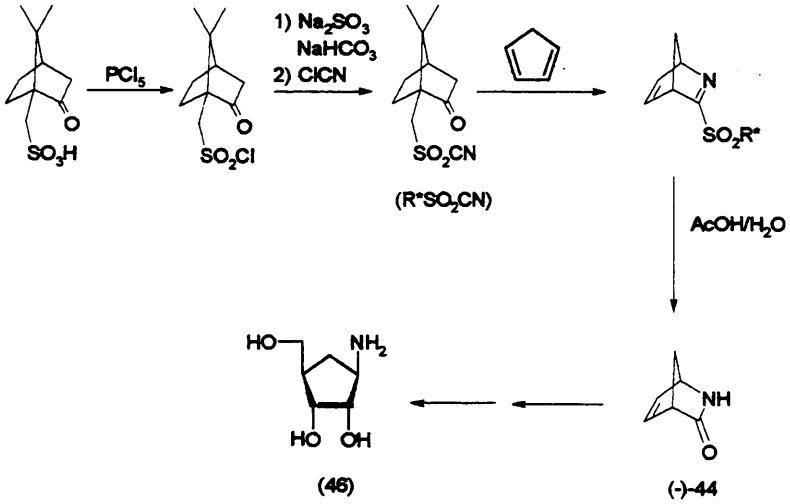
>100gl^{-1}. (+)-Lactam (44) was obtained with >98% e.e., 45% yield from the fermentation with ENZA-1, and (-)-lactam (44) was similarly obtained with >98% e.e., 45% yield from the fermentation with ENZA-20.[118]



Scheme 2.10

The optically active amino acids (+) or (-)(45) can be recovered from the aqueous phase after removal of the corresponding lactam (44) by acidification (HCl) and removal of the water to give the hydrochloride salt. The optically active (-)-lactams (44) and (-)-amino acids (45) are useful starting materials for the synthesis of carbocyclic nucleosides.[118,119,120]

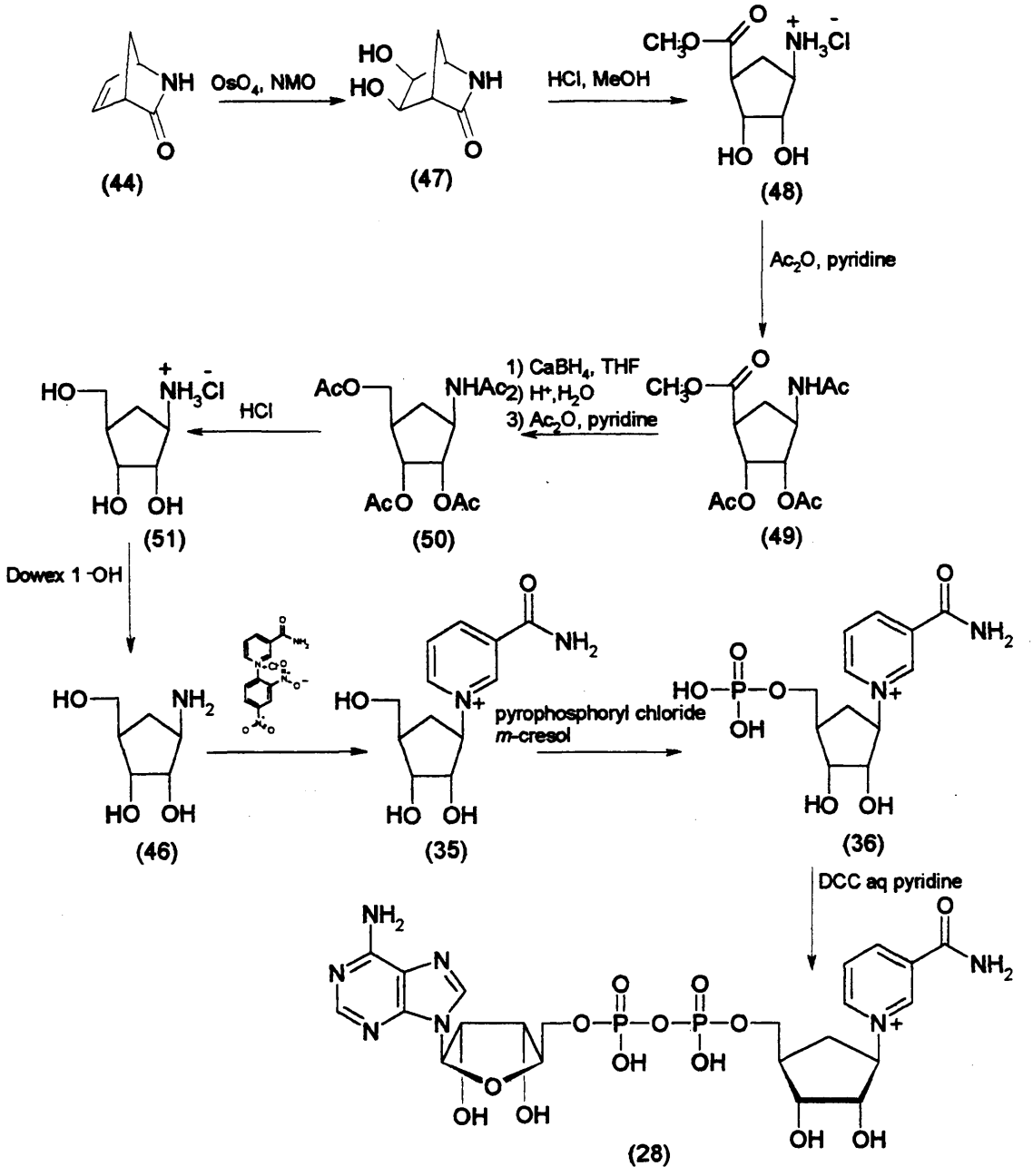
The lactam (44) can also be synthesised enantioselectively from an asymmetric Diels-Alder reaction between cyclopentadiene and a chiral dienophile, usually a chiral sulphonyl cyanide R*-SO_2-CN (scheme 2.11).[121] Both the racemic and chiral (-)-lactam (44) are commercially



Scheme 2.11

available. The chiral lactam (44) used in this study was kindly donated by Chiros Ltd. The lactam (44) was used as the starting material for the synthesis of carbocyclic NAD^+ (28).

Racemic lactam (44) was used to prepare a mixture of carbocyclic NAD^+ (28) and pseudo carbocyclic NAD^+ (29) which are separable by HPLC,[87] the chiral (-)-lactam (44) gives only one diastereoisomer (28). Scheme 2.12 shows the proposed route to the carbocyclic analogue of NAD^+ (28) from the lactam (44).

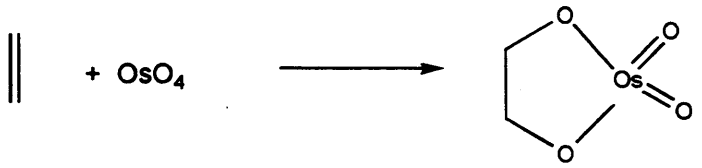


Proposed route to caba NAD^+ (28)

Scheme 2.12

2.2.2.1 Preparation of *exo-cis*-5,6-Dihydroxy- 2-azabicyclo[2.2.1] heptan-3-one (47)

The diol (47) was prepared from the lactam (44) by dihydroxylation using a catalytic amount of osmium tetroxide with N-methyl morpholine N-oxide (NMO) as the cooxidant.[122] Osmium tetroxide (OsO_4) is a very reliable reagent for the *cis* hydroxylation of alkenes to the corresponding *cis* diol,[123] unfortunately it is highly toxic and consequently it is rarely used stoichiometrically. However, OsO_4 can be used catalytically in the presence of a secondary oxidant such as NMO,[122,124] hydrogen peroxide,[125,126,127] metal chlorates[128,129] or sodium hypochlorite.[130] The *cis* hydroxylation of alkenes by OsO_4 is well established to take place via an osmium (VI) intermediate which on reductive or oxidative hydrolysis yields the *cis* diol[123,131,132] (scheme 2.13).



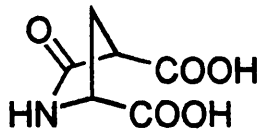
Scheme 2.13

The role of the cooxidant is to oxidise the osmium (VI) intermediate back to Os(VIII) in order to oxidise more of the alkene. The use of OsO_4 for the *cis* hydroxylation of alkenes has been reviewed by Schroder.[133]

In this study the diol (47) was liberated using sodium metabisulphite[134,135] to reductively hydrolyse the Os(VI) intermediates to give lower forms of osmium which were removed by filtration. The N-methyl morpholine by-product was removed by azeotroping the residue with water.[122] The crude diol (47) was purified by precipitation from THF and methanol.

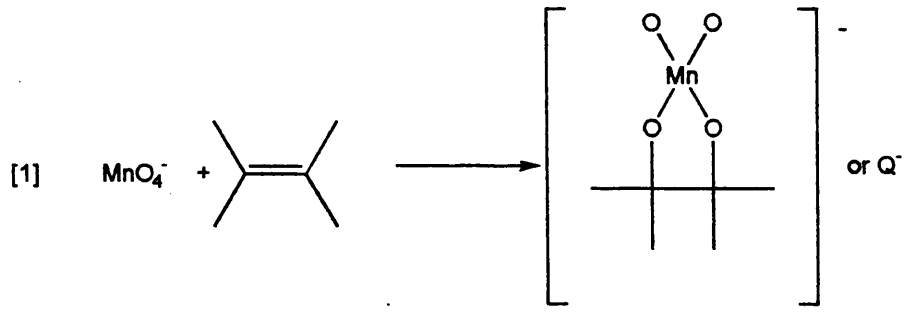
It was claimed in the literature[136] that the dihydroxylation reaction could be carried out using potassium permanganate as the oxidant with greater than 90% yield of diol (47). The use of $KMnO_4$ instead of OsO_4 would have been preferable as $KMnO_4$ is much less toxic than OsO_4 and much cheaper. Unfortunately when the reaction was attempted in this study the best yield of diol (47) obtained was 30% compared to 80% for the OsO_4 reaction.

Potassium permanganate is a strong oxidising agent and the diol (47) could be oxidised to give the ring opened diacid compound (52). This should always happen in acidic or neutral solutions, but even in alkaline solution the yields were still less than 30%. It has been suggested in the literature[137] that increased stirring improves the yield of diols from $KMnO_4$ oxidations, but again this failed to increase the yield to above 30%. The permanganate reaction like the osmium tetroxide reaction is believed to proceed via a cyclic ester

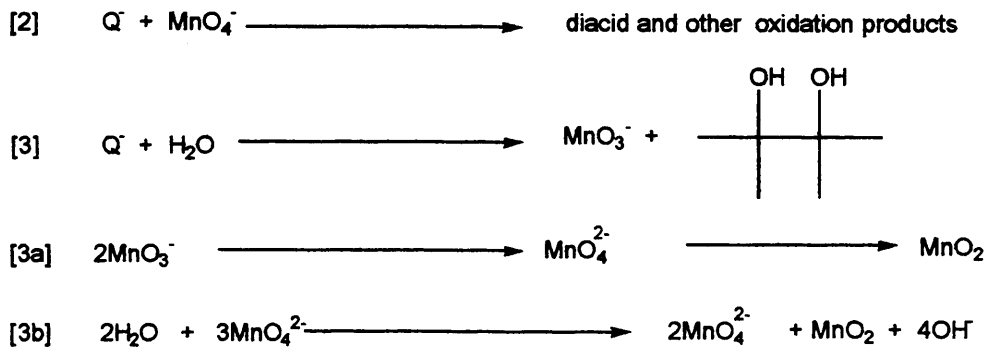


(52)

intermediate. The following mechanism was proposed to explain why increased stirring is expected to improve the yield of diol[137] (scheme 2.14).



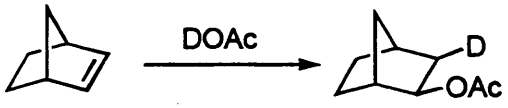
Once formed, Q^- may then undergo either of two alternative decompositions, one with xs MnO_4^- and the other with solvent water.



Scheme 2.14

Increased stirring reduces the probability of further oxidation by increasing the dispersion of the alkene and lowering the local concentration of permanganate. The use of $KMnO_4$ as an oxidant in organic chemistry has been reviewed by Fatiadi.[138] The permanganate reaction was abandoned in favour of the osmium tetroxide reaction as the latter reliably gave the diol (47) with >80% yield.

Dihydroxylation gave only the *exo* addition product. The OsO_4 approaches from the least hindered face of the double bond. In norbornene (53), electrophilic additions are mainly syn additions[139] and attack in these cases is always from the *exo* face[140,141,142,143] (e.g. scheme 2.15), unless the *exo* face is blocked by substituents in the 7 position, in which case *endo* attack may predominate.[144,145] The lactam (44) is similar to norbornene (53) and would be expected to react in a similar way to give only the *exo* addition product.

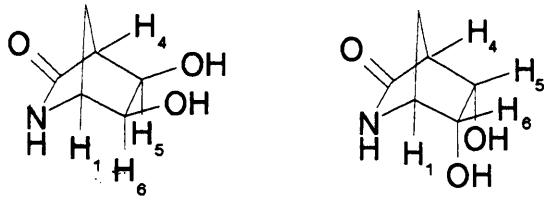


(53) (54)

exo addition product

Scheme 2.15

The Karplus equation relates the vicinal proton coupling constants to the dihedral angle therefore in a simple case if the dihydroxylation was *endo*, then the 1,6 and 4,5 protons of the diol (47) would have dihedral angles of approximately 0°; and the vicinal coupling constants $J_{1,6}$ and $J_{4,5}$ would be about 5-6Hz, similar to that for the *cis* 5,6 protons ($J_{5,6}$=5.8Hz). An *exo* hydroxylation would give rise to a *trans* orientation for the 1,6 and 4,5 protons which gives a dihedral angle ca. 90°, the coupling constant would be <2Hz. The observed $J_{1,6}$ and $J_{4,5}$ are <2Hz consistent with *exo* attack (figure 2.5). However, there are limitations to the use of the Karplus equation which must be considered. The vicinal coupling constants are dependent upon the C-C bond length, the dihedral angle, the orientation of substituents and the electronegativity of substituents. The most difficult factor to deal with is the effect of substituent electronegativity, and in this case the two hydroxyl substituents may cause problems with the simple Karplus relationship.



exo dihydroxylation *endo* dihydroxylation

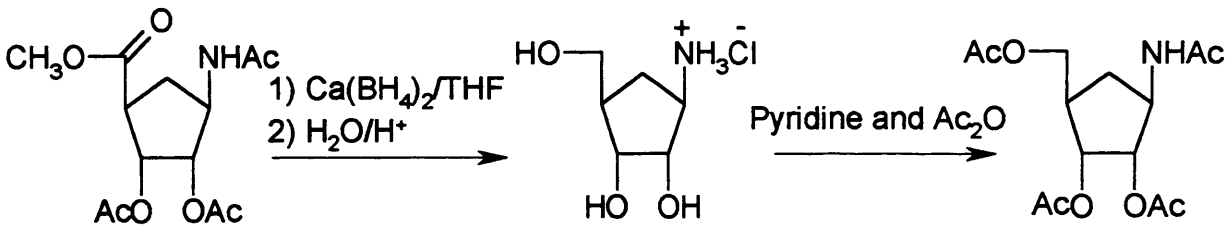
Figure 2.5

2.2.2.2 Preparation of Methyl 4β-Amino-2α,3α-dihydroxy-1β-cyclopentanecarboxylate Hydrochloride (48) and Methyl 4β-Acetamido-2α,3α-diacetoxy-1β-cyclopentanecarboxylate (49)

Ester (48) was readily prepared from the diol (47) by refluxing with methanolic HCl,[122] the residual syrup could be purified by recrystallisation from THF and methanol. The yields were quantitative. Ester (48) was readily converted to the triacetate (49) using pyridine and acetic anhydride.[122] This conversion was necessary as previous attempts to reduce the unprotected form (48) were unsuccessful. The ester (48) was converted to the triacetate (49) which made the compound more readily soluble in organic solvents and therefore increased the range of reducing agents which could be used.

2.2.2.3 Preparation of Methyl 4β-Acetamido-2α,3α-diacetoxy-1β-(acetoxymethyl)cyclopentane (50)

Tetracetate (50) was prepared from triacetate (49) using calcium borohydride in THF as the reducing agent (scheme 2.16). The calcium borohydride was produced *in situ* from sodium borohydride and anhydrous calcium chloride.[146] To ensure complete conversion to the calcium borohydride the calcium chloride needed to be very finely ground and the reaction mixture had to be stirred vigorously at room temperature for 8 hours. If complete conversion to calcium borohydride had not occurred before the addition of triacetate (49) then a mixture of tetracetate (50) and unreacted triacetate (49) was obtained, as sodium borohydride is not an efficient reagent for the reduction of esters to alcohols. The borates can be removed by evaporation with methanol, and conversion of the crude triol (51) to the tetracetate (50) allows the product to be extracted into organic solvent leaving behind the rest of the inorganic matter in the aqueous layer. (50) was purified using flash chromatography on silica gel 60, the column was eluted with CH_2Cl_2 followed by 1% MeOH in CH_2Cl_2, then 2% MeOH in CH_2Cl_2, then 5% MeOH in CH_2Cl_2, and finally 10% MeOH in CH_2Cl_2. This gave (50) as a yellow oil in 75% yield.



(49) (51) (50)

Scheme 2.16

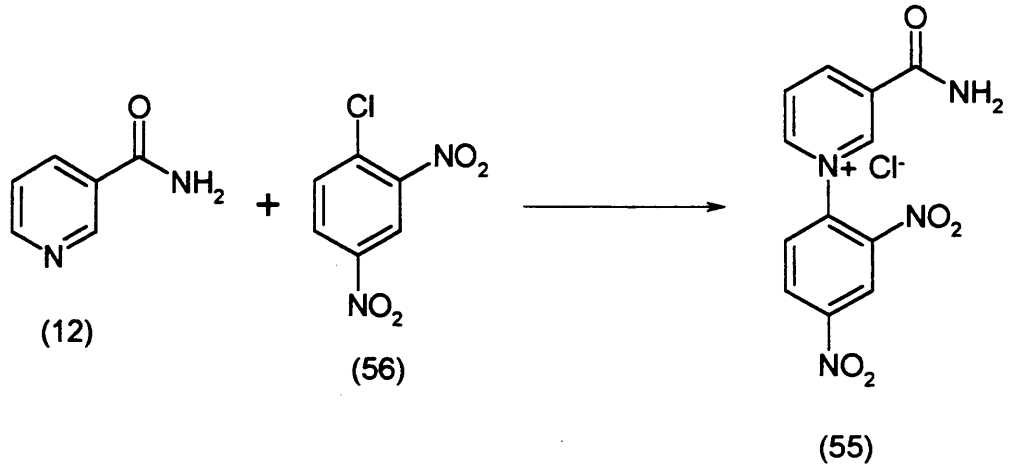
Other attempts to perform the reaction were carried out using different reducing agents, "super hydride" or lithium triethyl borohydride was used according to the method of Oppenheimer.[136] Very low yields of reduced material (51) were obtained (<20%) and this material was very crude, although conversion to the tetracetate (50) would have helped to separate the product from the inorganic residue. Lithium aluminium hydride was also used, but there was incomplete conversion to the triol (51); and even after conversion to the tetracetate (50) followed by extraction with an organic solvent the product was still contaminated by inorganic material. In this study the best reagent for the reduction step was found to be calcium borohydride in THF.

2.2.2.4 Preparation of 4β-Amino-2α,3α-dihydroxy-1β-(hydroxymethyl)cyclopentane Hydrochloride (51) and 4β-Amino-2α,3α-dihydroxy-1β-(hydroxymethyl)cyclopentane (46)

The triol (51) was prepared in quantitative yield by acidic hydrolysis of tetracetate (50).[122] This was readily converted into the free amine (46) by ion exchange. Next the triol (51) was dissolved in MeOH and passed through a column of Dowex 1 OH^- form to give amine (46), which can be readily converted into a variety of carbocyclic nucleosides.[87,114,115,116]

2.2.2.5 Preparation of N1 (2,4-dinitrophenyl) 3 carbamoyl-pyridinium chloride (55)

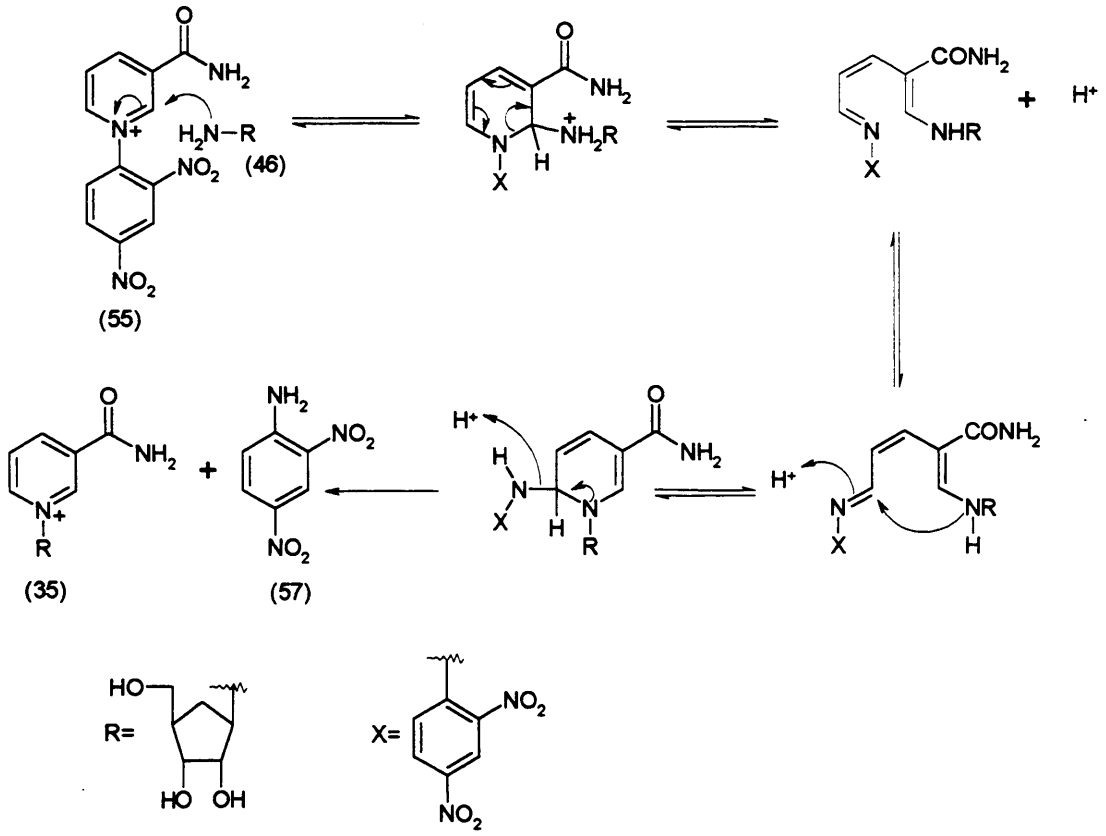
The nicotinamide addition product (55) was prepared from the addition reaction between nicotinamide (12) and 2,4-dinitrochlorobenzene (56) (scheme 2.17).[147] The nicotinamide addition product (55) was purified by precipitation from methanol and diethyl ether.



Scheme 2.17

2.2.2.6 Preparation of the Carbocyclic Nicotinamide Nucleoside (35)

The carbocyclic nicotinamide nucleoside (35) was prepared by the method of Sicsic *et al*,[147] nicotinamide nucleoside has also been prepared by this method.[85] This is an example of a Zinke reaction between N1 (2,4-dinitrophenyl) 3 carbamoyl-pyridinium chloride (55) and the amine (46) (scheme 2.18). This reaction is analogous to a base catalysed reaction of NAD^+ (9) (scheme 2.2).

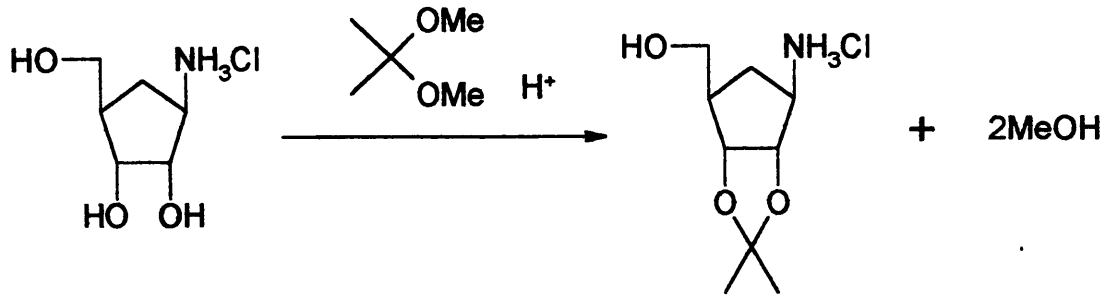


Scheme 2.18

The nicotinamide addition product (55) and the amine (46) were stirred together at room temperature and pressure for 48 hours. The 2,4-dinitroaniline by-product (57) was removed by extraction with ether. Ion exchange chromatography (Dowex 50 H^+ form, HCl 0 to 0.8M) gave a pure sample of the carbocyclic nucleoside (35).

2.2.2.7 Preparation of the Carbocyclic Nicotinamide Nucleotide (36)

Initially attempts were made to protect the 2' and 3' hydroxyl positions of the triol (51) with an isopropylidene group, the idea being that the protected triol could then be converted to the protected nucleoside and then phosphorylated to give the protected nucleotide. The method used was acetal exchange using 2,2-dimethoxypropane, as this method liberates two moles of methanol there is no need to use a dehydrating agent (scheme 2.19). Unfortunately none of

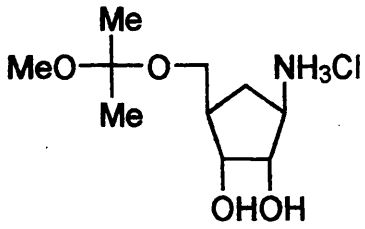


(51) (58)

Proposed route to isopropylidene derivative (58)

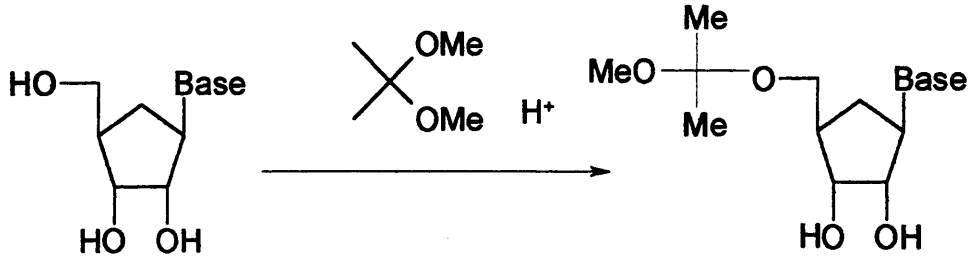
Scheme 2.19

the isopropylidene derivative (58) was detected by ^{1}H nmr spectroscopy, the main product appeared to be (59). This was demonstrated by a singlet in the ^{1}H nmr spectrum at 3.2ppm with an integration of 3H (MeO) and a singlet at 1.3ppm with an integration of 6H (2xMe).



(59)

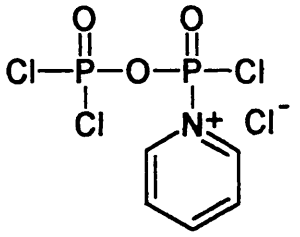
Hampton[149] reported that nucleosides react selectively with 2,2-dimethoxypropane at the 5'-hydroxyl group (scheme 2.20).



Scheme 2.20

However, direct phosphorylation of an unprotected nucleoside at the 5'-hydroxyl position is possible. Several methods have been reported, these include the use of phosphoryl chloride in wet trialkyl phosphate,[54] di(2-*tert*-butyl phenyl) phosphorochloridate,[150] pyrophosphoryl chloride in *m*-cresol,[55] phosphoryl chloride in the presence of water, pyridine and

acetonitrile,[151] and the method used by Todd and co-workers in their synthesis of NAD^+ (9) which is phosphoryl chloride in nitromethane and water.[83] Sowa and Ouchi[151] claim that direct phosphorylation of the 5'OH using phosphoryl chloride in the presence of water, pyridine and acetonitrile involves the formation of an adduct between pyrophosphoryl chloride and pyridinium chloride to which the structure of trichlorophosphopyridinium chloride (60) was assigned. Most of the above methods claim >90% yield and selectivity for the formation of the 5'-nucleotide, although later work[151] has cast doubt on the yields and selectivity claimed for the method using wet trialkyl phosphate as the solvent.[54]



(60)

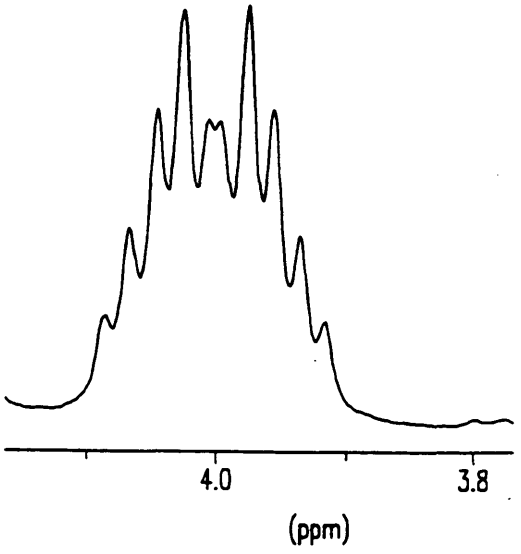
Phosphoryl chloride is usually a very reactive phosphorylating agent which phosphorylates all of the 2'-,3'- and 5'- hydroxyl positions indiscriminantly, to a mixture of all three mononucleotides.

Three of the above methods were applied to the synthesis of the carbocyclic nicotinamide nucleotide (36).[55,83,151] The method using phosphoryl chloride in pyridine and acetonitrile[151] yielded some material which was phosphorylated at the 6'- position, but unfortunately the yields were low and the material was very impure.

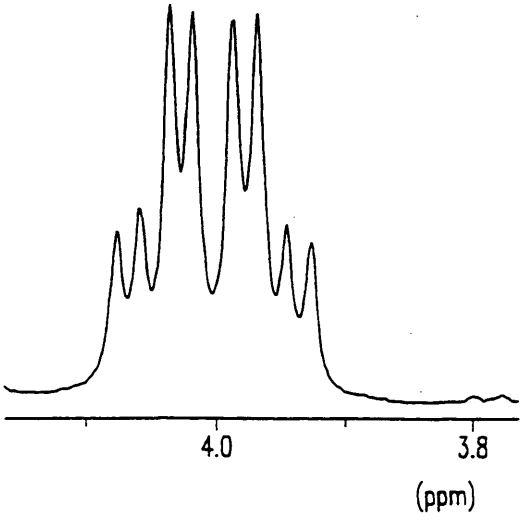
The method of Todd and co-workers[83] appeared to be giving material which was phosphorylated at the 2'- and 3'- hydroxyl positions, this was demonstrated by ^1H-{^{31}P} nmr spectroscopy which showed a loss of coupling to the 2'- and 3'- protons on ^{31}P decoupling, again the material was very impure.

The best method found for the direct phosphorylation at the 6'- position of the carbocyclic nucleoside (35) in this study was the use of pyrophosphoryl chloride in *m*-cresol.,[55] followed by hydrolysis. The crude material was purified by ion exchange chromatography. The material was dissolved in water and the pH adjusted to 5, then it was passed through a column of Dowex-2 (acetate form) to remove the chloride and phosphate ions. The last traces of

carbocyclic nucleotide (36) were washed from the column with 0.001M acetic acid. Unreacted carbocyclic nicotinamide nucleoside (35) and free nicotinamide (12) were removed by passage through a column of Dowex-50 (H^+ form), and finally a purified sample of the carbocyclic nucleotide (36) was obtained as the triethylammonium salt by passage through a column of DEAE sephadex eluted with a gradient of 25mM→200mM TEAB. The triethyl ammonium salt was obtained in 26% yield after removal of excess TEAB by evaporation with methanol. The position of phosphorylation was confirmed by ^1H-{^{31}P}nmr spectroscopy, the proton signal at 3.76ppm for 6'-H and 6"-H collapsed from a 10 peak multiplet to an 8 peak multiplet after ^{31}P decoupling, none of the other proton signals were affected (figure 2.6). The 6'- and 6''- protons are prochiral and diastereotopic and therefore they are chemically non equivalent. After ^{31}P decoupling the observed splitting pattern is ABX where A and B are the 6'- and 6''- protons and X is the 4'- proton of the cyclopentane ring. Further evidence was obtained from the ^{13}C nmr spectra, the signal at 68.0ppm for the 6'- carbon atom was split into a doublet by the ^{31}P and shifted downfield from the 6'-C signal in the nucleoside (62.9ppm). The signal for the adjacent atom 4'-C at 46.0ppm was also split into a doublet and shifted slightly downfield. The signals for the 2'-C and 3'-C were not split by the ^{31}P. Although direct phosphorylations of unprotected nucleosides generally give lower yields than phosphorylations of the corresponding protected nucleosides, the overall yield for the conversion may be better since the route involving introduction and removal of protecting groups increases the synthetic route by two steps (protection and deprotection), with the expected losses at each stage.



^{1}H NMR signal for 6'-H and 6''-H in carbocyclic nicotinamide nucleotide (36)



^{1}H $\{^{31}P\}$ NMR signal for 6'-H and 6''-H in carbocyclic nicotinamide nucleotide (36)

Figure 2.6

2.2.2.8 Preparation of Carbocyclic NAD^+ (28) and Pseudo Carbocyclic NAD^+ (29)

In the original synthesis of NAD^+ (9),[83] nicotinamide mononucleotide (13) and AMP (1) were coupled using dicyclohexylcarbodiimide (DCC) in aqueous pyridine. There are three possible products from this reaction, the AMP (1) can undergo self condensation to give AP^1P^2A, the NMN (13) can undergo self condensation to give P^1P^2- di-(nicotinamide mononucleoside) pyrophosphate, or the AMP (1) and NMN (13) can react with each other to give NAD^+ (9) as the desired product. In practise very little of the P^1P^2- di-(nicotinamide mononucleoside) pyrophosphate was formed. Hughes *et al.*[83] suggested that this may be due to the way the charges in a dipolar ion interact affecting participation in a carbodiimide condensation. A similar effect was observed in the DCC coupling reaction between choline phosphate and cytidine-5' phosphate.[153]

In this study when carbocyclic NMN (36) and AMP (1) were coupled using DCC in aqueous pyridine, no P^1P^2- di-(carbocyclic nicotinamide mononucleoside) pyrophosphate was observed. The product was isolated using ion exchange chromatography (DEAE Sephadex), unreacted carbocyclic NMN (36) starting material was recovered and could be recycled. The only other compounds to be detected were AMP (1) and AP^1P^2A.

When racemic carba NMN (36) was used as the starting material both diastereoisomers carba NAD^+ (28) and pseudo carba NAD^+ (29) were produced. When (-)-carba NMN (36) was used in the homochiral synthesis carba NAD^+ (28) was produced as a single diastereoisomer.

2.2.2.9 Separation of the Diastereoisomers Carbocyclic NAD^+ (28) and Pseudo Carbocyclic NAD^+ (29)

The diastereoisomers (28) and (29) from the synthesis using racemic lactam (44) were separated by HPLC[87] on a preparative scale using a reverse phase C-18 column. When the conditions of Slama and Simmons[87] were used the product remained on the column for over 2 hours and the peaks were very broad and poorly resolved. In this study the diastereoisomers were separated using a buffer of 20mM sodium dihydrogen phosphate and 2mM

tetrabutylammonium phosphate adjusted to pH 6.0 with tetrabutylammonium hydroxide, a gradient of methanol (10-30%) was applied over a period of 40 minutes. The diastereoisomers came off as two separate peaks, carba NAD^+ (28) eluted from the column first with a retention time of 24.3 minutes, immediately followed by pseudo carba NAD^+ (29) with a retention time of 25.0 minutes.

2.3 Summary

Both carbocyclic NAD^+ (28) and pseudo carbocyclic NAD^+ (29) were prepared and isolated starting from the racemic lactam (44). Carbocyclic NAD^+ (28) was prepared as a single diastereoisomer when (-)-(44) was used as the starting material. The single diastereoisomer of carbocyclic NAD^+ (28) is 99+% diastereomerically pure. This can be seen from the ^1H nmr data. In the spectra of the diastereoisomeric mixture of carbocyclic NAD^+ (28) and pseudo carbocyclic NAD^+ (29) two signals are visible representing the two distinct types of anomeric protons, whereas in the spectra of the single diastereoisomer of carba NAD^+ (28) only one doublet is seen in the anomeric region. Also when a sample of the single diastereoisomer (28) is run through an HPLC column using the same conditions that were employed to separate (28) and (29) (section 2.2.2.9) only one peak was observed.

CHAPTER 3

EVALUATION OF CARBA NAD$^+$ (28)

Chapter 3

Evaluation of Carba NAD^+ (28)

3.0 Introduction

3.0.1 Stereospecificity of Dehydrogenases

Ever since the pyridine coenzymes NAD^+ (9) and $NADP^+$ (10) were discovered,[58,62] there has been much speculation about the mechanism of hydrogen transfer. Karrer suggested that hydrogen transfer involved the pyridine ring[154] but he did not establish the actual site of reduction. It was Westheimer and Vennesland[155,156] who showed that hydrogen transfer took place *para* to the nitrogen atom of the nicotinamide ring (figure 3.1). This was established in the following way; chemically deuterated NAD^+ was oxidized enzymically and then cleaved by

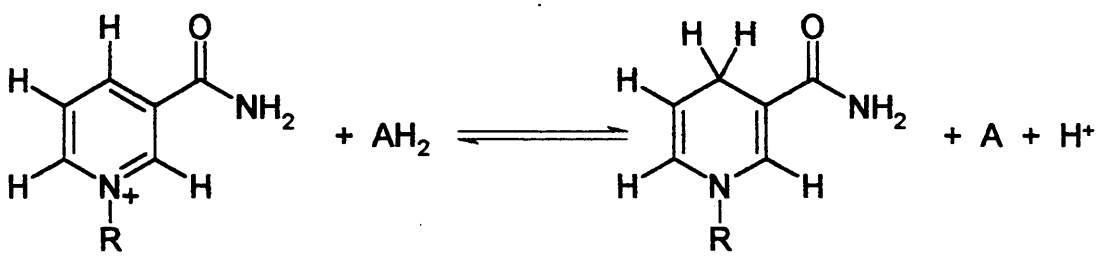
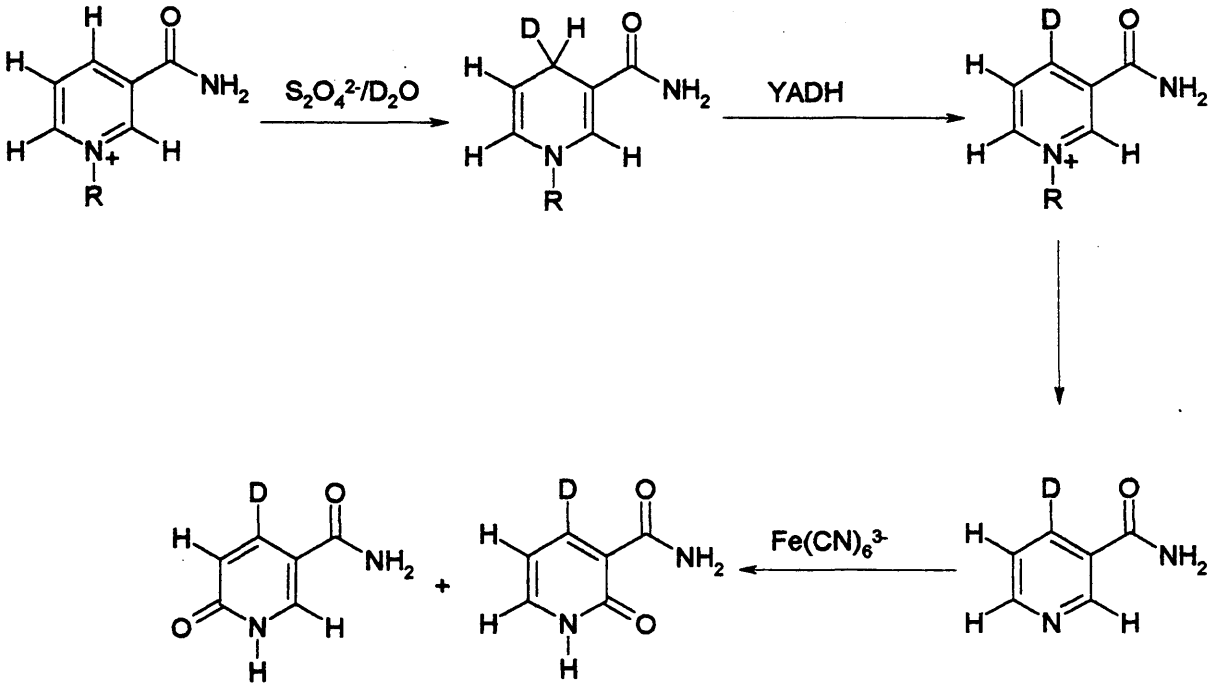


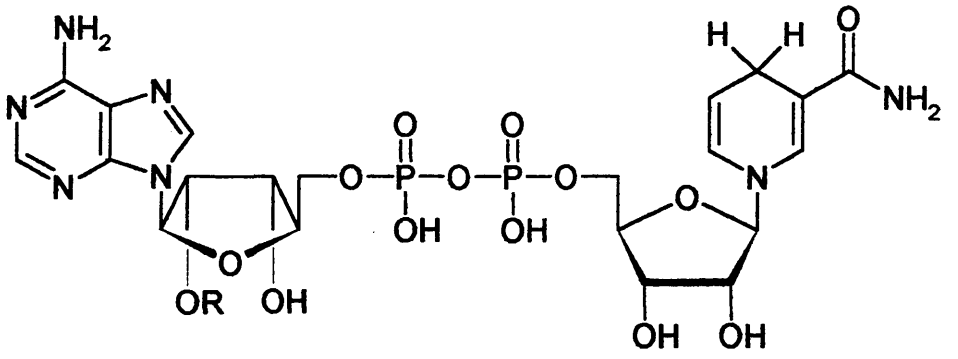
Figure 3.1

means of a nicotinamide adenine dinucleotidase to deuterated nicotinamide. This was oxidised with alkaline ferricyanide with no loss of deuterium. Alkaline ferricyanide oxidises N-methyl nicotinamide to a mixture of 2 and 6-pyridones,[157] therefore neither the 2- nor the 6- position was involved in the reduction of NAD^+ (9) (scheme 3.1).[158] Further evidence was provided by Westheimer, who showed that only N-benzyl 4-deutero nicotinamide transferred deuterium to a model hydrogen acceptor; neither the 2- nor the 6- deuterated N-benzyl nicotinamides transferred deuterium.[159] Nmr spectra of dihydro N-methyl nicotinamide and the deuterated analogue confirmed that reduction takes place at the 4-position of the nicotinamide ring,[160,161] therefore NADH may be formulated as (30). In 1951 it was discovered that the hydride transfer process was direct[162] and a year later Fischer and coworkers were the first to report that the yeast alcohol dehydrogenase catalysed reaction between NAD^+ (9) and ethanol was stereospecific with respect to the coenzyme.[155]



Scheme 3.1

The 4- position of the dihydronicotinamide ring is prochiral and therefore two diastereoisomers of NADD (62) are possible (figure 3.2). Fisher prepared NADD (62) by two methods, one chemical and the other enzymatic. The enzymatic method involved YADH and deuterated ethanol (equation 3.1) and the chemical method involved reduction with sodium



R=H NADH (30)

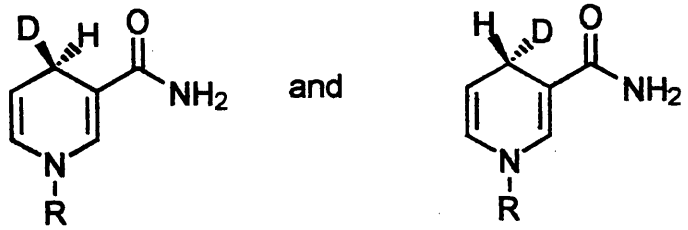
$R=PO_3H_2$ NADPH (61)

dithionite in D_2O. Both samples of NADD (62) were then oxidised with YADH and acetaldehyde, the chemically prepared NADD (62) gave a mixture of NAD^+ (9) and $NAD(D)^+$ (63) whereas the enzymatically prepared NADD (62) gave only NAD^+ (9). This demonstrates that the chemically prepared NADD (62) is a mixture of diastereoisomers $NADD_R$ (62a) and $NADD_S$ (62b) and that the enzymatically prepared NADD (62) was a single diastereoisomer. The two diastereoisomers were not produced in a 1:1 mixture by the chemical reduction of NAD^+ (9), the $NADD_R$ (62a) was preferred to the $NADD_S$ (62b), at the

$$CH_3CD_2OH + NAD^+ \rightleftharpoons NADD + CH_3CDO$$

Equation 3.1

time this was thought to be within experimental error, however later work by Pullman[158] found that the preference for the *re* face of the ring was even greater than originally thought. This phenomenon is due to the coenzyme adopting a folded conformation in solution in which the adenine ring blocks the *si* face of the nicotinamide ring and makes it more hindered to attack.



$NADD_R$ (62a) $NADD_S$ (62b)

carba $NADD_R$ (64a) carba $NADD_S$ (64b)

Figure 3.2

There are two classes of enzyme (class A and class B) which require NAD^+ (9) as a cofactor, they differ only in terms of the direction of attack on the pyridine ring by the hydride ion during the reduction step. Class A enzymes transfer to the *re* face of NAD^+ (9) or $NADPH^+$ (10) and use the *pro-R* hydrogen of NADH (30) or NADPH (61). Class B enzymes transfer to the *si* face and use the *pro-S* hydrogen (figure 3.3).

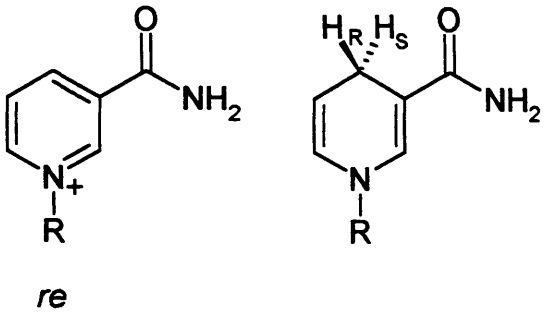


Figure 3.3

The stereochemical ambivalence in dehydrogenases is unusual, since stereochemical uniformity is arguably more common for most classes of enzymes. Many theories and generalisations have been proposed to explain the stereochemical choices made by dehydrogenases and most of these have been summarized by You.[163] A lot of theories have fallen down when tested with other enzymes and many others have been very restrictive. The controversial theory proposed by Benner and coworkers in 1983[164] is described below in more detail. Benner suggested that the stereoselectivity of NAD^+ (9) dependent alcohol dehydrogenases (transferring either the *pro-R* or *pro-S* hydrogen of NADH (30)) correlates with the thermodynamic stability of their substrates, and appears to reflect evolutionary pressure to adjust the conformation of NADH (30) in the active site so as to match the cofactor's reducing power to the oxidizability of the substrate. He postulated that the stereochemical preferences of dehydrogenases reflect their evolution under selective pressure to become optimal catalysts; and that they can be understood by consideration of the following points:

(1) Enzymatic transfer of the *pro-R* hydrogen of NADH (30) occurs when the nicotinamide ring is in the *anti* conformation, and transfer of the *pro-S* hydrogen occurs when the ring is in the *syn* conformation (figure 3.4).

(2) *Anti*-NADH is a weaker reducing agent than *syn*-NADH.

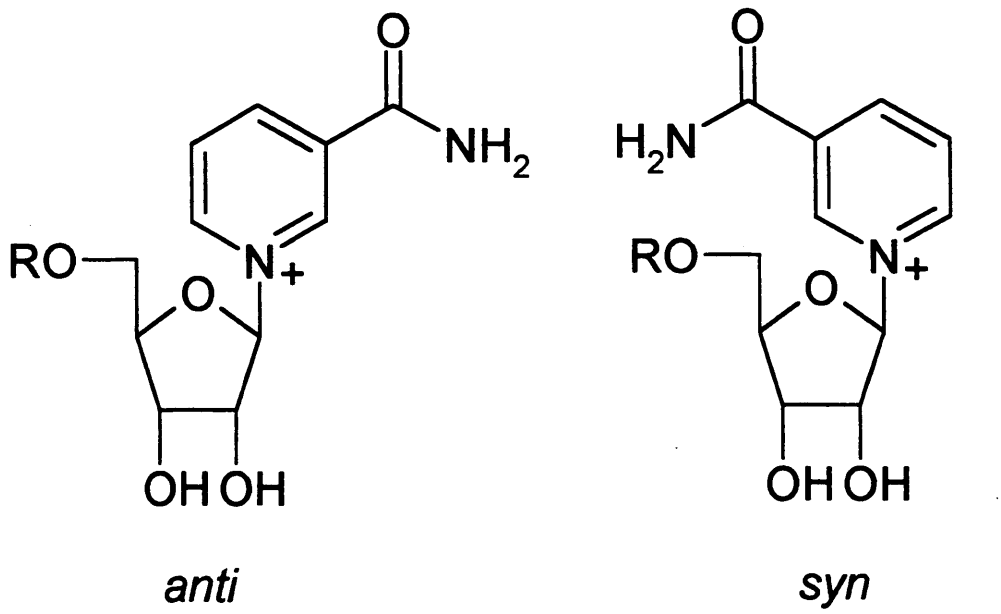


Figure 3.4

(3) Enzymes are optimally efficient when the intermediate states along the reaction coordinate are of equal free energies.

(4) Dehydrogenases have evolved to be optimally efficient catalysts.

Crystal structures of four dehydrogenases[164] led to suggestions[165,166] that *pro-R* dehydrogenases bind the NAD^+ (9) cofactor in an *anti* conformation in the catalytically important complexes and *pro-S* dehydrogenases bind the cofactor in a *syn* conformation. This hypothesis was first proposed by You in 1978.[165]

Benner suggested that the nicotinamide ring is twisted into a boat configuration to optimise electron donation of the nitrogen lone pair into the antibonding orbital of the C-O bond in the ribose ring, this is a "reverse anomeric effect".[167] (figure 3.5) When in the *anti* conformation this makes the *pro-R* hydrogen axial and in the *syn* conformation the *pro-S* hydrogen is axial. Principles of orbital overlap suggest that the axial hydrogen is most easily transferred. A boat conformation would make the nitrogen pyramidal and this would enhance the carbonium ion character at the 4-position of the nicotinamide ring. This is consistent with the large ^{15}N isotope effects which have been observed.

The equilibrium constants between the *syn* and *anti* forms of both the oxidised and reduced cofactors were estimated from NMR data on nicotinamide mononucleotide,[168,169] this can be

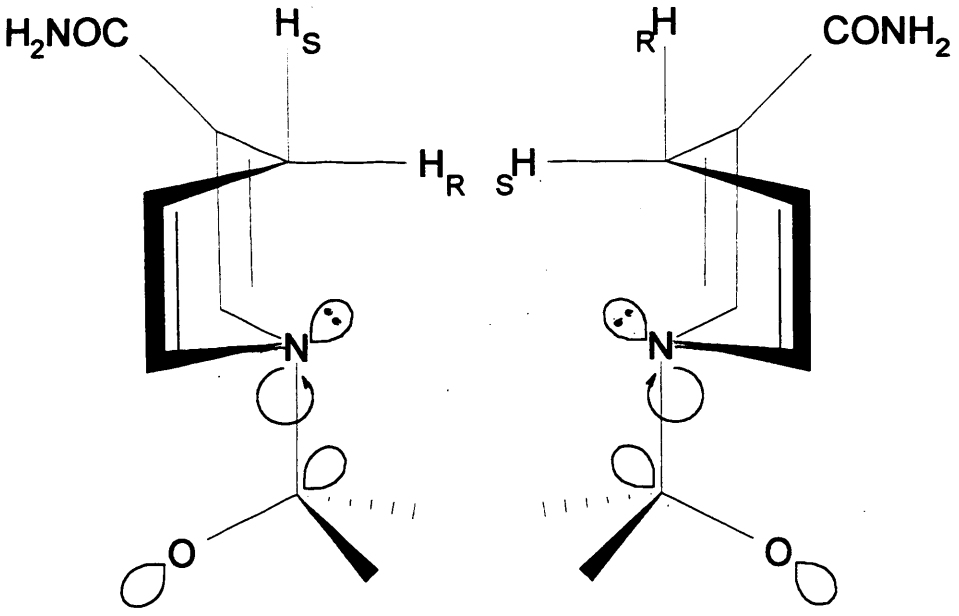


Figure 3.5

expressed qualitatively as in figure 3.6. There has been experimental data for almost a dozen enzymes which showed that the 'internal' equilibrium constant (that between the enzyme bound species) is close to unity, even when the 'external' equilibrium constant (between free species) favours reactants or products by 3 orders of magnitude or more.[170,171] Albery and Knowles have suggested that such an arrangement might be expected for an efficient catalyst, especially one that acts reversibly.[171] Benner summarised his findings thus; "Dehydrogenases

select the weaker reducing agent (*anti* NADH, and hence the *pro-R* hydrogen) to reduce more reactive carbonyls, and the stronger reducing agent (*syn* NADH, and hence the *pro-S* hydrogen) to reduce less reactive carbonyls."

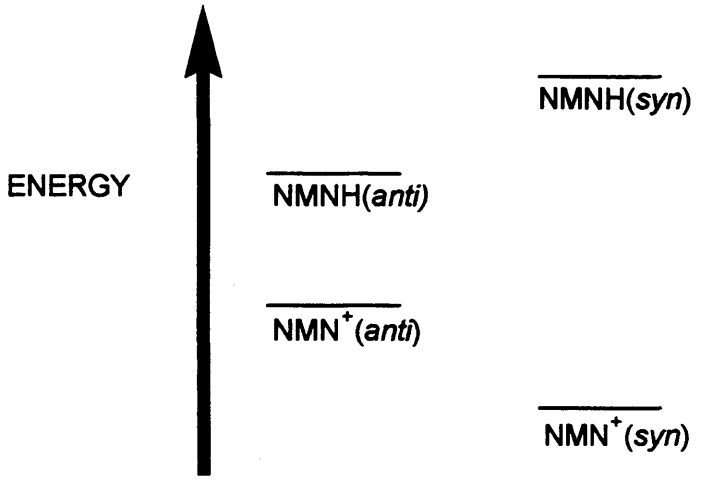


Figure 3.6

Benner used his findings to predict that lactaldehyde reductase from pig kidney specifically transfers the *pro-R* hydrogen of NADPH (61), he then proved that this was correct using stereochemical experiments.[157] However, Oppenheimer reviewed published data for three dehydrogenases that met Benner's criteria[173] but did not fit the correlation. He concluded "the dehydrogenases, however remain as intractable as ever and have yet to yield the secrets of their underlying principles." In later work Benner and co-workers[174] noted the challenge to their theory and claimed that it reflected a misstatement of their thesis and overlooked most of the available data. They also claimed that the challenge overlooked the general requirement that the physiological role of an enzyme must be "well defined" if data from that enzyme is to be used to test a functional theory in bioorganic chemistry. You[163] has also criticised Benner's theory because to demonstrate their theory Benner and coworkers used lactaldehyde reductase, the natural substrate of this enzyme is not known and is exactly the sort of enzyme which they wanted to avoid. Also the stereospecificity of this enzyme has been known since 1966.

The carbocyclic analogue of NAD^+ (28) is of interest to probe the stereoelectronics of reductases since the C-O σ^* orbital would no longer be available for the nitrogen lone pair to overlap with and therefore no reverse anomeric effect could take place.

3.0.2 Mechanism of Dehydrogenation Reactions with NAD^+ (9)

It is generally thought that the hydrogen transfer process occurs via a hydride ion H^- transfer, the oxidation-reduction potential of the system:

$$NAD^+ + H^- \rightleftharpoons NADH$$

has been measured as -0.320V at pH7 and 25°.[176] The redox potential of $NADP^+$/NADPH does not differ significantly from that of the NAD^+/NADH system.[177]

Dehydrogenases catalyse the oxidation of alcohols to carbonyl compounds, they use either NAD^+ (9) or $NADP^+$ (10) as cofactors. Some dehydrogenases are specific for one of the coenzymes whilst others will use both. The reactions are reversible so carbonyl compounds can be reduced by NADH (30) or NADPH (61). The reaction rates can be measured by the appearance or disappearance of the reduced coenzyme as it has a characteristic absorbance at 340nm. NADH (30) and NADPH (61) also fluoresce when excited at 340nm, this provides an even more sensitive means of assay.

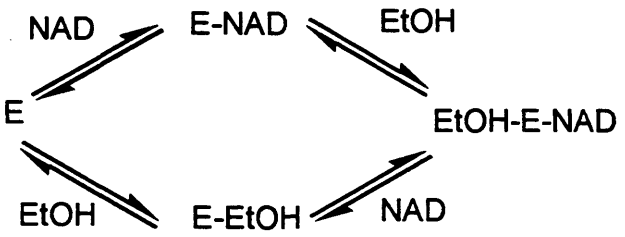
Alcohol dehydrogenases are zinc metalloenzymes of broad specificity, they oxidise a wide range of aliphatic and aromatic alcohols to the corresponding carbonyl compounds. The two most studied alcohol dehydrogenases are horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase, and they both use NAD^+ (9) as a cofactor. Crystal structures of the apo and holo horse liver enzymes have been solved at 2.4 and 4.5A respectively.[178]

LADH is a dimer consisting of two chains of molecular weight 40,000. Each chain contains one NAD^+ (9) binding site and two Zn^{2+} sites, but only one of the Zn^{2+} ions is directly involved

with catalysis. In the unliganded enzyme the coordination of the zinc is a distorted tetrahedron with three protein ligands, cysteines 46 and 176 and histidine 67, and one water molecule. YADH is a tetramer of molecular weight 145,000. In this case each chain has only one NAD^+ (9) and one Zn^{2+} binding site. However it is assumed that both enzymes have the same overall reaction mechanism.[179]

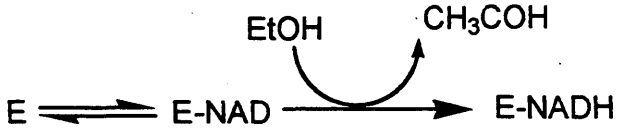
The 3D structure of LADH was reported in 1973,[180] and since then various binary and ternary complexes with NAD^+ (9), NADH (30), inhibitors and poor substrates have been studied.[181] It has been observed that when LADH binds NAD^+ (9), the LADH adopts a closed conformation which binds the alcohol more strongly than the open conformation. The binding of the coenzyme causes a conformational change and this increases the affinity of the enzyme for the other substrate.[182]

LADH has been studied using both steady state and stopped flow kinetics, and it has been shown that the oxidation of alcohols is an ordered mechanism, the coenzyme binds first and the rate determining step is the dissociation of the enzyme-NADH complex.[183,184,185] With YADH the enzyme-product complexes are rapidly dissociated and therefore the chemical steps are rate determining.[186] Kinetic isotope effects have been used to give information about the nature of the bond formation and bond breaking steps in the transition state. For YADH the kinetic isotope effects have been measured for the oxidation of RCD_2OH with NAD^+ (9) and the reduction of RC_6H_4COH with NADD (62).[186,187] The reaction rates are much slower with deuterated substrates giving isotope effects with values of k_H/k_D =3-5. Primary isotope effects with values of 2-15 are good evidence that a C-H bond is being broken during the transition state as C-D bonds are cleaved more slowly than C-H bonds. Pre steady state kinetics have been used to study the hydride transfer process of LADH, similar isotope effects were observed.[188,189] The LADH redox reactions are believed to occur by the Theorell Chance Mechanism[179] whereas the YADH reactions are believed to occur via a random sequential mechanism (figure 3.7).[189]



(1) Random Sequential Mechanism

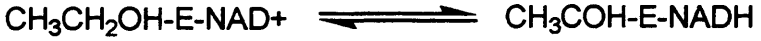
(2) Ordered Mechanism



(3) Theorell-Chance Mechanism

Figure 3.7

The Theorell Chance mechanism is an example of an ordered mechanism where the ternary complex does not accumulate. In this mechanism exchange of the ternary complexes



exchange of ternary complexes

Figure 3.8

(figure 3.8) involves both proton and hydride transfer. Three proposals have been made to explain the mechanism of this transfer (figure 3.9):

(1) The zinc ion binds directly to the reduced substrate and acts as a Lewis acid.[182]

(2) The substrate only enters into the secondary coordination sphere of the zinc ion and it is the zinc bound water molecule which acts as the catalyst, however the geometry of the complex does not seem to be compatible with the crystal structure.[182]

(3) This proposal still has the zinc bound water molecule as the catalyst but this time the substrate is directly coordinated to the zinc.[179]

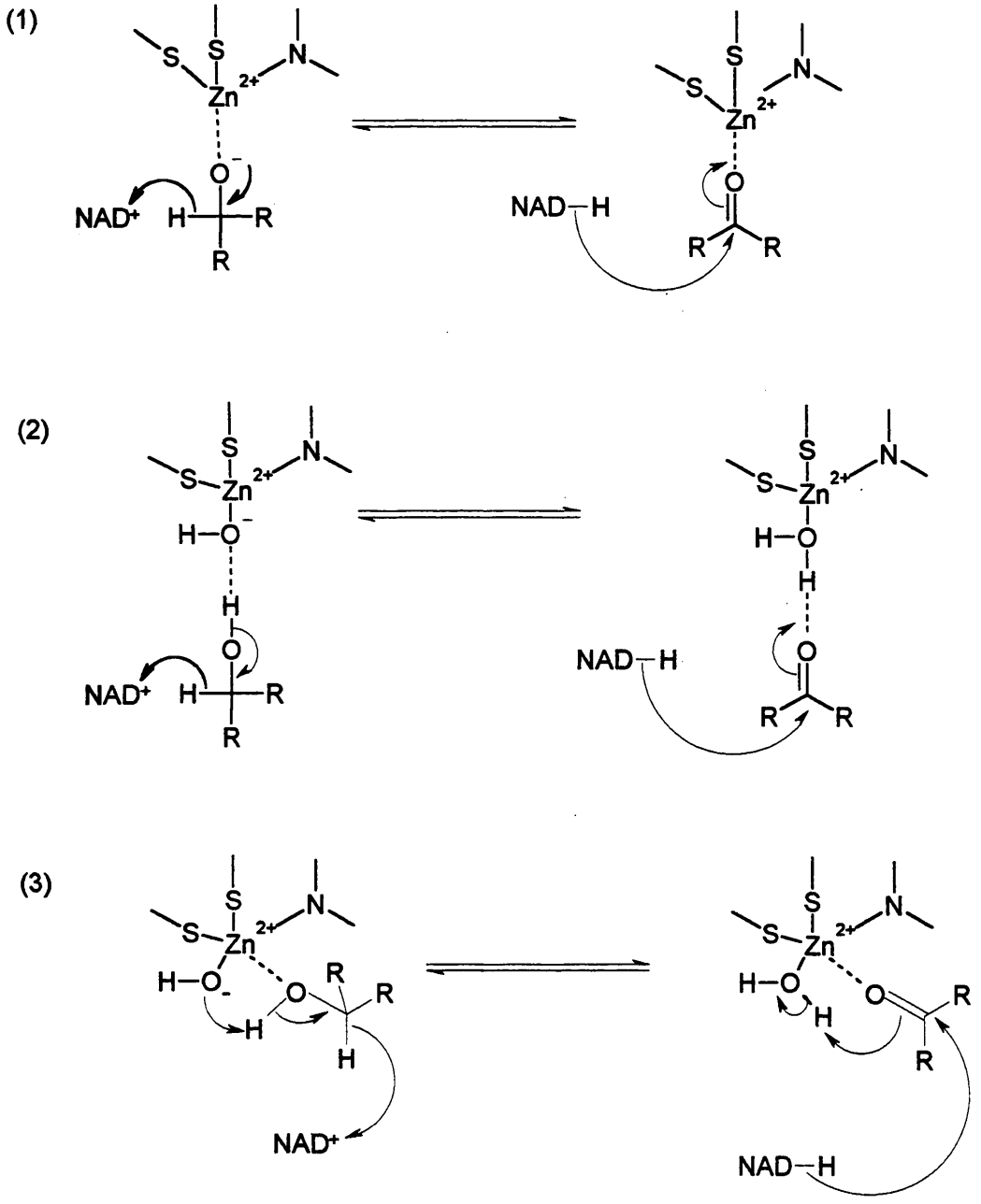


Figure 3.9

There is much less dispute about the hydride transfer step than there is about the proton transfer process. It is believed that the nicotinamide ring adopts a boat conformation which enhances the carbonium ion character of the *para* position. It has been shown that the LADH reaction occurs via hydride transfer rather than via a radical mechanism.[191]

Glycerol-3-phosphate dehydrogenase (1.1.1.8) is a class B dehydrogenase which catalyses the formation of dihydroxyacetone phosphate from L glycerol-3-phosphate with NAD^+ (9) as the cofactor. It was first discovered in mammalian systems by Ichihara and Greenberg in 1957.[192] The main source is rabbit muscle although it can also be obtained from chicken muscle and chicken liver. It has been reported that NADH (30) unfolds to the open chain form when bound to glycerol-3-phosphate dehydrogenase (GPDH)[190] as it does when bound to alcohol dehydrogenase.

In the case of GDPH an ordered mechanism is observed where the coenzyme binds first.[193,194] The glycerophosphate binds after the coenzyme and is bound by the ester group. It has been suggested that the -SH groups are involved in the active site[195] as are the histidyl residues.[190] Vallee and coworkers have also suggested that Zn may be present in the enzyme as in YADH and LADH.[196] The activity of the enzyme is affected by the sulphate ion concentration, and the reduction of dihydroxyacetone phosphate is inhibited by phosphate ions. The enzyme is also inhibited by high concentrations of substrate below the pH optimum. An abortive complex is probably formed, which involves binding of the substrate to the binding site of the product. This binding occurs via the phosphate group which is not involved in the normal binding of the substrate in the active complex, but it is involved in binding the product.[194]

In solution the free coenzyme can be thought of as being in a rapid, dynamic equilibrium between an open extended conformation and a folded conformation in which the nicotinamide ring is associated with the adenine. However, the enzyme bound form of the coenzyme appears to be in the open form in the case of most dehydrogenases. It is assumed that the open form is important for redox activity.

3.1 Aims

The aim of this project was to investigate the stereoelectronics and the stereospecificity of the dehydrogenses using carba NAD^+ (28) to probe Benner's theories. This study also aims to investigate the conformation of carba NAD^+ (28) and the mechanism of the reaction between carba NAD^+ (28) and dehydrogenases and compare this data with data for NAD^+ (9).

3.2 Results and Discussion

3.2.1 Kinetic Properties of Carbocyclic NAD^+ (28)

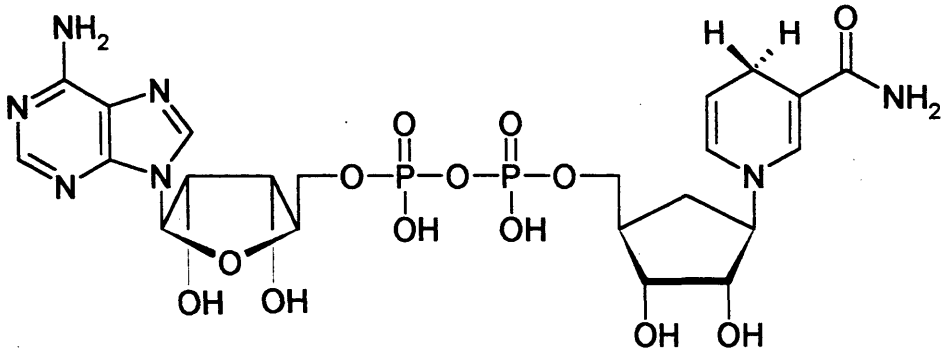
The kinetic properties of carba NAD^+ (28) compared to NAD^+ (9) were measured using two enzymes YADH (1.1.1.1) (class A) and glycerol-3-phosphate dehydrogenase (1.1.1.8) (class B). The K_m, V_{max} and K_{eq} were examined for both enzymes (table 3.1). The reactions were monitored spectrophotometrically to detect the change in concentration of the reduced cofactor. The dihydronicotinamide chromophore absorbance appears at a longer wavelength

		NAD^+ (9)		Carba NAD^+ (28)	
		Observed	Literature	Observed	Literature
YADH (1.1.1.1)	K_m/ M	(4.1±0.3) $\times10^{-4}$	4.5×10^{-4} ref 197	(1.8±0.2) $\times10^{-3}$	1.7×10^{-3} ref 87
	V_{max}/ $\mu Mmin^{-1}unit^{-1}$	(1.6±0.03) $\times10^{-1}$	3.8×10^{-1} ref 197	(1.6±0.5) $\times10^{-1}$	2.8×10^{-1} ref 87
	K_{eq}/M	(1.17±0.2) $\times10^{-11}$	1.15×10^{-11} ref 198	(6.0±0.3) $\times10^{-13}$	3.1×10^{-13} ref 87
GPDH (1.1.1.8)	K_m/ M	(8.8±2)$\times10^{-4}$	3.8×10^{-4} ref 199	(2.5±0.3) $\times10^{-3}$	/
	V_{max}/ $\mu Mmin^{-1}unit^{-1}$	(1.5±0.5) $\times10^{-1}$	/	(0.9±0.5) $\times10^{-1}$	/
	K_{eq}/M	(5.4±0.6) $\times10^{-12}$	1.0×10^{-12} ref 200	(1.9±0.3) $\times10^{-13}$	/

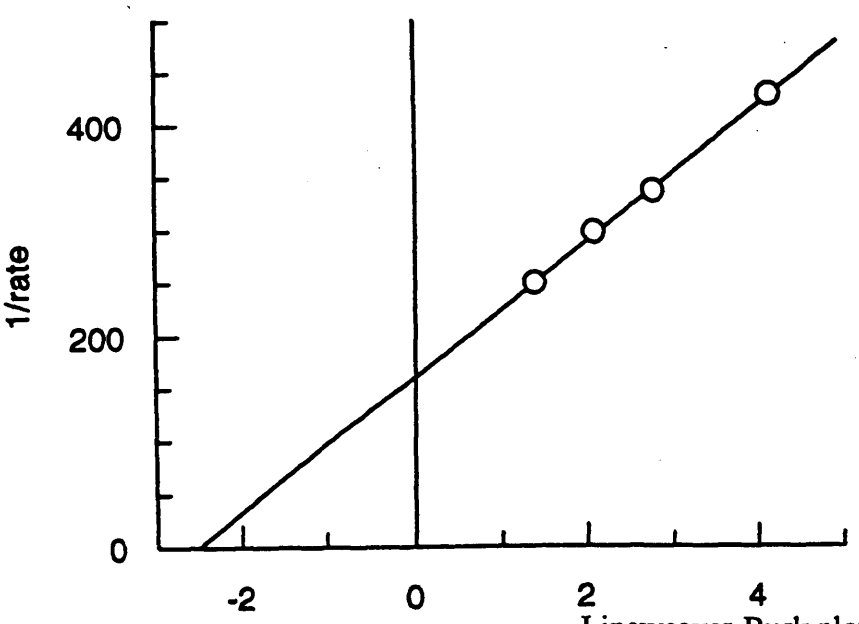
Table 3.1 Kinetic properties of NAD^+ (9) and carba NAD^+ (28) with YADH and GPH

for carba NADH (65) than NADH (30), the NADH (30) absorbance is at 340nm, whereas carba NADH (65) absorbs at 360nm, this is consistent with simple 1-alkylated 1,4-dihydronicotinamides, where the absorption maxima also occurs at 360nm.[87] The results

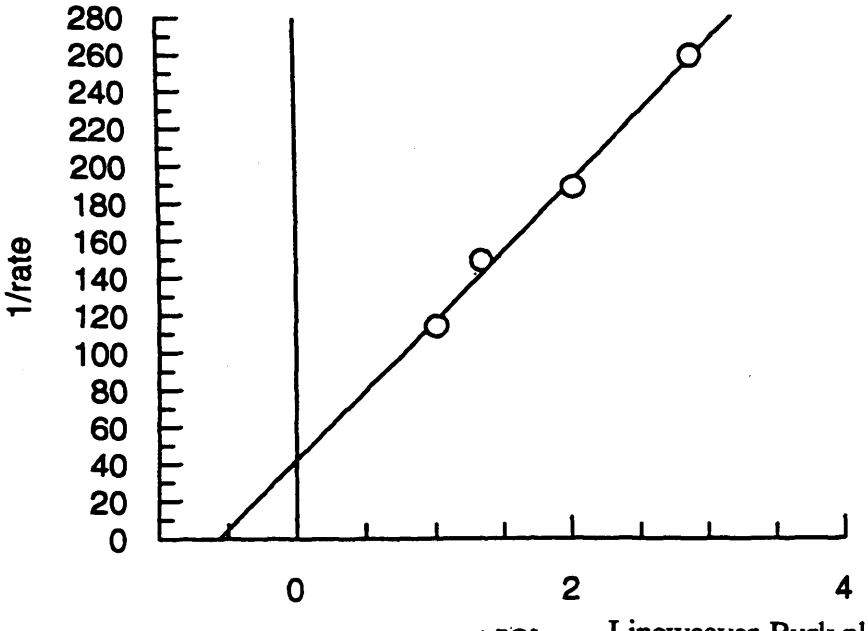
were plotted according to the method of Lineweaver and Burk[201] and straight lines were obtained (figures 3.10a and 3.10b). The K_m value for carba NAD^+ (28) with YADH differs by only a factor of five from the corresponding value for NAD^+ (9) and the K_m value for carba NAD^+ (28) with GPDH differs by only a factor of three from the corresponding value for NAD^+ (9). The V_{max} values are also close, the V_{max} values with YADH are almost identical and only differ by a factor of two for GPDH. It has already been shown that carba NAD^+ (28) is a substrate for YADH and that it can occupy the dinucleotide binding site of these enzymes and make all of the intermolecular contacts which are necessary for substrate recognition.[87] The kinetic data obtained for carba NAD^+ (28) with glycerol-3-phosphate dehyrogenase indicates that this is also true with this enzyme.



carba NADH (65)



Lineweaver-Burk plot for the oxidation of ethanol by NAD^+ (9) catalysed by YADH [S]=[NAD^+] [EtOH]=[0.33M]

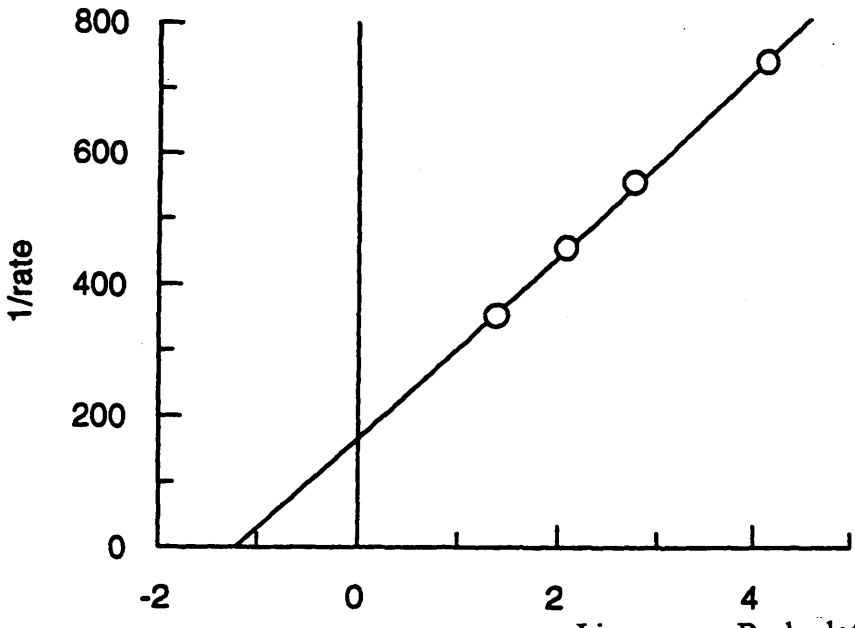


Lineweaver-Burk plot for the oxidation of ethanol by carbaNAD$^+$ (28) catalysed by YADH [S]=[carbaNAD$^+$] [EtOH]=[0.33M]

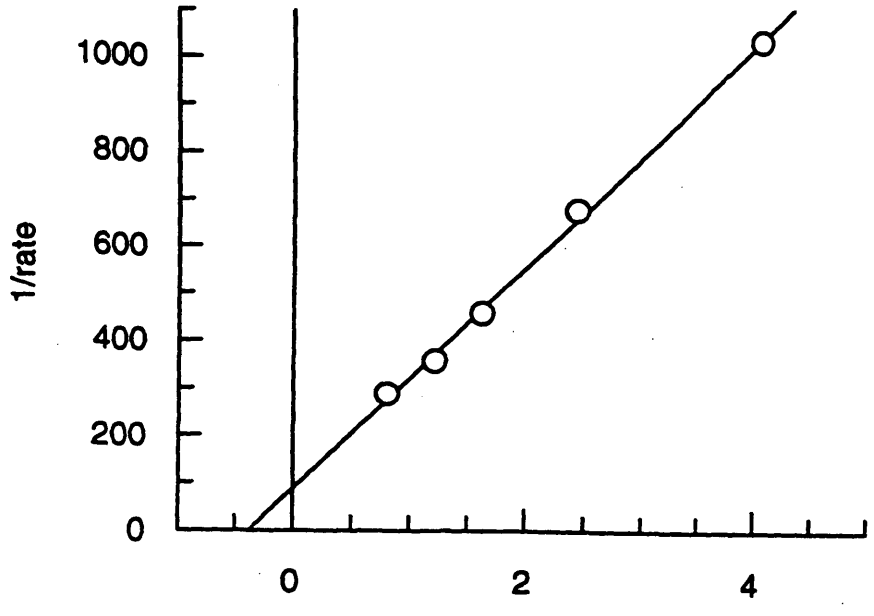
Figure 3.10a

[S] in mM

ν in μmoles min^{-1}



Lineweaver-Burk plot for the oxidation of glycerol-3-phosphate by NAD^+ (9) catalysed by GPDH [S]=[NAD^+] [glycerol-3-phosphate]=[42mM]



Lineweaver-Burk plot for the oxidation of glycerol-3-phosphate by carbaNAD$^+$ (28) catalysed by GPDH [S]=[carbaNAD$^+$] [glycerol-3-phosphate]=[42mM]

[S] in mM

Figure 3.10b

ν in μmoles min^{-1}

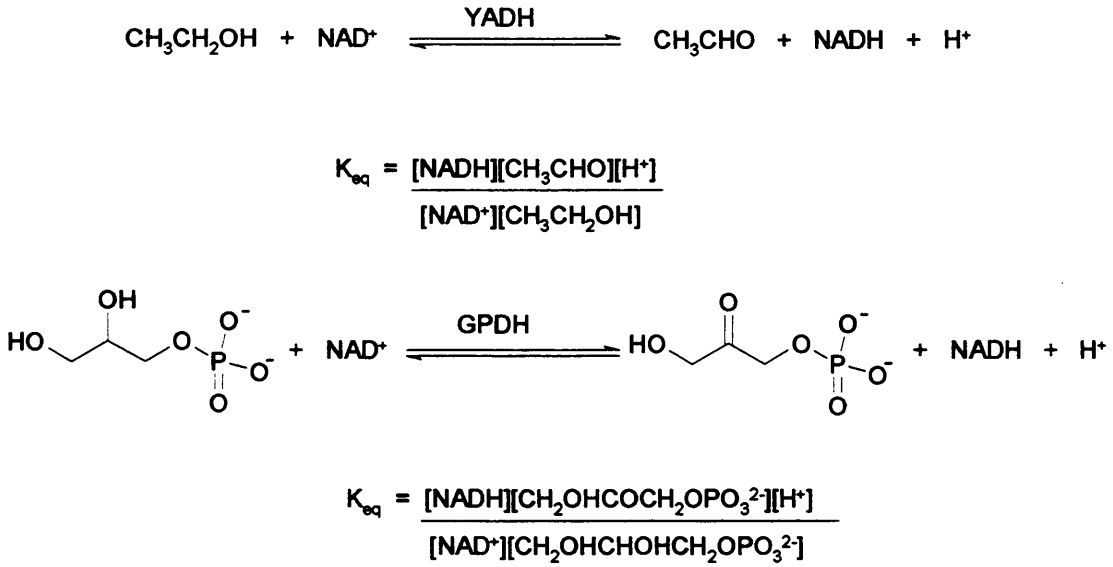


Figure 3.11

The equilibrium constants were determined at pH 8.0 with a mixture of oxidised cofactor, alcohol and excess dehydrogenase (figure 3.11). The concentrations were calculated from absorbance of reduced cofactor at equilibrium, the concentrations of the carbonyl compounds were assumed to be equal to that of the reduced cofactor because of the stoichiometry of the reactions. The observed value for K_{eq} of carba NAD^+ (28) with YADH is twice the literature value,[87] this is because the literature used a mixture of carba NAD^+ (28) and pseudo carba NAD^+ (29). Pseudo carba NAD^+ (29) does not function as a substrate for YADH. The K_{eq} of carba NAD^+ (28) with YADH is twenty times less than the corresponding K_{eq} value of NAD^+ (9) and the K_{eq} value of carba NAD^+ (28) with GPDH is twenty eight times less than the corresponding K_{eq} value of NAD^+ (9) therefore the carbocyclic analogue is less oxidising than NAD^+ (9).

The equilibrium constants for a reaction can be related to the Gibbs free energy for the reaction using the following formula:

$$\Delta G^0 = -RT\ln K_{eq}$$

where $R=8.3144 JK^{-1}mol^{-1}$ and $T=298K$

The Gibbs free energy ΔG^0 values for NAD^+ (9) and carba NAD^+ (28) are shown in table 3.2.

	NAD^+ (9)	carba NAD^+ (28)
YADH (1.1.1.1)	62.4	69.7
GPDH (1.1.1.8)	64.3	72.6

ΔG^0 values in $kJmol^{-1}$

Table 3.2 ΔG^0 values for NAD^+ (9) and carba NAD^+ (28) with YADH and GPDH

The ΔG^0 values for carba NAD^+ (28) are 12% larger than the corresponding values with NAD^+ (9) and for both carba NAD^+ (28) and NAD^+ (9) the values with YADH are 3% smaller than the corresponding values with GPDH. All of the reactions have a positive ΔG^0 value, this means that the reactions will not occur spontaneously, the enzymes are necessary for the reactions to occur. The ΔG^0 values for NAD^+ (9) are smaller and therefore less positive than the corresponding values for carba NAD^+ (28) and therefore the NAD^+ (9) reactions occur more readily than the carba NAD^+ (28) reactions. When a system is at equilibrium the ΔG^0 value is zero. The ΔG^0 values for the natural cofactor NAD^+ (9) and the analogue carba NAD^+ (28) are very similar, differing by less than 10 $kJmol^{-1}$, this indicates that the carba NAD^+ (28) can act as a very good substrate for dehydrogenases.

The ΔG^0 values can be related the reduction potentials for the redox reactions using the following equation:

$$\Delta G^0 (kcal) = -nEF$$

n=number of moles of electrons involved in the reaction

E=cell potential V

$F=23.06 kcalV^{-1}mol^{-1}$

Each redox reaction can be considered to be made up of two half cell reactions and the cell potential E is the sum of the reduction potentials for both half cell reactions. The reduction potentials for the NAD^+ (9) and carba NAD^+ (28) reactions are shown in table 3.3.

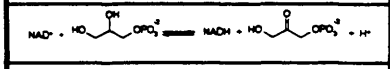
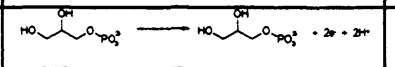
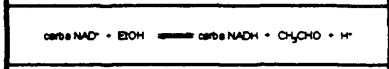
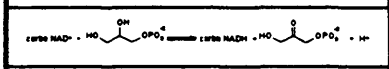
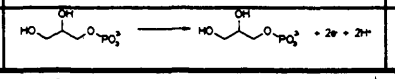
Redox Reaction	Half Cell Reaction	Reduction Potential (V)	Cell Potential E (V)
$NAD^+ + EtOH \rightleftharpoons NADH + CH_3CHO + H^+$	$NAD^+ + H^+ + 2e^- \longrightarrow NADH$	-0.32	-0.323
	$CH_3CH_2OH \longrightarrow CH_3CHO + 2e^- + 2H^+$	-3×10^{-3}	
	$NAD^+ + H^+ + 2e^- \longrightarrow NADH$	-0.32	-0.333
		-13×10^{-3}	
	carbaNAD$^+$ + H$^+$ +2e$^-$ ⟶ carbaNADH	-0.359	-0.362
	$CH_3CH_2OH \longrightarrow CH_3CHO + 2e^- + 2H^+$	-3×10^{-3}	
	carbaNAD$^+$ + H$^+$ +2e$^-$ ⟶ carbaNADH	-0.362	-0.375
		-13×10^{-3}	

Table 3.3 Reduction potentials for the reactions of NAD^+ (9) and carba NAD^+ (28) with YADH and GPDH

The observed reduction potential for the carba NAD^+ (28) half cell reaction is -0.36V the reduction potential for the NAD^+ (9) half cell is known to be -0.32V, this is less negative than the carba NAD^+ (28) value. This indicates that the carba NADH (65) is a stronger reducing agent than NADH (30) and conversely that carba NAD^+ (28) is a weaker oxidising agent than NAD^+ (9), this is consistent with the rest of the observed kinetic data. This was the expected result as it was assumed that the natural cofactor would be a better oxidising agent than the analogue.

According to Benners third hypothesis "enzymes are optimally efficient when they bind intermediate states along the reaction coordinate so that they have equal or nearly equal energies",[164] it is the energies of the bound and not the unbound species which should be considered. However in the measurement of the external K_{eq} it is the unbound species which are being considered. The internal equilibrium constant (K_{eq} internal) has been measured for NAD^+ (9) with YADH and EtOH.[167] This measurement is based on the fact that if there is a stoichiometric excess of enzyme over coenzyme then most of the coenzyme (>90%) will be

enzyme bound. In the literature experiments the K_{eq} internal were measured with the enzyme concentration at least ten times greater than the dissociation constant of the NAD^+ (9).[167] However if the same conditions were used in the determination of K_{eq} internal of carba NAD^+ (28) with YADH it would require an enzyme concentration of 2×10^{-2}M. Having such a high enzyme concentration leads to solubility problems.

In this study the K_{eq} internal of NAD^+ (9) with YADH was measured using the literature conditions[167] and the apparent K_{eq} internal of carba NAD^+ (28) was measured using the same enzyme concentration as in the NAD^+ (9) experiment. The internal equilibrium constant measures the equilibrium between the two ternary complexes (figure 3.8). The observed K_{eq} internal are shown in table 3.4.

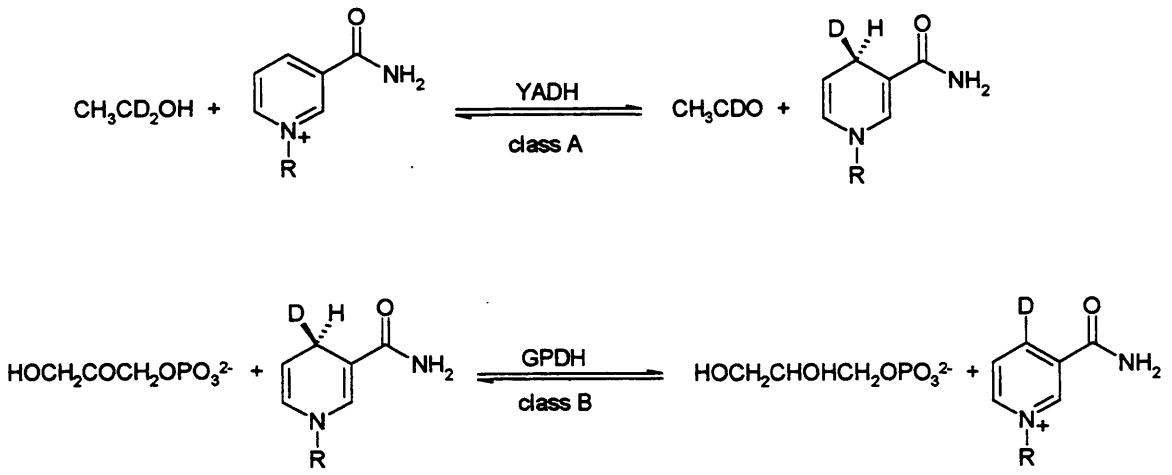
	NAD^+ (9)	carba NAD^+ (28)
literature internal K_{eq}	0.2 ref 167	/
observed internal K_{eq}	0.3	0.4

Table 3.4 Apparent internal equilibrium constants of NAD^+ (9) and carba NAD^+ (28) with YADH

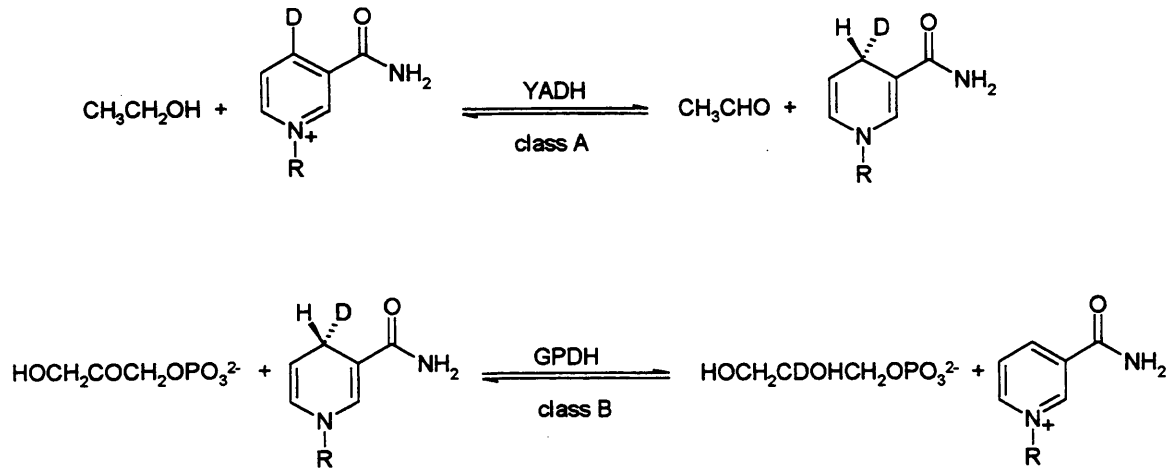
Although this observed internal equilibrium constant with carba NAD^+ (28) can only be taken as a rough estimate of the actual value, it can be seen that the internal equilibrium constants with both NAD^+ (9) and carba NAD^+ (28) are showing the same trend, in other words they are both tending towards unity. This is consistent with the suggestion made by Albery and Knowles and Benners third hypothesis that in order for an enzyme to be optimally efficient it must have an internal equilibrium constant of close to unity i.e. the energies of the two ternary complexes must be almost equal.

Again this is more evidence which confirms the ability of carba NAD^+ (28) to act as a good substrate for dehydrogenases and the similarity in behaviour between the unnatural substrate carba NAD^+ (28) and the natural substrate NAD^+ (9).

3.2.2 Stereospecificity of Dehydrogenases



Experiment 1

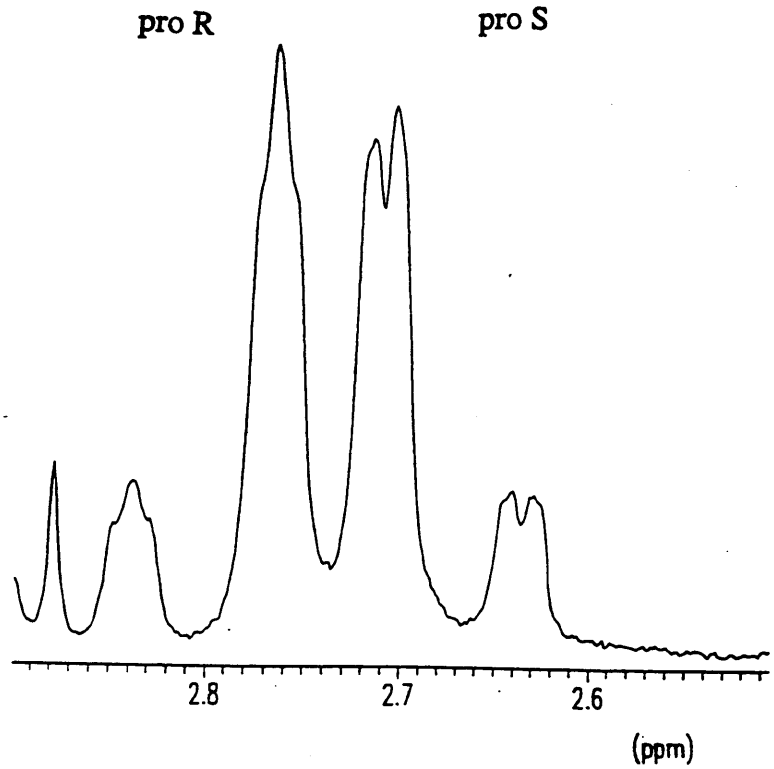


Experiment 2

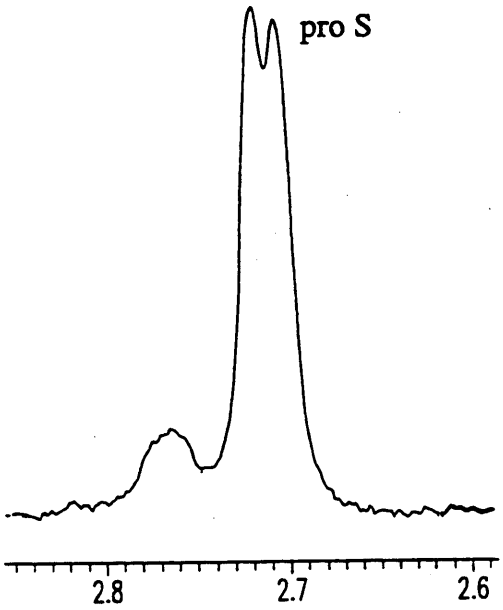
Figure 3.12

Two experiments were set up to examine the stereospecificity of the carba NAD^+ (28) reactions with the class A and class B enzymes (figure 3.12). The first experiment involves the reaction of oxidised cofactor with CH_3CD_2OH in the presence of YADH (class A), the products are separated using ion exchange chromatography and examined by proton nmr spectroscopy. The reduced deuterated cofactor is then oxidised with dihydroxyacetone and glycerol-3-phosphate dehydrogenase. The second experiment is the reduction of deuterated cofactor with ethanol and YADH to give the reduced deuterated cofactor which was then oxidised with dihydroxyacetone and glycerol-3-phosphate dehyrogenase. Two experiments were done to ensure that results were not simply due to a kinetic isotope effect.

With NAD^+ (9) the stereochemistry experiments gave the expected results, NAD^+ (9) with CH_3CD_2OH and YADH gave $NADD_R$ (62a) which was then oxidised by dihydroxyacetone and glycerol-3-phosphate dehyrogenase to give NAD^+ deuterated in the para position $NAD(D)^+$ (63). The deuterated NAD^+ (63) upon reduction with YADH and ethanol gave $NADD_S$ (62b) which was oxidised to give undeuterated NAD^+ (9). As expected the class A enzyme YADH is delivering the hydride (or D) to the *re* face of the NAD^+ (9) and the class B enzyme is transferring the *proS* hydrogen (or deuterium). The results were examined by proton nmr spectroscopy as the N4 methylene protons of the dihydronicotinamide ring are nonequivalent (figure 3.13). The two N4 protons are diastereotopic and could therefore be non-equivalent anyway, but in the reduced mononucleotide NMNH they appear to give a single resonance. The non-equivalence is believed to be due to the fact that in solution the reduced pyridine cofactors are in a rapid dynamic equilibrium between an open extended conformation and a folded stacked conformation in which the dihydropyridine ring is puckered and the B face of the ring is associated with the adenine ring.[202] The *pro R* proton is downfield of the *pro S* proton and the vicinal coupling constant for $J_{4R,5}$ is 3.1Hz whereas $J_{4S,5}$ is 3.9Hz. This indicates that the dihedral angle *4R*-5 is displaced towards 90^0 and *4S*-5 is displaced towards 0^0. In NMNH the coupling constants $J_{4R,5}$ and $J_{4S,5}$ are identical, which provides evidence that it is the adenine ring which causes puckering of the dihydronicotinamide ring. The rapid equilibrium between the two forms means that sharp signals are observed. In the fluoresence excitation spectrum of carba NADH (65)[87] the intensity of excitation at 260nm has been interpreted to mean that carba NADH (65) also adopts a folded stacked conformation in solution. It was therefore assumed that the two N4 methylene protons in carba NADH (65) would also be non-equivalent. This proved to be the case. The presence or absence of



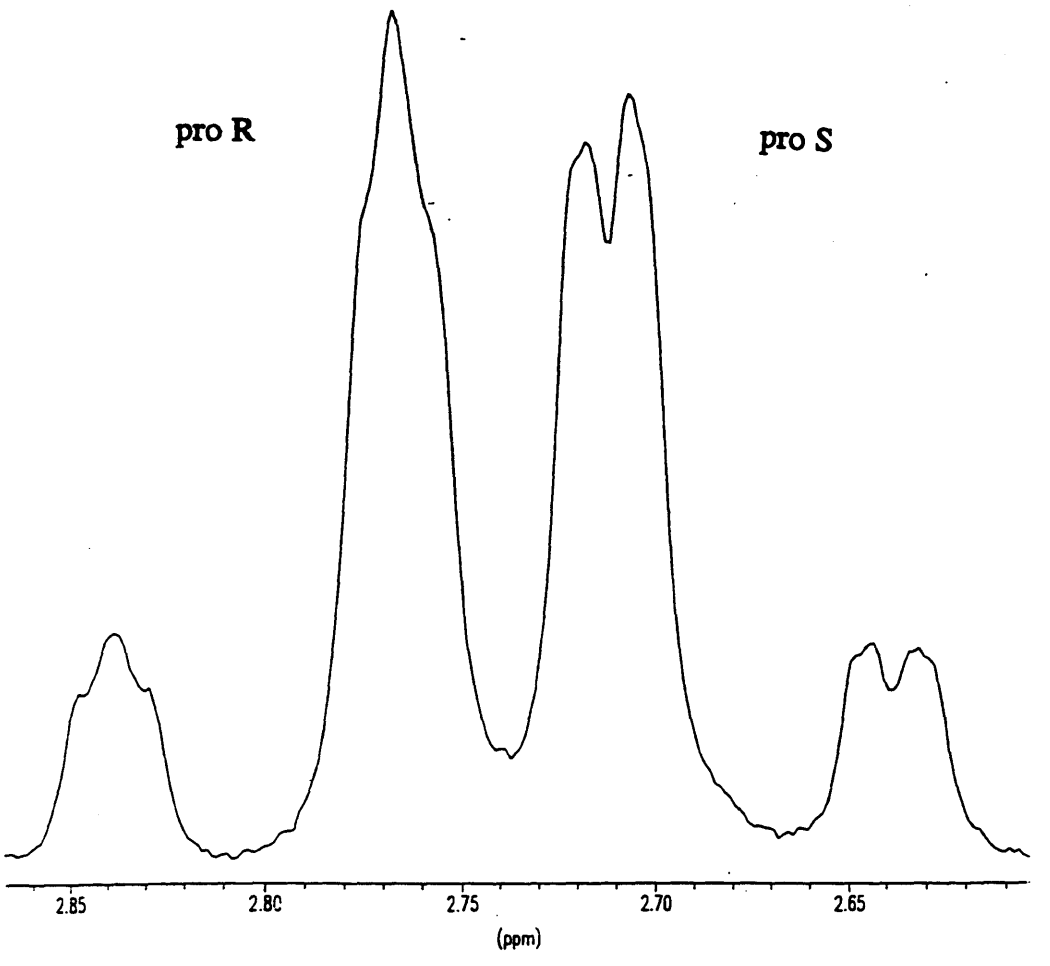
(a) NADH (30)



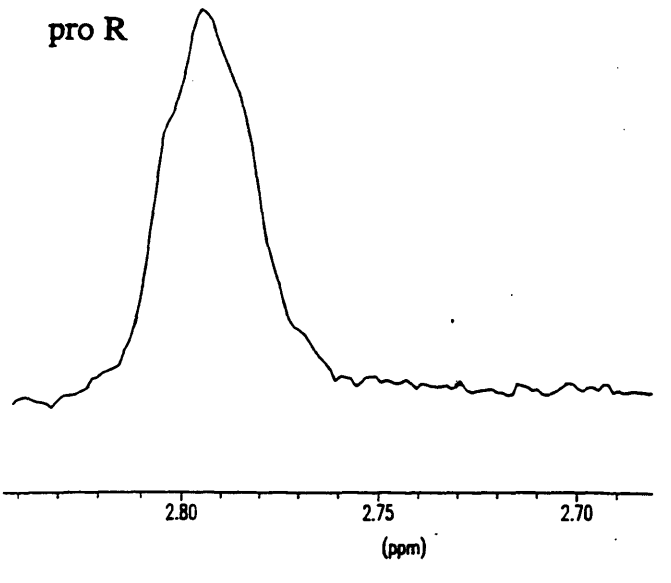
(b) $NADD_R$ (62a)

N-4 methylene proton region of the ^1H nmr spectra of (a) NADH (30) and (b) $NADD_R$ (62a)

Figure 3.13a



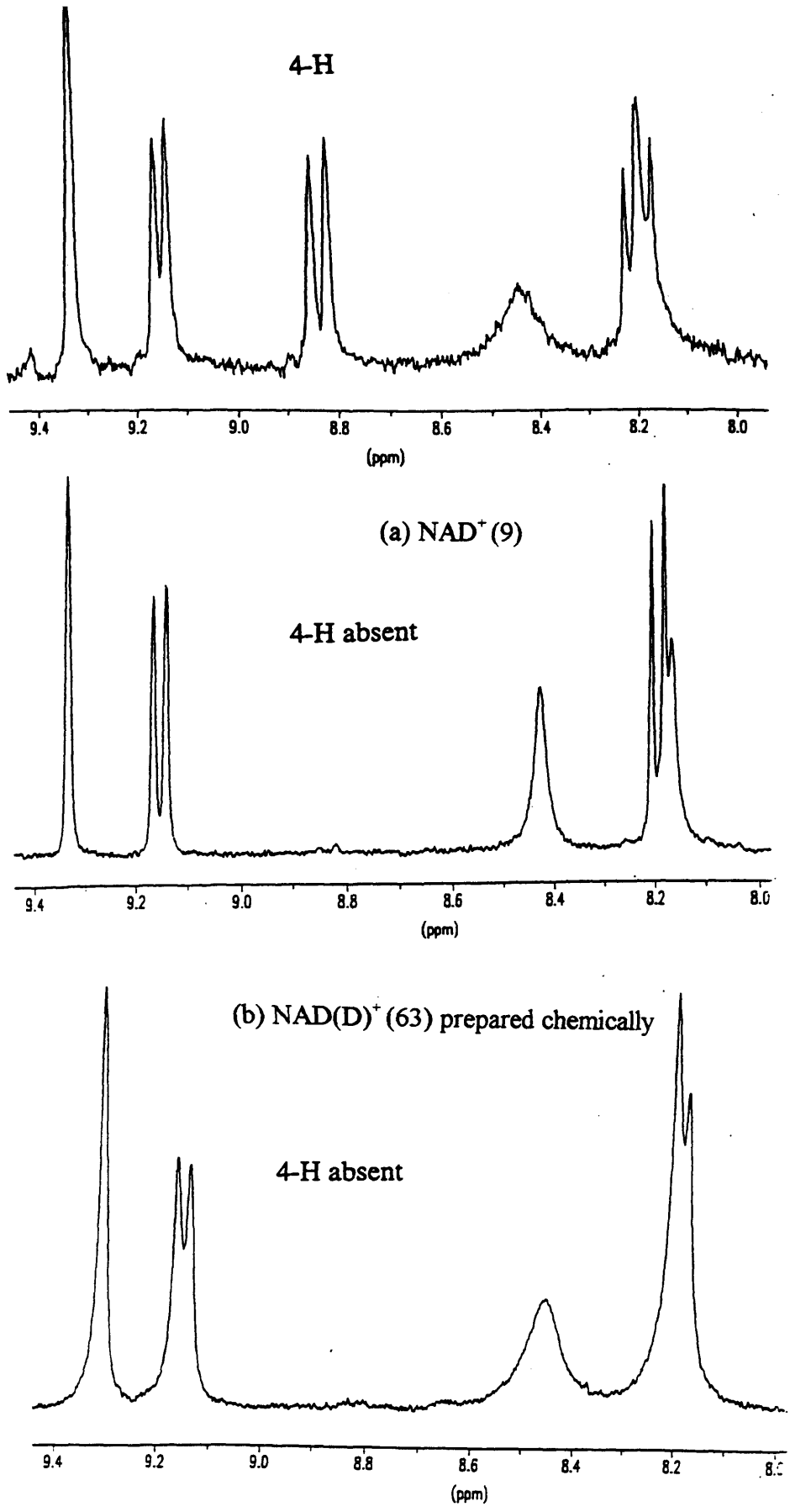
(a) NADH (30)



(b) $NADD_S$ (62b)

N-4 methylene proton region of the ^1H nmr spectra of (a) NADH (30) and (b) $NADD_S$ (62b)

Figure 3.13b



(c) $NAD(D)^+$ (63) prepared enzymatically

Aromatic region of the ^1H nmr spectra of (a) NAD^+ (9), (b) $NAD(D)^+$ (63) prepared chemically and (c) $NAD(D)^+$ (63) prepared enzymatically

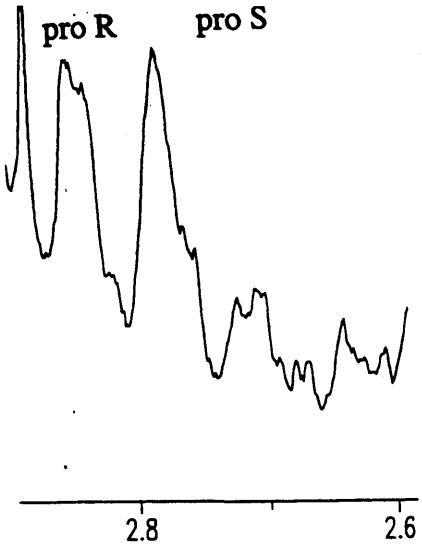
Figure 3.14

deuterium in the oxidised form of the cofactors was readily determined from the presence or absence of an N4 aromatic proton signal in the proton nmr spectrum (figure 3.14).

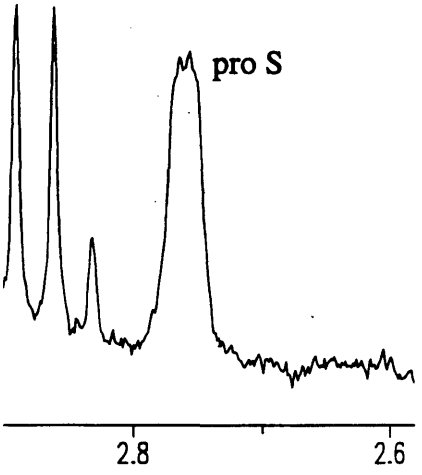
The analogous two stereochemical experiments (figure 3.12) were then carried out with carba NAD^+ (28). The unfavourable equilibrium position of the YADH conversion of carba NAD^+ (28) to carba NADH (65) was pulled towards the carba NADH (65) side by the addition of 0.01M semicarbazide to remove the acetaldehyde as it is formed. This is analogous to the method used by Winer and Schwert.[203] Reduction of carba NAD^+ (28) with CH_3CD_2OH in the presence of YADH resulted in the loss of the downfield N-4 dihydronicotinamide methylene signal and loss of multiplicity at the upfield signal (figure 3.15). In NADH (30) the *pro R* proton is downfield of the *pro S* proton,[202] and this has been found to be the case for all other analogues which have been examined.[204] It was therefore a reasonable assumption that in this case too the downfield signal is that of the *pro R* hydrogen. This means that the YADH is still showing class A stereoselectivity with the carbocyclic NAD^+ (28) and transferring to the re face of the nicotinamide ring to give carba $NADD_R$ (64a). The two N-4 methylene signals of carba NADH (65) are shifted downfield with respect to the corresponding signals in NADH (30). When carba $NADD_R$ (64a) was oxidised with dihydroxyacetonephosphate and GPDH the carba $NAD(D)^+$ (66) obtained showed no N-4 aromatic proton signal in the nmr spectrum (figure 3.16). This demonstrates nicely that the class B GPDH is transferring the *pro S* hydrogen with carba NADH (65) as it would with NADH (30).

Experiment 2 (figure 3.12) with carba $NAD(D)^+$ (66) confirms the above results. Carba $NAD(D)^+$ (66) with YADH and ethanol gives carba $NADD_S$ (64b), this time it is the upfield N-4 methylene signal which has disappeared (figure 3.15). Oxidation of this carba $NADD_S$ (64b) with GPDH and dihydroxyacetone gave carba NAD^+(28) which contained an N4 hydrogen in the aromatic ring (figure 3.16). Even without the reverse anomeric effect the enzymes are still acting with the same stereoselectivity.

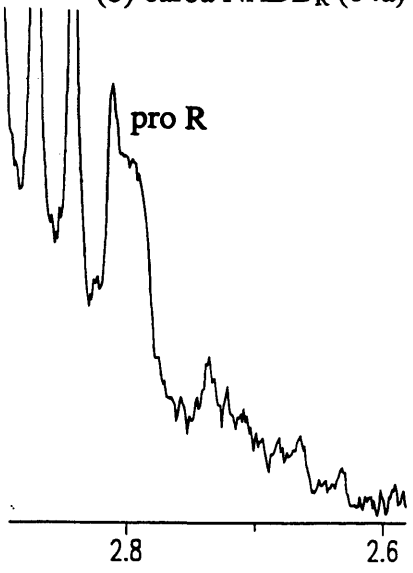
The stereochemical experiments were carried out in a pyrophoshate buffer and when the products were separated by ion exchange chromatography on DEAE Sephadex with TEAB buffer large amounts of pyrophosphate was also isolated with the coenzymes. Therefore the NMR spectra were swamped by triethylammonium peaks associated with pyrophosphate, the spectra of the carbocyclic NADH (65) and carbocyclic NADD (64) are not as well resolved as those of NADH (30) and NADD (62) as the N-4 methylene signals from the



(a) carba NADH (65)



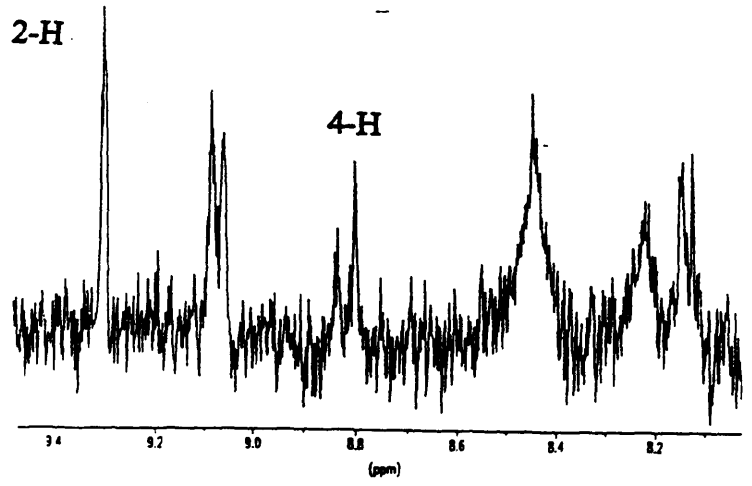
(b) carba $NADD_R$ (64a)



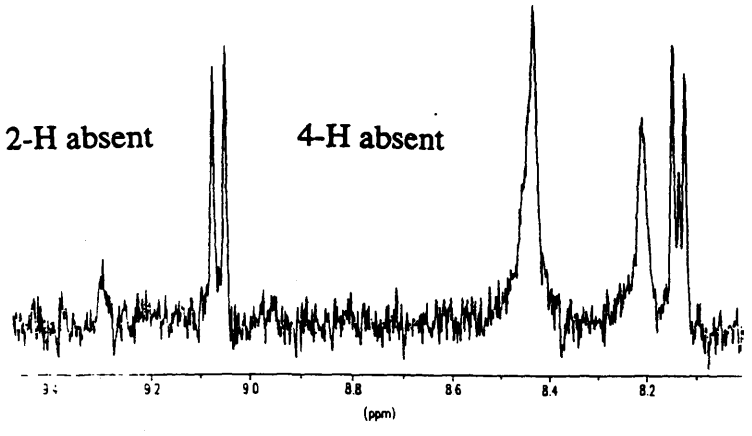
(c) carba $NADD_S$ (64b)

N-4 methylene proton region of the ^1H nmr spectra of (a) carba NADH (65), (b) carba $NADD_R$ (64a) and (c) carba $NADD_S$ (64b)

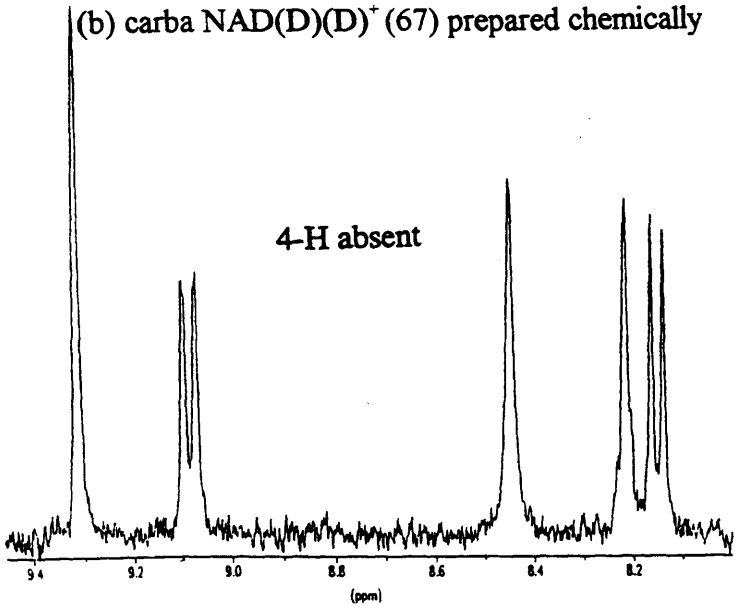
Figure 3.15



(a) carba NAD^+ (28)



(b) carba $NAD(D)(D)^+$ (67) prepared chemically



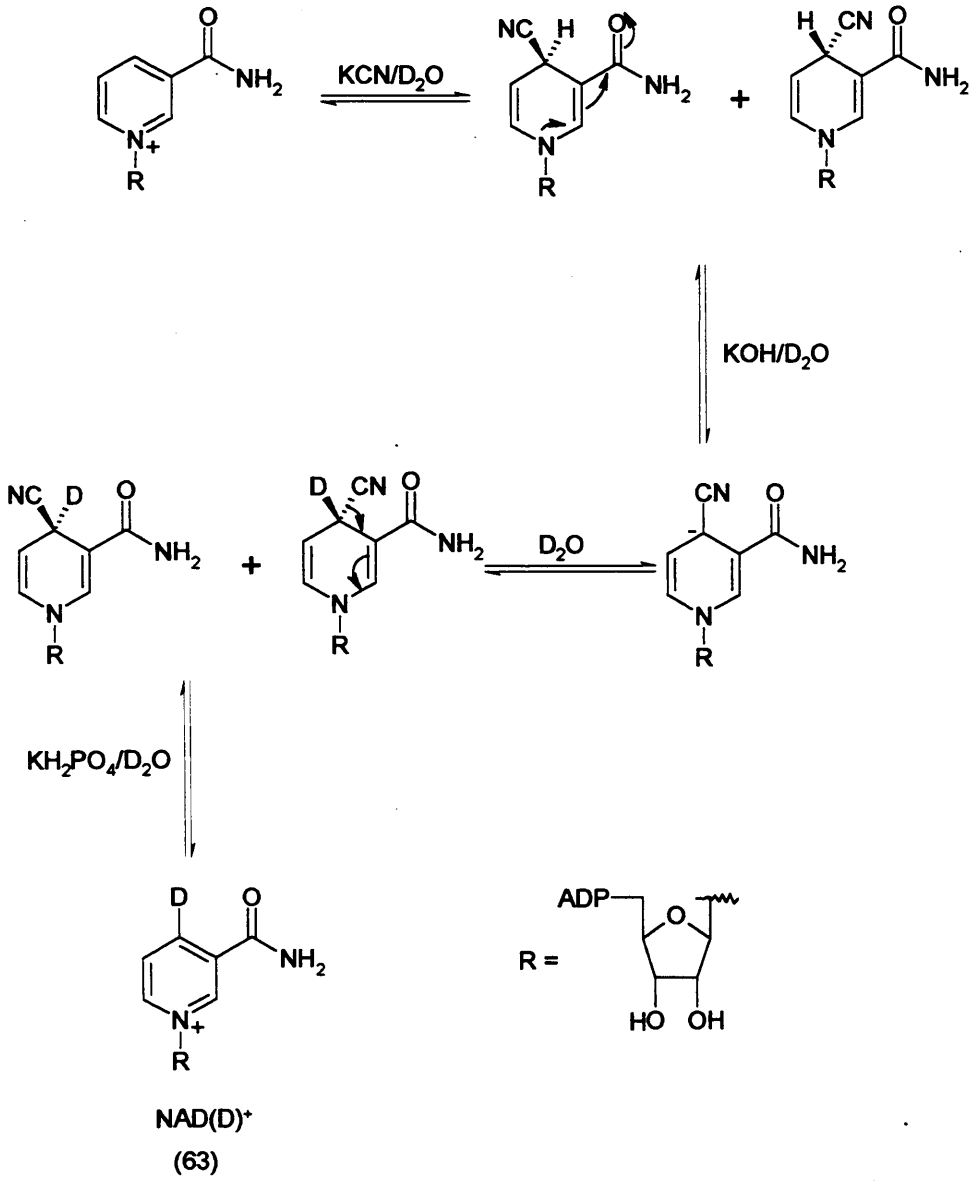
(c) carba $NAD(D)^+$ (66) prepared enzymatically

Aromatic region of the ^1H nmr spectra of (a) carba NAD^+ (28), (b) carba $NAD(D)(D)^+$ (67) prepared chemically and (c) carba $NAD(D)^+$ (66) prepared enzymatically

Figure 3.16

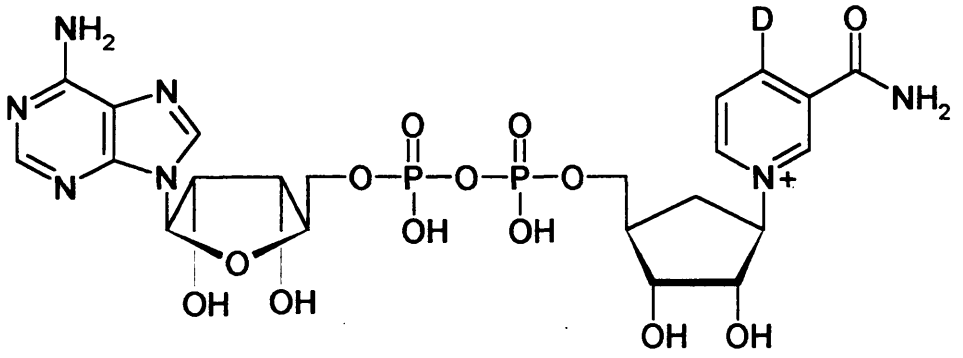
dihydronicotinamide ring are shifted downfield in carba NADH (65) compared with NADH (30) and this causes the carba NADH (65) signals to slightly overlap with the satellite peaks from the triethylammonium signals.

$NAD(D)^+$ (63) was also prepared chemically using the method of San Pietro[205] (scheme 3.2). This involves formation of the cyanide adducts, the N-4 proton in these adducts is acidic enough to be exhanged with D^+ in D_2O medium. The cyanide anion is removed by dilution and lowering of the pD to 6.7. This gives the oxidised coenzyme deuterated at the N-4 position of the nicotinamide ring The proton nmr data of the chemically prepared deuterated coenzyme matched the data for the enzymatically prepared sample (figure 3.14).



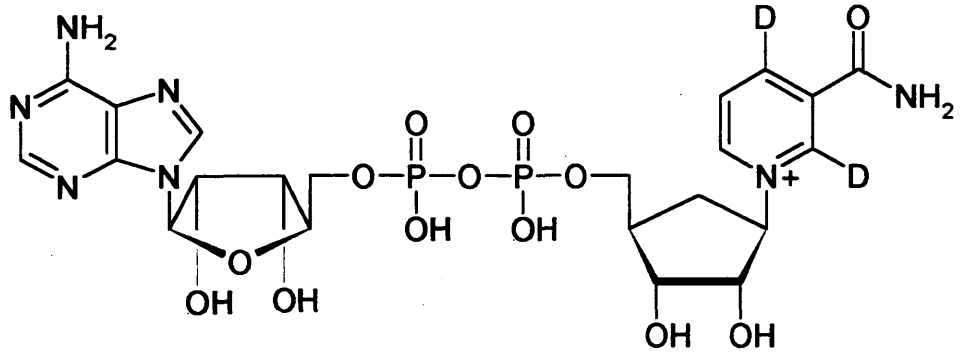
Scheme 3.2

However when carba NAD^+ (28) was treated under similar conditions the proton nmr spectrum showed that both N-4 and N-2 protons had ben replaced by deuterium to give $NAD(D)(D)^+$ (67). As an excess of cyanide was used a dicyanide adduct may be formed during the reaction to give the di-deuterated compound (67) (scheme 3.3). Replacing the ribose ring oxygen with carbon makes the 2 position of the nicotinamide ring more susceptible to nucleophillic attack.



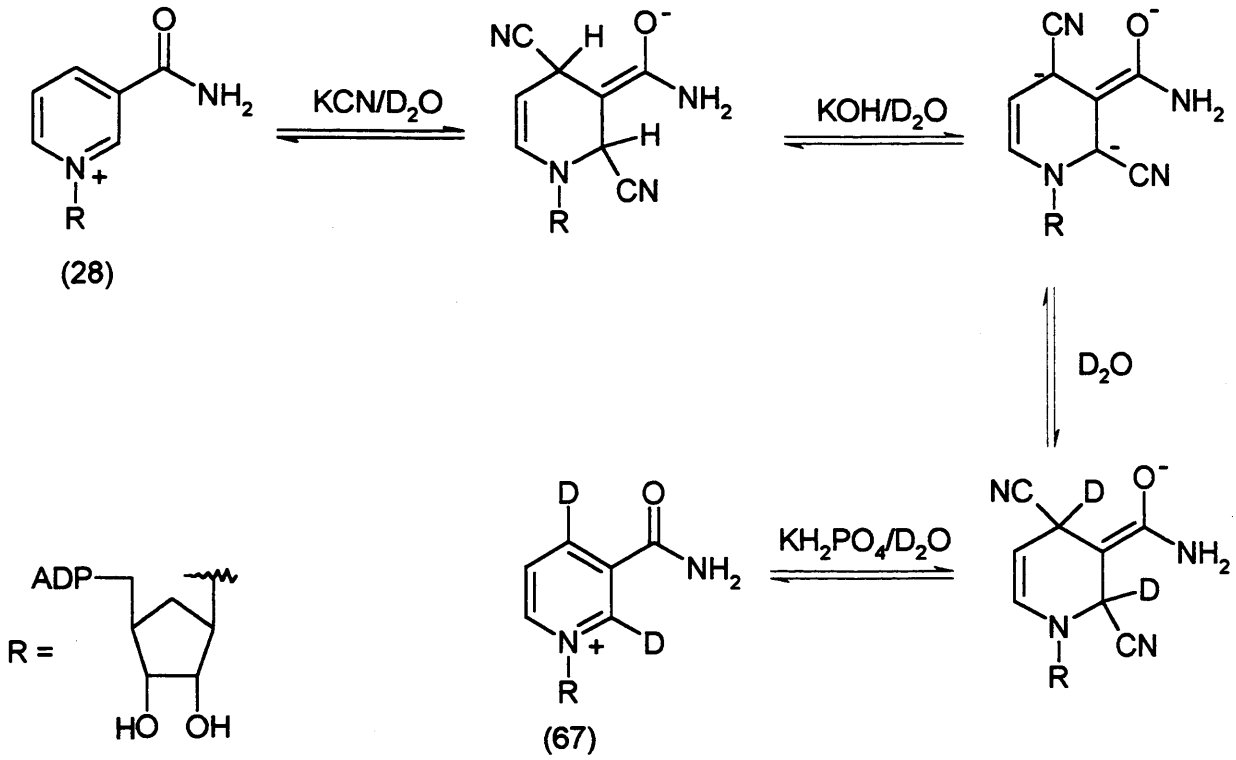
carba $NAD(D)^+$

(66)



carba NAD(D)(D)+

(67)



Scheme 3.3

3.2.3 Kinetic Isotope Effect

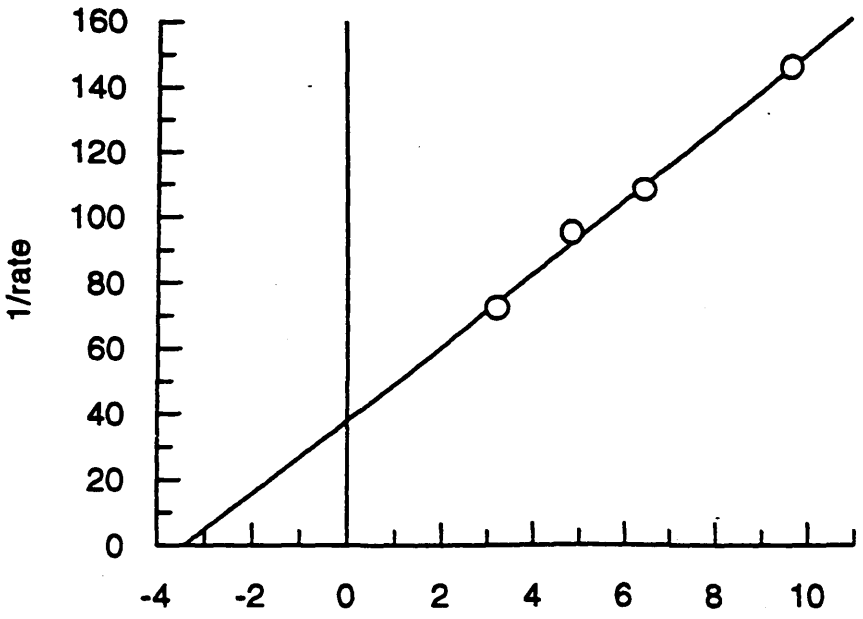
The isotope effects of YADH for the oxidation of $RCD_2OH$with NAD^+ (9) and the reduction of RC_6H_4CHO with NADD (62) have been measured.[186,187] Values of k_H/k_D =3-5 were obtained and theses are typical of a primary isotope effect. A primary isotope effect is good evidence that the transition state involves breaking of a C-H bond, C-D bonds are broken more slowly than C-H bonds. In this study the kinetic isotope effects of the reduction of NAD^+ (9) with CH_3CD_2OH and YADH were compared to those of carba NAD^+ (28) with CH_3CD_2OH and YADH. The K_M and V_{max} were examined with both deuterated and non deuterated ethanol (table 3.5). The reactions were monitored spectrophotometrically to detect the change in concentration of the reduced cofactor. The results were plotted according to the method of Lineweaver and Burk[201] and straight lines were obtained (figures 3.17a and 3.17b).

	NAD^+ (9)	carba NAD^+ (28)
K_{MH} /M	$(2.92\pm0.4)\times10^{-4}$	$(1.38\pm0.2)\times10^{-3}$
K_{MD} /M	$(1.94\pm0.2)\times10^{-4}$	$(1.12\pm0.3)\times10^{-3}$
$K_{M,H}/K_{M,D}$	1.50	1.23
$V_{max,H}$ /μmoles^{-1}min^{-1}unit	$(6.60\pm0.2)\times10^{-1}$	$(1.34\pm0.3)\times10^{-1}$
$V_{max,D}$ /μmoles^{-1}min^{-1}unit	$(3.30\pm0.5)\times10^{-1}$	$(0.51\pm0.3)\times10^{-1}$
$V_{max,H}/V_{max,D}$	2.0	2.6

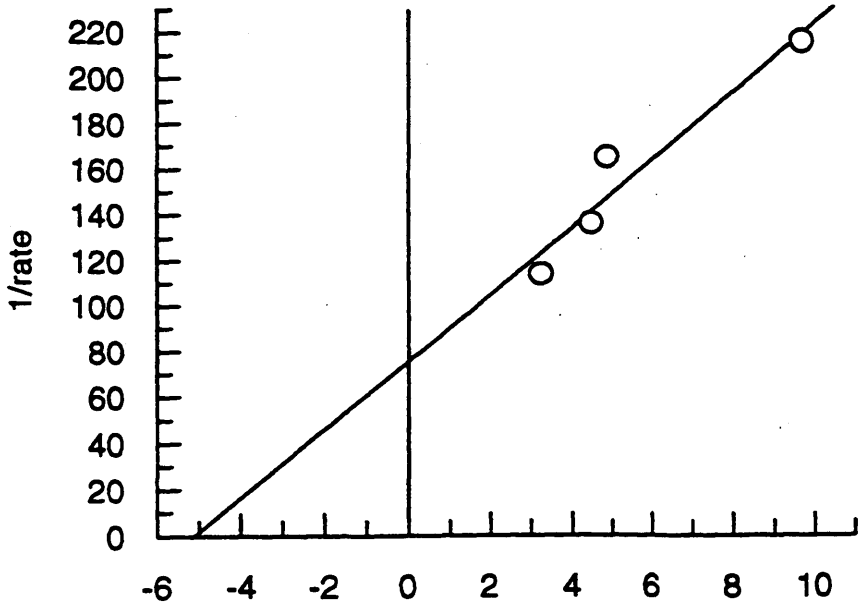
Table 3.5 Kinetic isotope effects for the reaction of carba NAD^+ (28) and NAD^+ (9) with YADH and ethanol

From $k_{cat}=V_{max}/[E_0]$ then $k_H/k_D = V_{max,H}/V_{max,D}$ if $[E_0]$ is the same in both cases. In the study of *para* substituted benzyl alcohols with YADH and NAD^+ (9) $K_{M,H}/K_{M,D}$ values of 1.1-1.6 were obtained.[186] Similar values were obtained in this study with $K_{M,H}/K_{M,D}$ = 1.50 for NAD^+ (9) and $K_{M,H}/K_{M,D}$ = 1.23 for carba NAD^+ (28). The $V_{max,H}/V_{max,D}$ values are about 2 for both NAD^+ (9) and carba NAD^+ (28) giving an isotope effect on k_{cat} of two. In the study with para substituted benzyl alcohols the isotope effect on k_{cat} was four.[186] In this study the observed primary isotope effect is indicative of a C-H bond being broken during the rate limiting step. The similarity of the observed isotope effects for NAD^+ (9) and carba NAD^+ (28) suggests that the reaction mechanism is the same in both cases.

The kinetic isotope effect of NAD^+ (9) with YADH and benzyl alcohol were also measured (figure 3.17c) and compared to the literature values.[186] In this study the commercially available $C_6D_5CD_2OH$ was used to measure the isotope effect whereas in the literature $C_6H_5CD_2OH$ was used, however the substitution of deuterium for hydrogen on the aromatic ring should have very little effect on the kinetic isotope effect. The results are shown in table 3.6.



Lineweaver-Burk plot for the oxidation of CH_3CH_2OH by NAD^+ (9) catalysed by YADH
[S]=[NAD^+] [CH_3CH_2OH]=[0.33M]

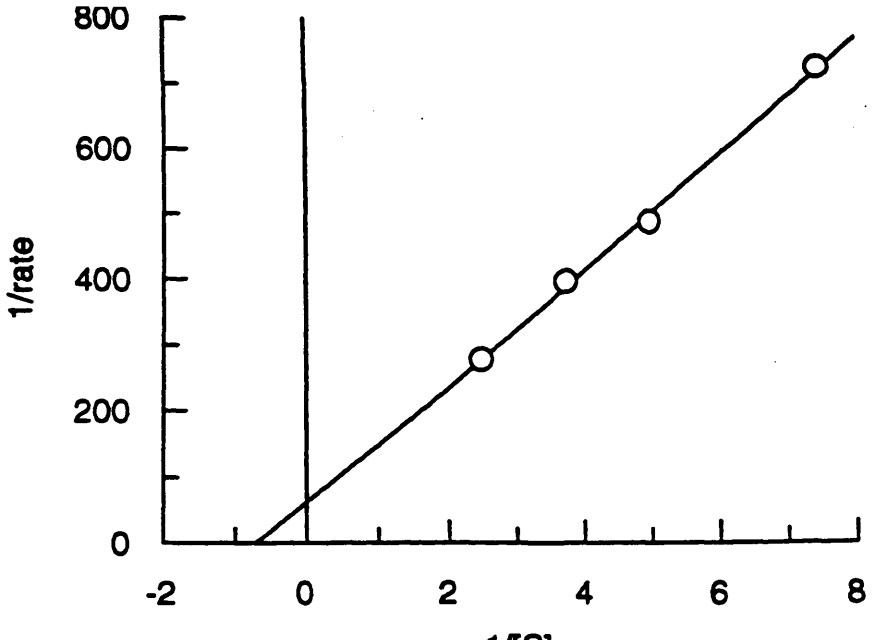


Lineweaver-Burk plot for the oxidation of CH_3CD_2OH by NAD^+ (9) catalysed by YADH
[S]=[NAD^+] [CH_3CD_2OH]=[0.33M]

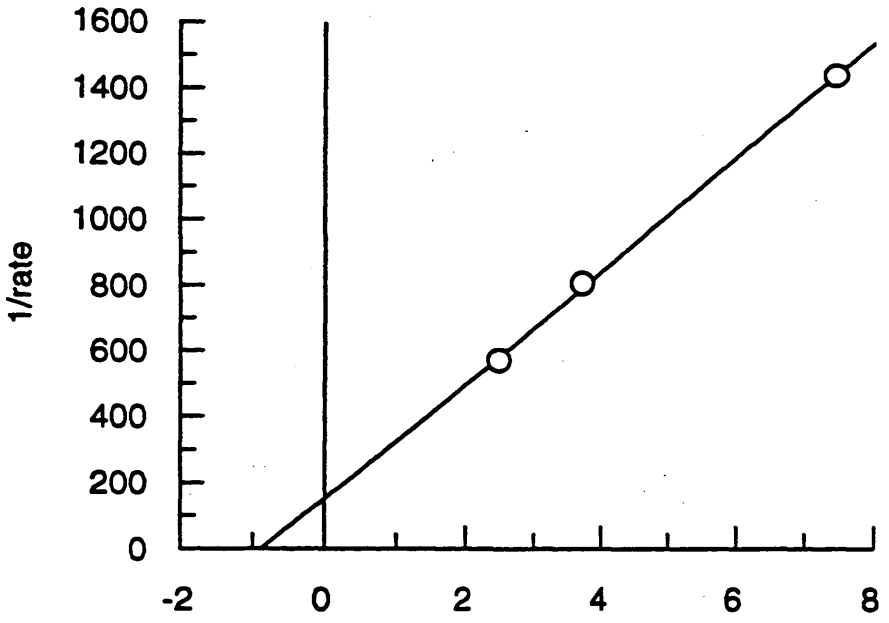
Figure 3.17a

[S] in mM

ν in μmoles min^{-1}



Lineweaver-Burk plot for the oxidation of CH_3CH_2OH by carbaNAD$^+$ (28) catalysed by YADH [S]=[carbaNAD$^+$] [CH_3CH_2OH]=[0.33M]

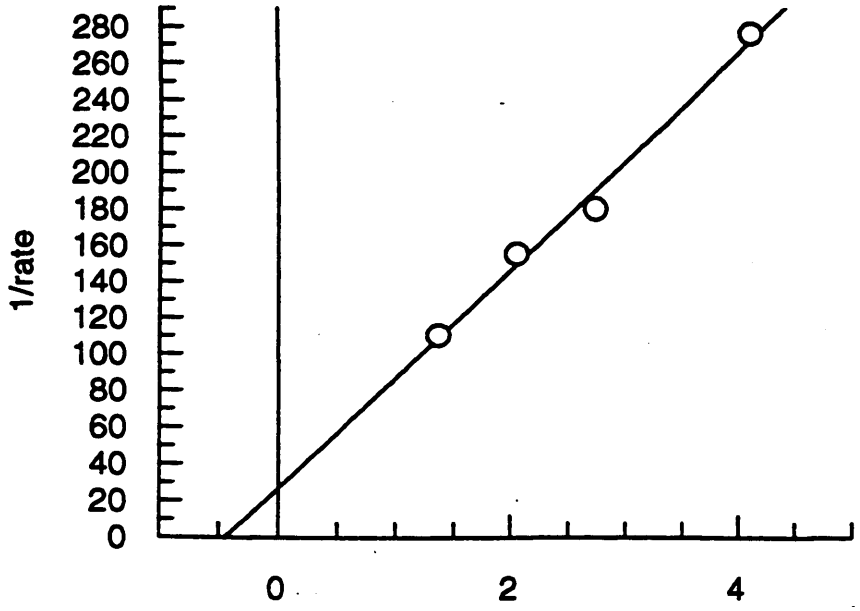


Lineweaver-Burk plot for the oxidation of CH_3CD_2OH by carbaNAD$^+$ (28) catalysed by YADH [S]=[carbaNAD$^+$] [CH_3CD_2OH]=[0.33M]

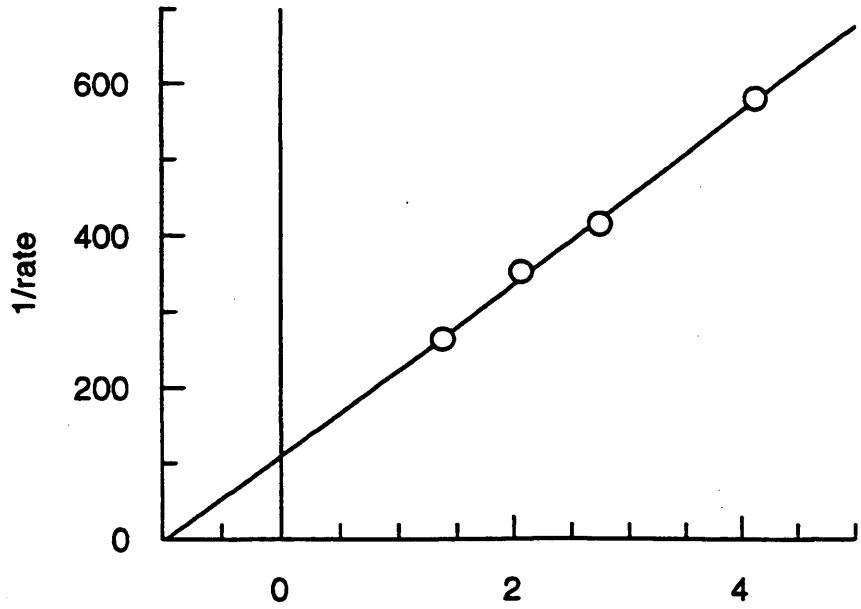
Figure 3.17b

[S] in mM

υ in μmoles min^{-1}



Lineweaver-Burk plot for the oxidation of $C_6H_5CH_2OH$ by NAD^+ (9) catalysed by YADH
[S]=[NAD^+] [$C_6H_5CH_2OH$]=[50mM]



Lineweaver-Burk plot for the oxidation of $C_6D_5CD_2OH$ by NAD^+ (9) catalysed by YADH
[S]=[NAD^+] [$C_6D_5CD_2OH$]=[50mM]

Figure 3.17c

[S] in mM

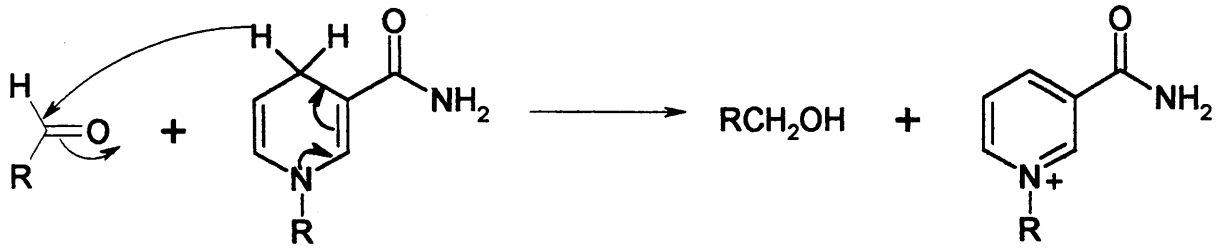
υ in μmoles min^{-1}

K_{MH} /M^{-1}	$(2.1\pm0.3)\times10^{-3}$
K_{MD} /M^{-1}	$(1.18\pm0.2)\times10^{-3}$
K_{MH} /K_{MD}	1.8
$V_{max,H}$ /μmoles^{-1}unit^{-1}unit	$(4.1\pm0.4)\times10^{-3}$
$V_{max,D}$ /μmoles^{-1}unit^{-1}unit	$(1.23\pm0.2)\times10^{-3}$
$V_{max,H}$ /$V_{max,D}$	3.3

Table 3.6 Kinetic isotope effects for the reaction of NAD^+ (9) with YADH and benzyl alcohol

In the literature[186] an isotope effect on k_{cat} of 3.3 was obtained and the isotope effect on K_M was 1.3. In this study the observed kinetic isotope effects with benzyl alcohol and NAD^+ (9) closely matched the literature values[186] (table 3.6). This result confirms the validity of the previous results with ethanol.

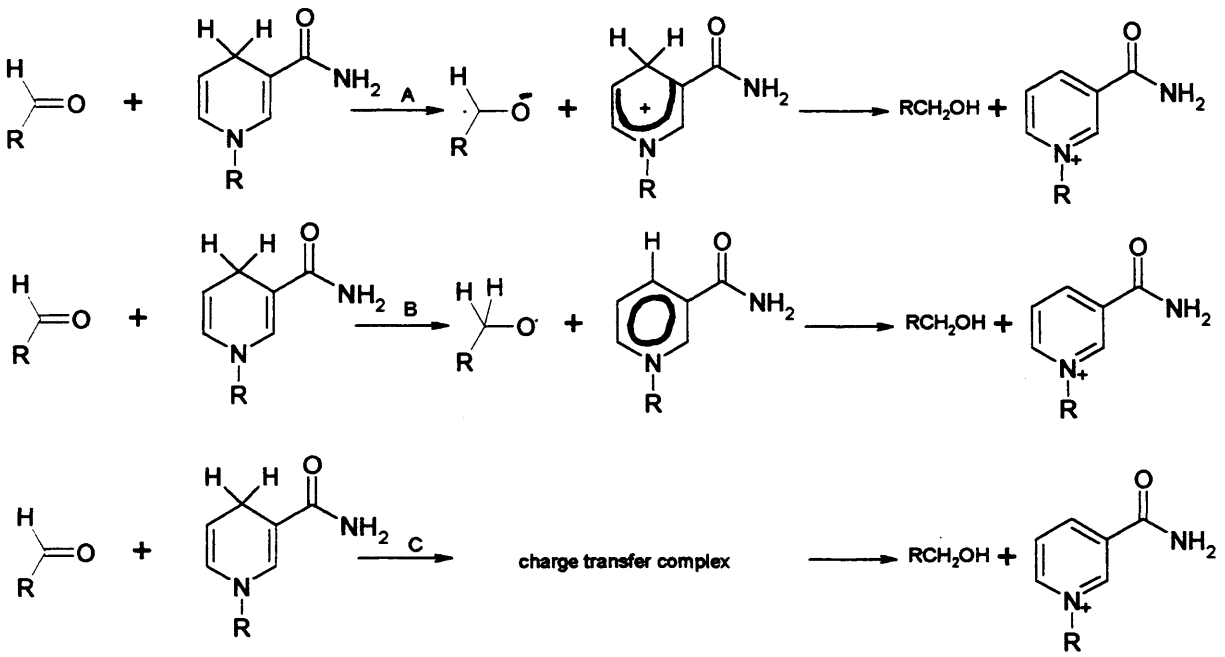
The enzyme catalysed NADH reductions are formally regarded as hydride transfer reactions, in all cases the reductions are characterized by the direct transfer of a hydride ion from the cofactor to the substrate. However, failure to observe any exchange with the solvent does mean that a direct hydride transfer process (scheme 3.4) can be distinguished from other mechanisms. Further evidence was necessary to establish the mechanism of this process.



Scheme 3.4

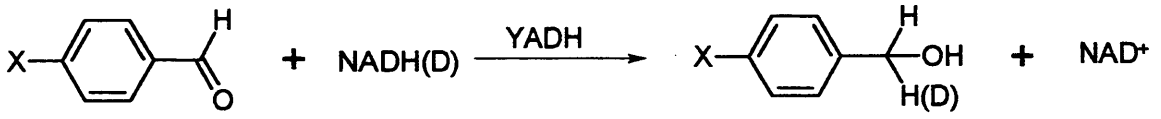
Three possible alternative mechanisms were suggested by Klinman[183] (scheme 3.5), all of these mechanisms involve the formation of an intermediate. Path A involves the formation of a free radical by an initial electron transfer, path B also has a free radical intermediate but this is formed by an atom transfer process. Path C involves the formation of a charge transfer complex. Path C can be regarded as a special case of scheme 3.4 and path A and path B as it

could be possible for the charge transfer complex to breakdown either by a radical process or by hydride transfer.



Scheme 3.5

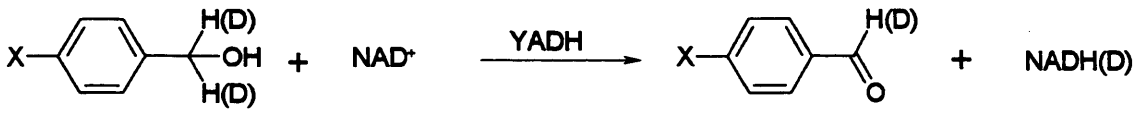
Klinman used the following system in an effort to distinguish among the possible mechanisms for the enzyme catalysed NADH dependent reductions (scheme 3.6).[186] This system was used



X= CH_3O, CH_3, Cl, Br, H

Scheme 3.6

to probe both the isotope and substituent effects. This system was chosen as the aromatic aldehydes are turned over slowly. The transfer of hydrogen from the coenzyme to the aromatic aldehyde was conducted to be rate limiting under steady state conditions. The observed substituent effects led to the suggestion that there is relatively little charge at C-1 of the aldehyde or alcohol in the transition state of this reaction. The following system was used by Klinman in an attempt to confirm the proposed transition state structure in the YADH catalysed reaction (scheme 3.7).



X= CH_3O, CH_3, Cl, Br, H

Scheme 3.7

The above reactions were characterised by large deuterium isotope effects. This suggests that the rate limiting step in both directions is the hydrogen transfer step. Klinmans results indicated that the mechanism involved hydrogen transfer in the rate limiting step and that the release of substrates from the ternary complex is not infinitely fast, relative to the chemical interconversion step.

The magnitude of the isotope effects on the Michaelis constants can be used as a measure of the rate of substrate release from the ternary complex compared to the interconversion of the ternary complexes. Klinman used the relative magnitudes of the isotope effects on the K_M for the coenzyme compared to the substrate as a measure of the relative rates of coenzyme versus substrate release from the ternary complex. When this data was interpreted in terms of preferential release of cofactor or substrate from the ternary complex followed by rapid release of the other substrate from the binary complex it was consistent with the rate limiting step being a hydrogen transfer process in both the oxidation and reduction directions.

The similarity between the results for the YADH catalysed reaction with NAD^+ (9) and carba NAD^+ (28) suggest that both processes occur by the same mechanism. Both NAD^+ (9) and carba NAD^+ (28) are showing large primary isotope effects and this along with the Klinman results points to both reactions involving a hydrogen transfer process as the rate limiting step.

3.2.4 Solution Conformation of Carbocyclic NAD^+ (28)

Reduced NADH (30) exists in solution in a folded conformation which is in rapid equilibrium with the open chain form.[202] In the folded conformation, the *si* face of the dihydronicotinamide ring is folded against the adenine ring (figure 3.18). The oxidised coenzyme NAD^+ (9) also appears to adopt this same folded conformation. The chemical shifts for all the protons and carbons of both pyridinium and dihydropyridinium rings move upfield with respect to those of the mononucleotides NMN and NMNH.[202] The proton and carbon

signals for the adenine ring in NAD^+ (9) are also shifted with respect to the mononucleotide AMP (1).[160] The same effect can be observed for carba NAD^+ (28), carba NMN (36) and AMP (1) (table 3.7), this suggests that the same intramolecular interactions are occurring in carba NAD^+ (28) which occur in NAD^+ (9).

	N-2	N-4	N-5	N-6	A-2	A-8
NMN (15)	9.475	8.999	8.319	9.303	/	/
NAD^+ (9)	9.444	8.955	8.312	9.297	8.414	8.597
AMP (1)	/	/	/	/	8.080	8.429
carba NMN (36)	9.468	8.990	8.301	9.251	/	/
carba NAD^+ (28)	9.311	8.805	8.153	9.084	8.218	8.451

values in ppm from tsp

Table 3.7 ^1H nmr data for the aromatic region of NMN (15), NAD^+ (9), AMP (1), carba NMN (36) and carba NAD^+ (28)

The chemical shift effect is due to the anisotropic shielding by the ring current originating from one aromatic ring influencing the chemical shifts in the ring facing it, whether this second ring is aromatic or not. The evidence for the folded conformation of NADH (30) can also be determined from the proton spectrum of the two N-4 dihydronicotinamide protons.[202] The two chemical shifts of these protons are non equivalent in NADH (30) with the *pro S* proton appearing upfield of the *pro R* proton, whereas in NMNH they are close together in chemical shift and appear as a single resonance. The two protons are not related by symmetry and therefore must be chemically and magnetically non equivalent but in order to observe discrete resonances the chemical or magnetic anisotropy generated by nearby chiral centres must be greater than the line width of the signals. In the mononucleotide NMNH the only chiral centres are those of the ribose ring, and the anisotropy must not be great enough to resolve the signals. This indicates that the resolution of the two N-4 proton signals in NADH (30) must be due to the anisotropic effect of the adenine ring (figure 3.18),[202] and this would not be

possible if the coenzyme adopted an open chain form as the adenine ring would be too far away from the dihydropyridine ring to have an effect.

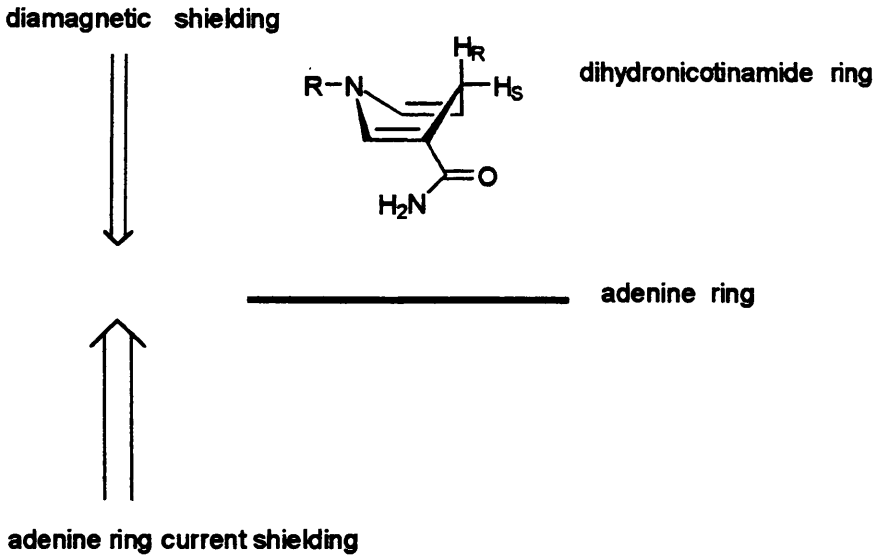


Figure 3.18

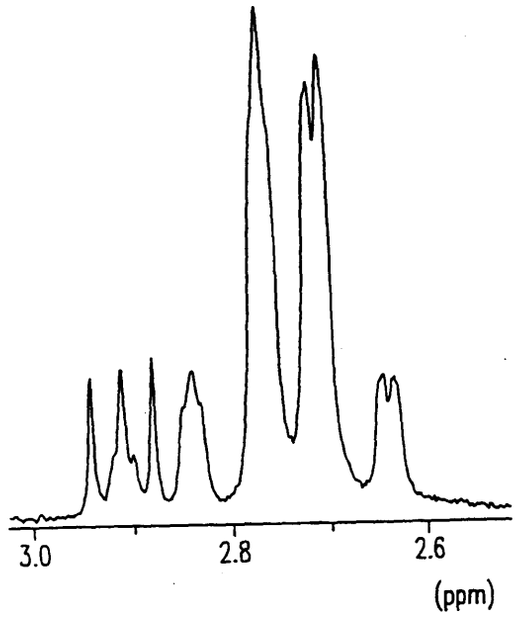
This effect can also be seen with carba NADH (65), the two N-4 methylene protons are non equivalent by proton nmr spectroscopy (table 3.8) and this indicates that carba NADH (65) is also adopting this folded conformation in which the adenine ring is associated with the dihydropyridine ring. This is good evidence that the carba NADH (65) is adopting the same solution conformation as NADH (30).

	N4 methylene protons	
NMNH	3.075	
NADH	2.802 (*pro R*)	2.674 (*pro S*)
carba NADH	2.883 (*pro R*)	2.748 (*pro S*)

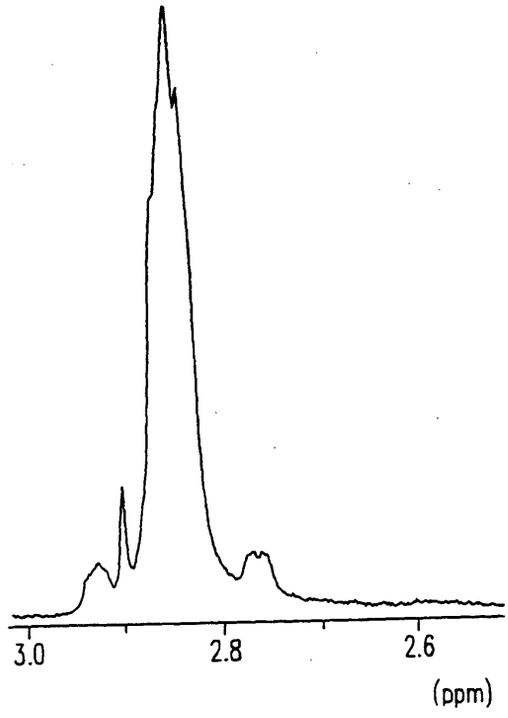
values in ppm from tsp

Table 3.8 ^{1}H nmr data for the N-4 methylene protons of NMNH, NADH (30) and carba NADH (65)

Methanol is a known destacking agent and running the proton nmr spectrum of NADH in a mixture of D_2O and CD_3OD causes the NADH (30) to unfold to the open chain form.[206] The anisotropic effect of the adenine ring is lost and the two N-4 methylene signals can be seen to lose resolution and move downfield towards the limiting mononucleotide values (figure 3.19). In the case of carba NADH (65), the addition of methanol again causes the N-4 methylene signals to lose resolution and shift downfield (figure 3.20). This provides further evidence for the folded nature of the solution conformation of carba NAD^+ (28).

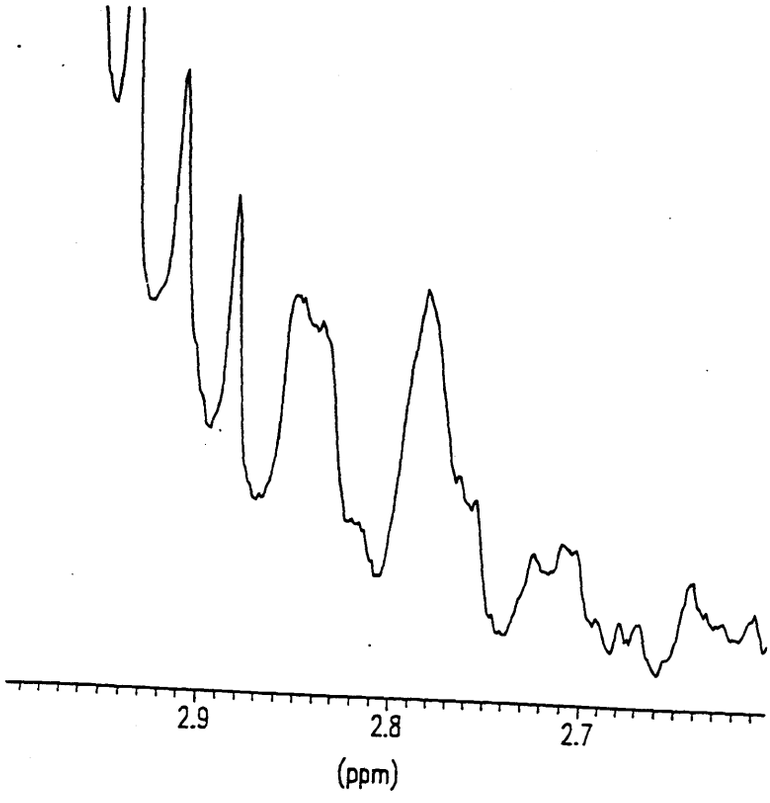


^{1}H nmr signal for the N-4 methylene protons of NADH (30) in D_2O

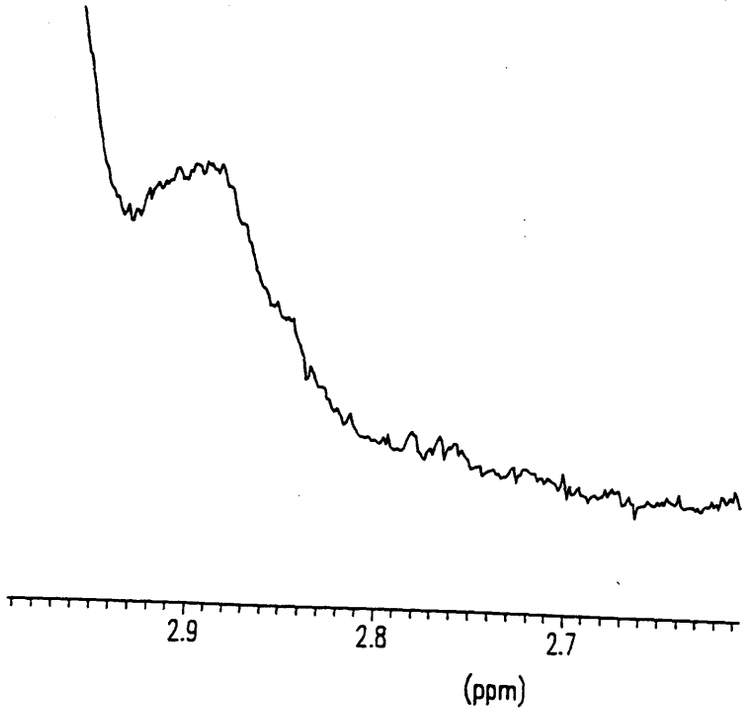


^{1}H nmr signal for the N-4 methylene protons of NADH (30) in D_2O:CD_3OD (7:3)

Figure 3.19



^{1}H nmr signal for the N-4 methylene protons of carba NADH (65) in D_2O

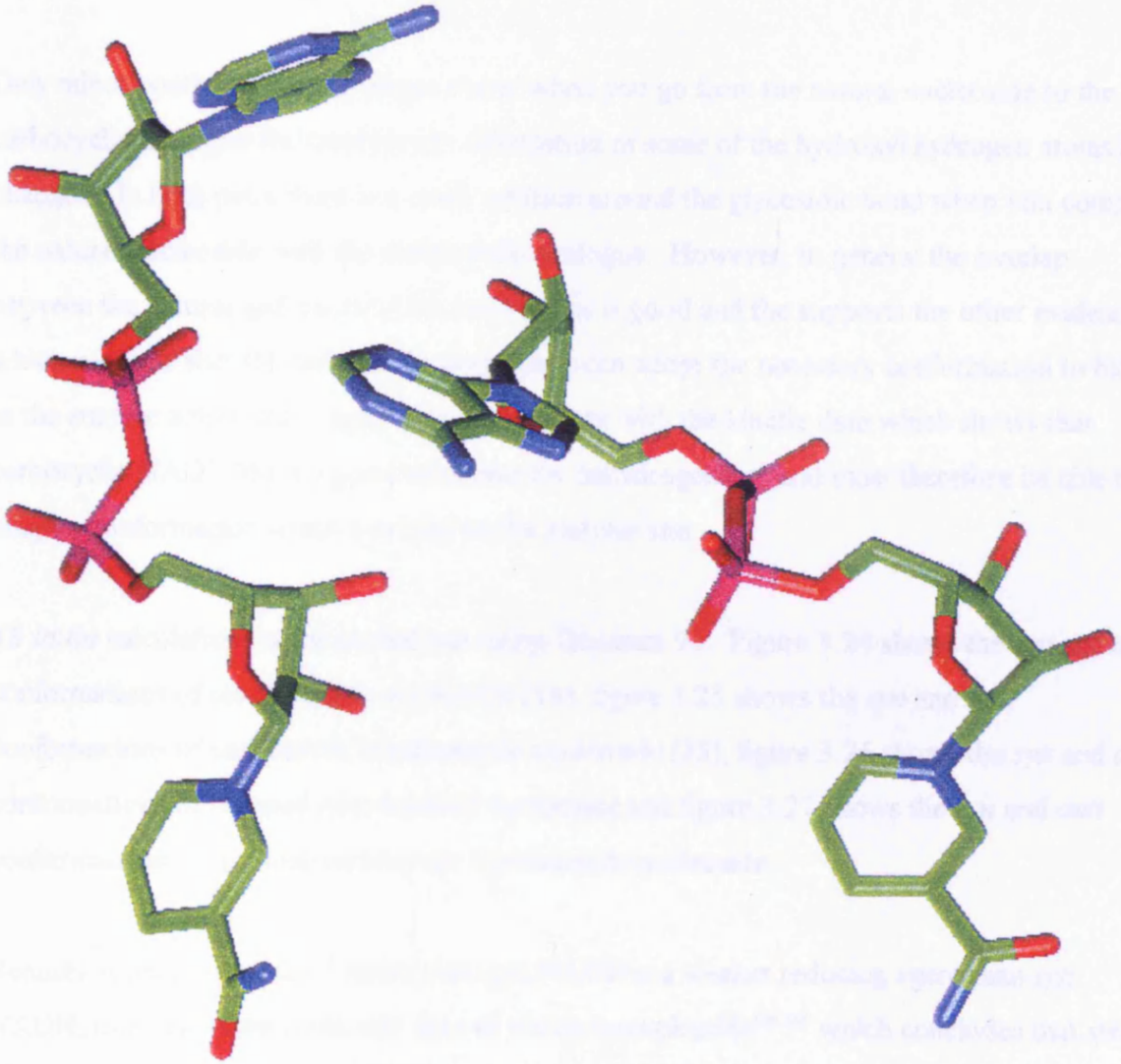


^{1}H nmr signal for the N-4 methylene protons of carba NADH (65) in D_2O:CD_3OD (7:3)

Figure 3.20

3.2.5 Crystal Structures of NAD^+ (9)

Figure 3.21 shows the NAD^+ (9) structure taken from alcohol dehydrogenase (ADH) (left) and the single crystal x-ray structure of NAD^+ (9) (right). The two structures have rather different conformations, the enzyme bound structure is more elongated than the structure from the Cambridge database which is more folded. The open chain form of the cofactor is assumed to be important for redox activity. The Cambridge database structure is a from a $Li^+.NAD^+$ complex, the ribose attached to the nicotinamide ring is in a $C_{3'}$ *endo* envelope conformation while the ribose attached to the adenosine ring is in a $C_{2'}$ *endo* twist conformation. In the structure from ADH both of the ribose rings have a $C_{2'}$ *endo* conformation. In both structures the two heterocyclic aromatic rings are in the *anti* conformation and the adenine and nicotinamide planes are almost perpendicular. There is a significant difference between the two structures, the conformation about the C-4' and the C-5' bond in the $Li^+.NAD^+$ complex is *gauche, gauche*, this is the preferred conformation, whereas in the structure from ADH this bond is *trans gauche,* and this corresponds to a very high energy state. Binding to the enzyme obviously causes the drastic conformational changes which are observed for the NAD^+ (9). As previously mentioned the enzyme bound NAD^+ (9) structure taken from ADH has the nicotinamide ring in an *anti* conformation this is consistent with the Benner hypothesis[164] which predicts that a *pro-R* (class A) dehydrogenase would bind the cofactor in the *anti* conformation. In carbocyclic NMN (36) the cyclopentane ring appears to be in a $C_{2'}$ *endo* conformation and in solution there is rapid equilibrium between the *syn* and *anti* forms, therefore the nicotinamide portion of the carbocyclic dinucleotide carba NAD^+ (28) is able to adopt the conformation necessary to bind at the enzyme site, and indeed the structures in figure 3.21 do not highlight any reason why replacing the ring oxygen with a methylene unit should cause a problem. This is consistent with the kinetic data which shows that carbocyclic NAD^+ (28) is a good substrate for dehydrogenases and must therefore be able to adopt the same conformation as NAD^+ (9) in order to bind at the enzyme binding site.



(a) (b)

Crystal Structures of NAD^+(9) (a) taken from alcohol dehydrogenase and (b) the single crystal structure.

Figure 3.21

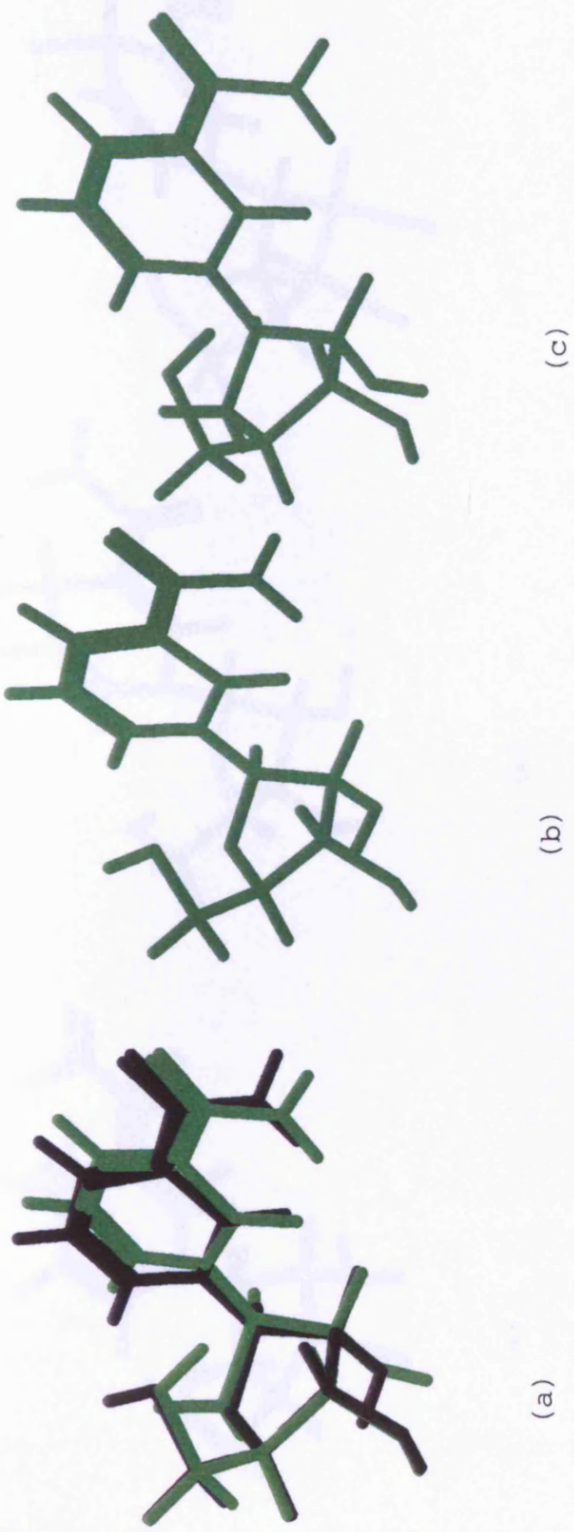
3.2.6 Molecular Modelling Studies

Molecular mechanics modelling was carried out on the nicotinamide nucleoside end of the coenzymes NAD^+ (9), carba NAD^+ (28), NADH (30) and carba NADH (65). These models were carried out using the DTMM programme. Figure 3.22 shows NAD^+ (9) and carba NAD^+ (28), (a) shows NAD^+ (9) (black) superimposed upon C-4, C-3 and C-2 of carba NAD^+ (28) (green), (b) is NAD^+ (9) and (c) is carba NAD^+ (28). Figure 3.23 shows NADH (30) and carba NADH (65), (a) shows NADH (30) (blue) superimposed upon C-4, C-3 and C-2 of carba NADH (65) (green), (b) is NADH (30) and (c) is carba NADH (65).

Only minor conformational changes occur when you go from the natural nucleoside to the carbocyclic analogue for example the orientation of some of the hydroxyl hydrogen atoms is changed. In both pairs there is a small rotation around the glycosidic bond when you compare the natural nucleoside with the carbocyclic analogue. However, in general the overlap between the natural and carbocyclic nucleosides is good and the supports the other evidence which suggests that the carbocyclic nucleoside can adopt the necessary conformation to bind at the enzyme active site. Again this is consistent with the kinetic data which shows that carbocyclic NAD^+ (28) is a good substrate for dehydrogenases and must therefore be able to adopt a conformation which can bind to the enzyme site.

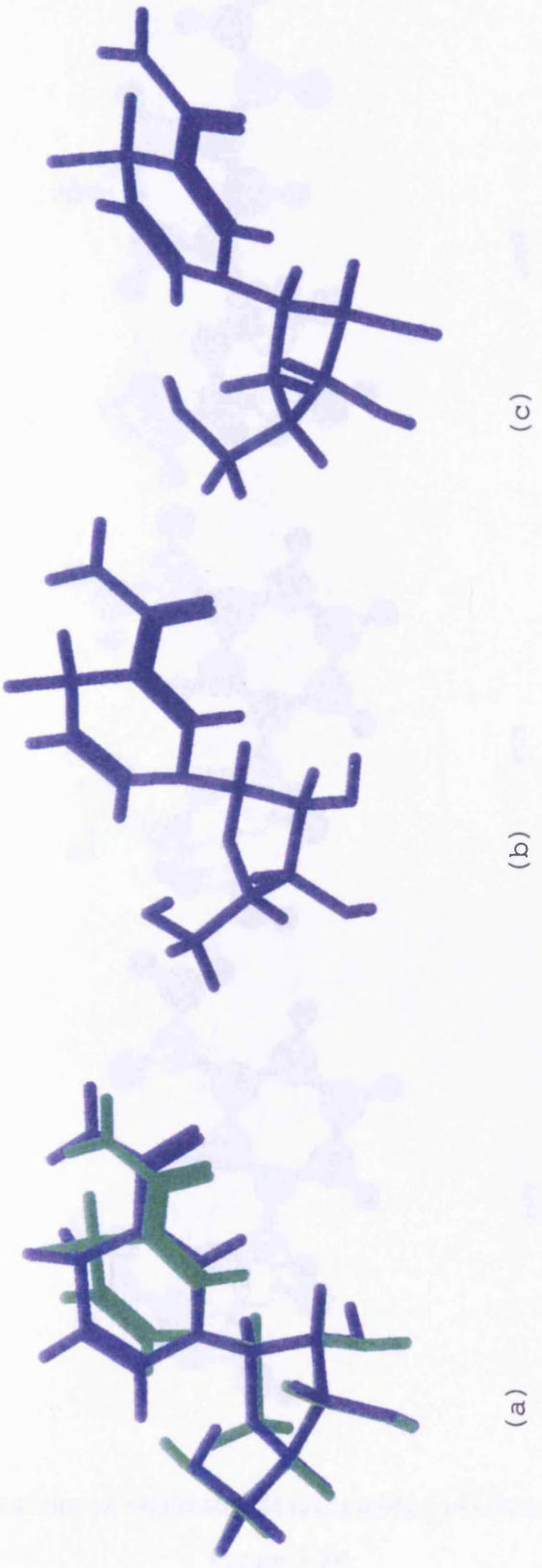
Ab initio calculations were carried out using Guassian 95. Figure 3.24 shows the *syn* and *anti* conformations of nicotinamide nucleoside (14), figure 3.25 shows the *syn* and *anti* conformations of carbocyclic nicotinamide nucleoside (35), figure 3.26 shows the *syn* and *anti* conformations of reduced nicotinamide nucleoside and figure 3.27 shows the *syn* and *anti* conformations of reduced carbocyclic nicotinamide nucleoside.

Benners second postulate[164] states that *anti* NADH is a weaker reducing agent than *syn* NADH, this was based upon nmr data of the mononucleotide[168,169] which concludes that *syn* NMN^+ is more stable than *anti* NMN^+ and that *anti* NMNH is more stable than *syn* NMNH. Benner concluded that dehydrogenases match the reducing power of the cofactor to the reactivity of the substrate. However, the nmr data upon which this was based may in itself be flawed.[234] The *ab initio* calculations do not support the conclusion that *syn* NMN^+ is lower in



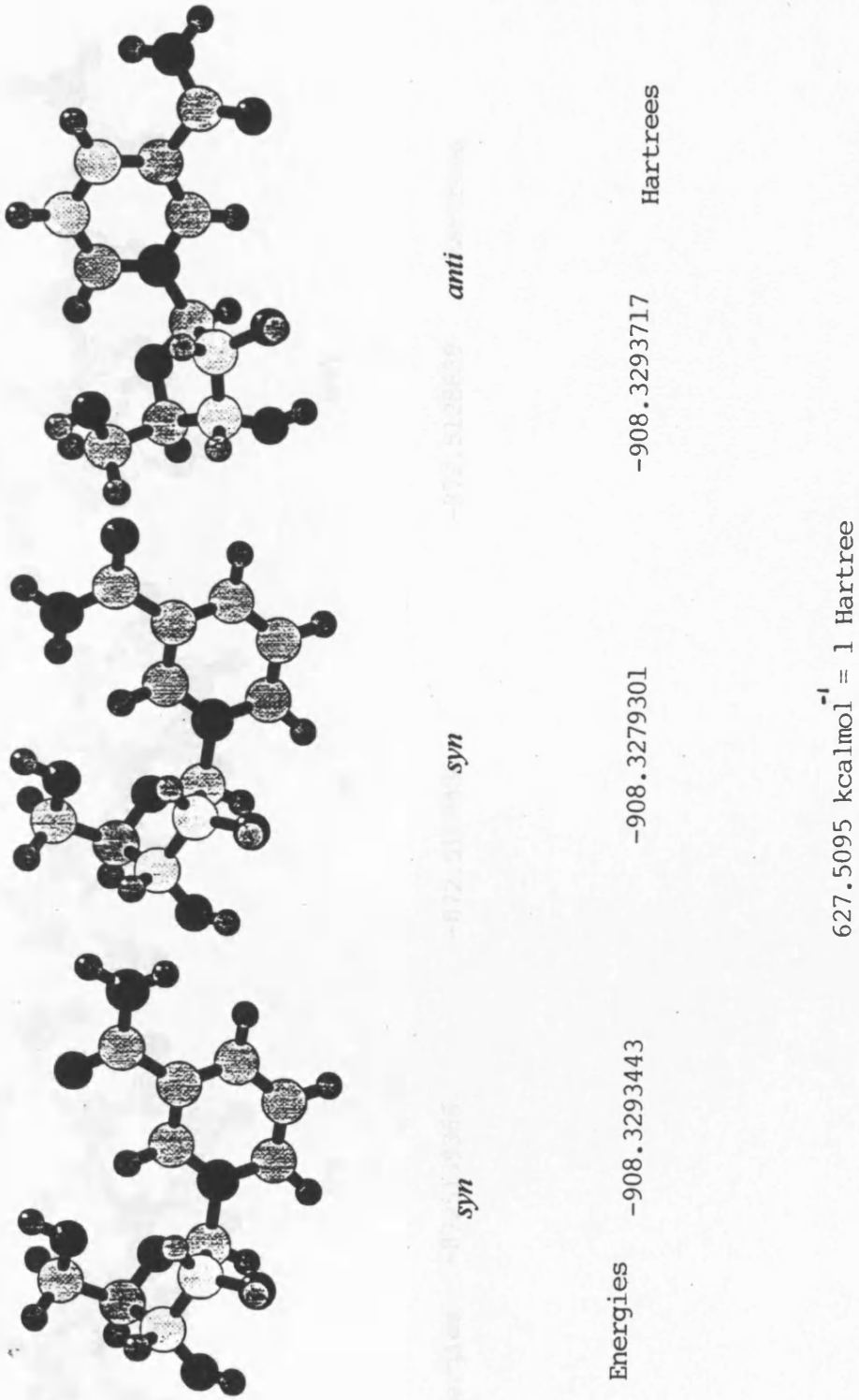
Molecular Models of (a) NAD^+ (9) (black) superimposed upon C-4, C-3 and C-2 of carba NAD^+ (28), (b) NAD^+ (9) and (c) carba NAD^+ (28).

Figure 3.22



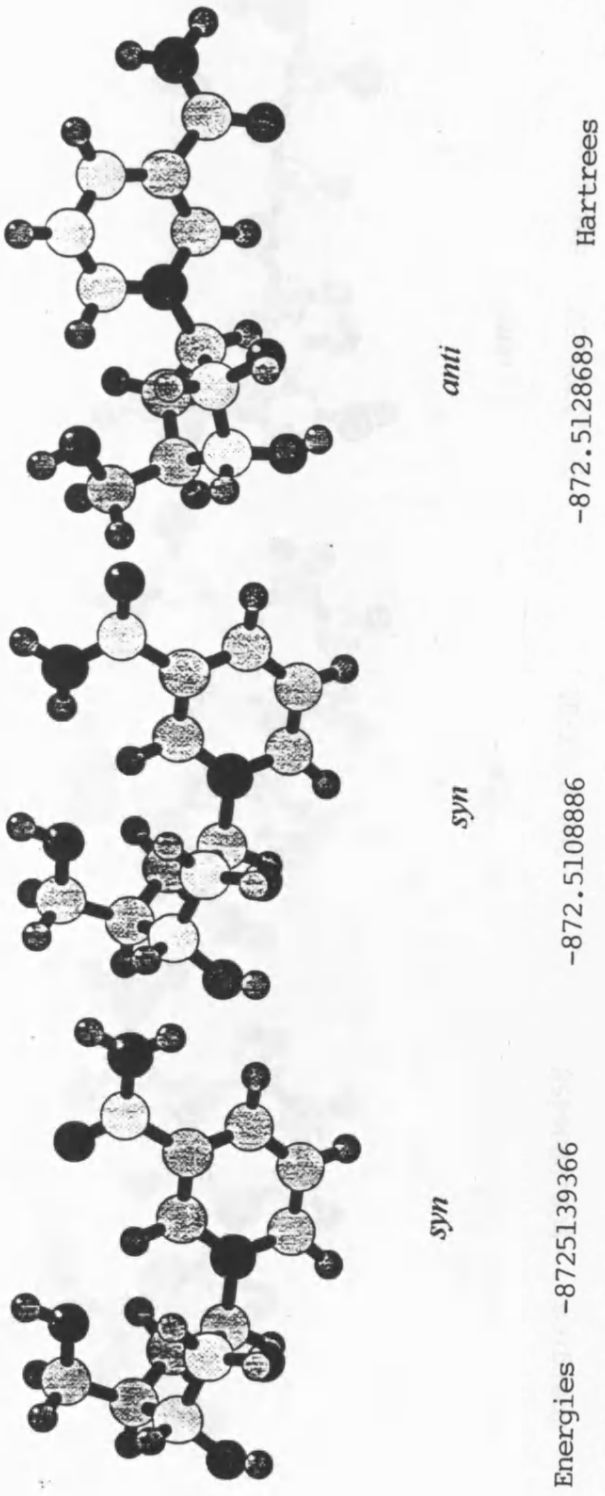
Molecular Models of (a) NADH (30) (blue) superimposed upon C-4, C-3 and C-2 of carba NADH (65), (b) NADH (30) and (c) carba NADH (65).

Figure 3.23



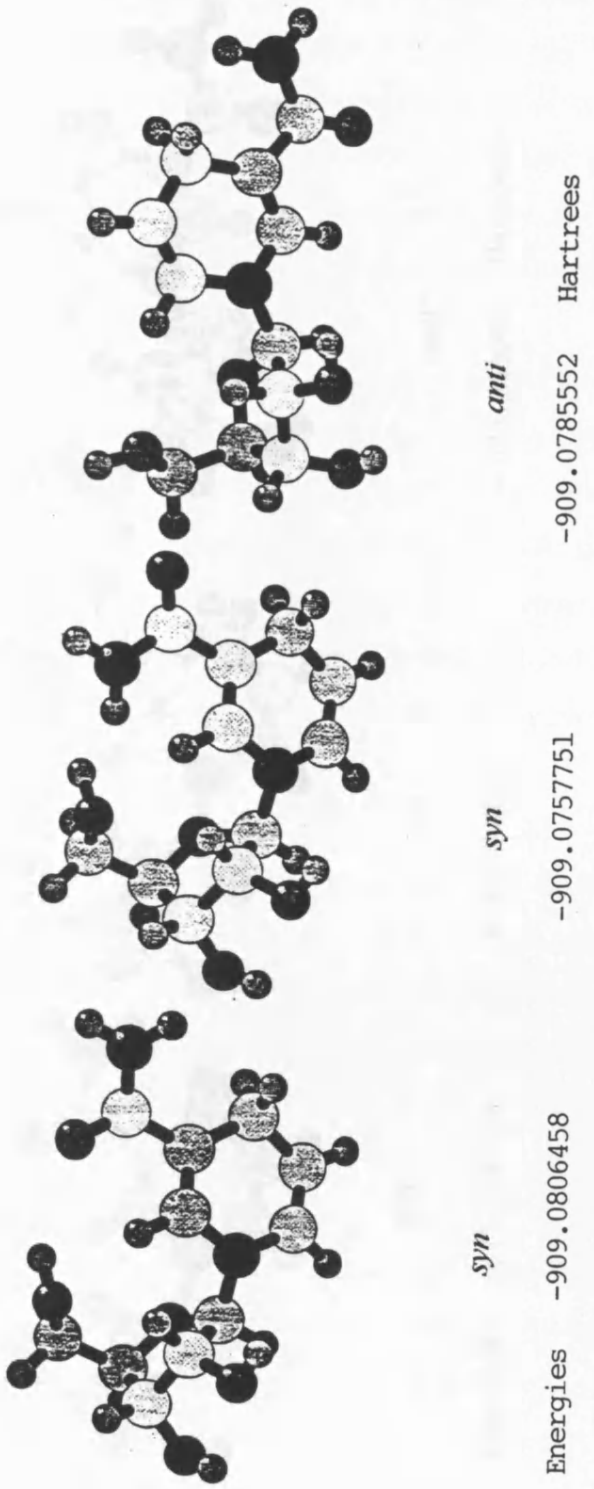
Syn and *anti* conformations of nicotinamide nucleoside (14) from *ab initio* calculations

Figure 3.24



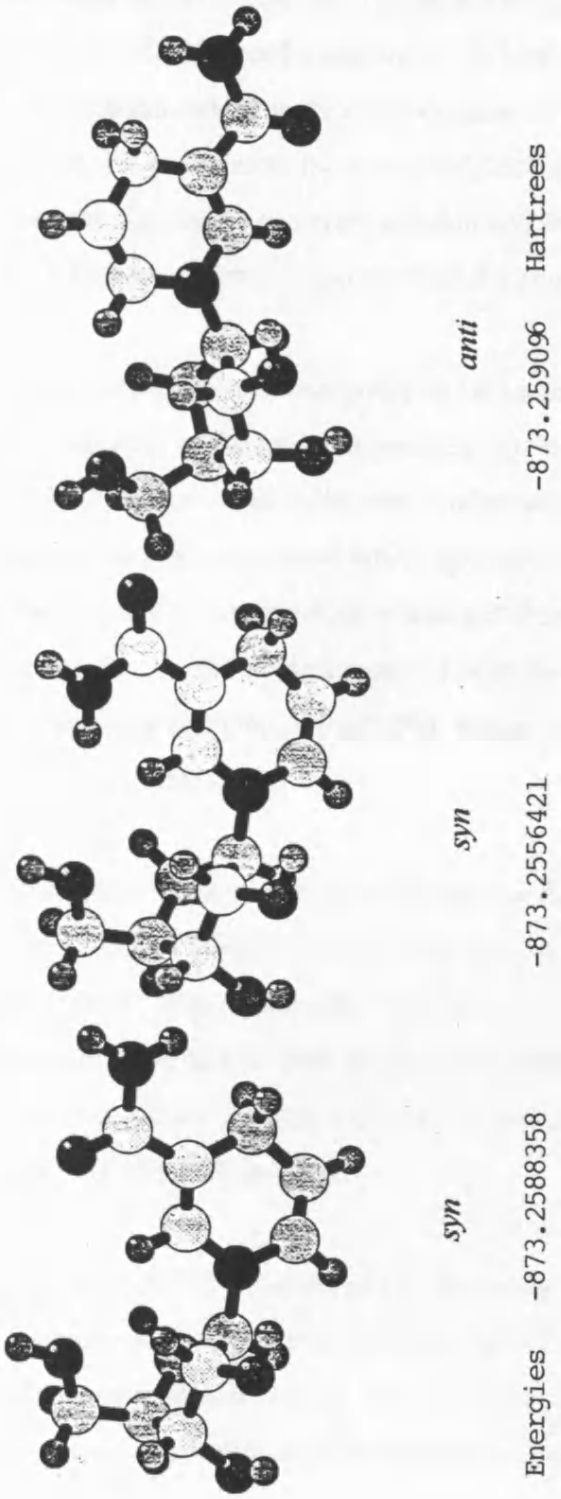
Syn and *anti* conformations of carba nicotinamide nucleoside (35) from *ab initio* calculations

Figure 3.25



Syn and *anti* conformations of reduced nicotinamide nucleoside from *ab initio* calculations

Figure 3.26



Syn and *anti* conformations of reduced carba nicotinamide nucleoside from *ab initio* calculations

Figure 3.27

energy than *anti* NMN^+, or that *anti* NMNH is lower in energy than *syn* NMNH. In fact the *anti* NMN^+ conformation is 0.017 kcal mol^{-1} lower in energy than the equivalent *syn* conformation and the *syn* NMNH conformation is 1.31 kcal mol^{-1} lower in energy than the equivalent *anti* conformation, which is the exact opposite of the Benner postulate. However in the carbocyclic case this is reversed, the *syn* carbaNMN^+ conformation is 0.67 kcal mol^{-1} lower in energy than the equivalent *anti* conformation and the *anti* carba NMNH conformation is 0.16 kcal mol^{-1} lower in energy than the equivalent *syn* conformation.

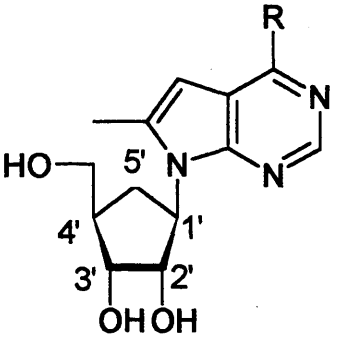
Benner suggested that the dihydronicotinamide ring twists into a boat configuration in order to optimise electron donation of the nitrogen lone pair into the C-O anti bonding orbital. This would make the *pro-R* hydrogen axial in the *anti* conformation and the *pro-S* hydrogen axial in the *syn* conformation and thus determine which hydrogen was transferred. In the carbocyclic case there is no C-O antibonding orbital and therefore no reverse anomeric effect could occur. However, the *ab initio* calculations of both the NMNH and carba NMNH have the dihydronicotinamide ring as being virtually flat, which suggests that the reverse anomeric effect is not playing an important role.

In all cases the sugar ring or carbocyclic ring is $C_{2'}$ *endo* with the 1'-H and 2'-H *trans* diaxial. In the ribose ring the 1'-H 2'-H dihedral angle is 158° and in the cyclopentane ring the 1'-H 2'-H dihedral angle is 166°. This is consistent with the crystal structures of NAD^+ (9) which show a $C_{2'}$ *endo* conformation and the nmr data for the carbocyclic nucleoside (35) and nucleotide (36) (table 3.9) which suggests a $C_{2'}$ *endo* conformation with a *trans* diaxial configuration of the 1'-H and 2'-H protons.

This evidence casts doubt upon the validity of the Benner hypothesis. The results of the *ab initio* calculations directly contradict the second postulate suggesting that in fact *syn* NADH is a weaker reducing agent than *anti* NADH. Also the shape of the dihydropyridine ring from the calculations is not consistent with a reverse anomeric effect.

3.2.7 NMR Studies on Carbocyclic Nicotinamide Mononucleoside (35) and Carbocyclic Nicotinamide Mononucleotide (36)

The puckering of the cyclopentane ring can be estimated from the nmr coupling constants. The Karplus equation[207] relates the vicinal coupling constants to the dihedral angle, however the coupling constants are also dependant on the nature of the substituents. Studies on the carbocyclic nucleosides (68 and 69)[208] led to the conclusion that the carbocyclic ring had a $C_{2'}$ *endo* conformation where the 2'-carbon is out of the plane formed by the other four carbon atoms.



(68) R=Cl

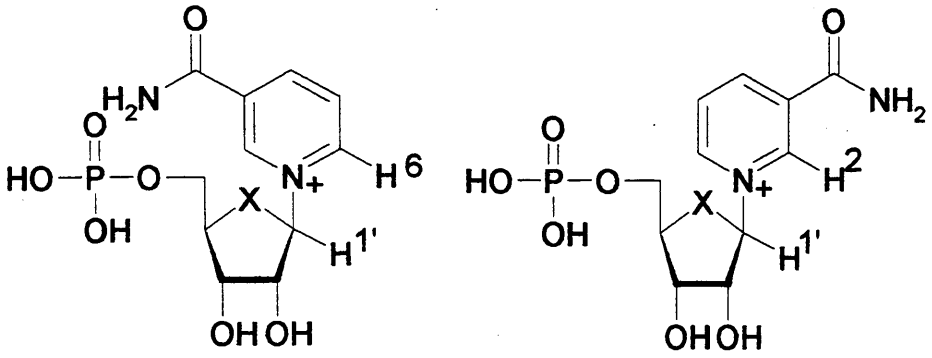
(69) R=NH_2

The coupling constants for (35), (36), (68) and (69) are shown in table 3.9. The large $J_{1',2'}$ values for (68) and (69) correspond to a *trans* diaxial configuration of the 1'-H and 2'-H protons, this is consistent with a $C_{2'}$ *endo* conformation. The $J_{2',3'}$ values and the $J_{3',4'}$ values are also consistent with this conformation. It would be reasonable to assume that the carbocyclic nicotinamide nucleoside (35) and nucleotide (36) would also adopt this $C_{2'}$ *endo* conformation, and indeed the $J_{1',2'}$, $J_{2',3'}$ and the $J_{3',4'}$ values support this assumption. This conclusion is consistent with the results of the *ab initio* calculations which show a $C_{2'}$ *endo* conformation for the cyclopentane ring with a dihedral angle between 1'-H and 2'-H of 166°. As the coupling constants are also dependent upon the nature of the substituents they are an unreliable method of predicting the ring conformation. However, as the results from the *ab initio* calculations support the conclusions from the ^1H nmr coupling constant experiments it seems reasonable to assume that the cyclopentane ring can adopt a $C_{2'}$ *endo* conformation.

	$J_{1',2'}$ values (Hz)	$J_{2',3'}$ values (Hz)	$J_{3',4'}$ values (Hz)
(68)	9.0	4.5	3.5
(69)	8.5	4.5	not observed
(35)	8.9	5.9	3.1
(36)	9.5	5.0	not observed

Table 3.9 ^{1}H nmr coupling constants for (68), (69), (35) and (36)

Sarma and Mynott[169] originally suggested that nicotinamide mononucleotide NMN (13) existed exclusively as the *syn* conformer in solution, whereas the reduced mononucleotide NMNH existed as the *anti* conformer. This conclusion was based on the sugar-base torsion angle. However, later work by Egan[234] based on the nuclear Overhauser effect (NOE) concluded that the *syn* and *anti* conformers were equally or nearly equally populated and interconverting rapidly on the NOE timescale. Benners second postulate[164] which states that *anti* NADH is a weaker reducing agent than *syn* NADH is based upon the Sarma and Mynott[169] findings. The Egan results[234] cast doubt upon the validity of those results and thus the validity of the whole Benner hypothesis. The *ab initio* calculations (figures 3.24, 3.25, 3.26, 3.27) also directly contradict the postulate suggesting that in fact *anti* NADH is a stronger reducing agent than *syn* NADH.



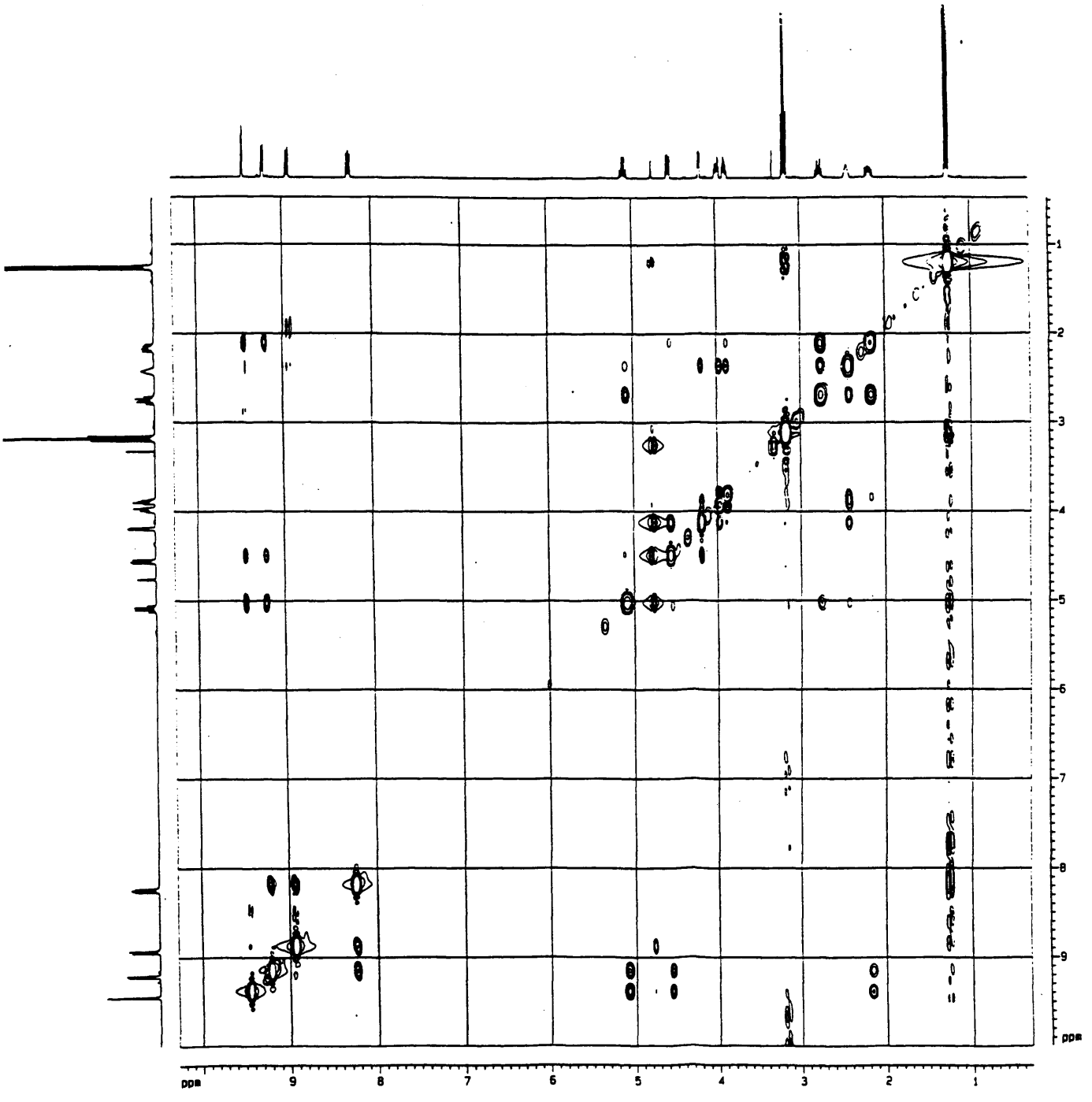
syn *anti*

X=CH_2 carbocyclic NMN (36)

X=O NMN (13)

An exclusive *syn* geometry in NMN (13) would lead to a large enhancement at 1'-H upon irradiation at 6-H and negligible enhancement at 1'-H upon irradiation of 2-H. The experimental findings[234] gave a large enhancement at 2-H upon irradiation at 1'-H and equal enhancements at 1'-H upon irradiation at 2-H and 6-H. This led to the conclusion that the *syn* and *anti* conformers are equally populated.

A NOESY experiment of carba NMN (36) shows approximately 50:50 NOE values from both 2-H and 6-H to 1'H, 2'-H and 5"-H (figure 3.28), this is consistent with rotation about the cyclopentane-nicotinamide bond, causing an averaging of the signals. In other words, as far as nmr is concerned the *syn* and *anti* orientations are indistinguishable. This is consistent with the data for NMN (13).[234] The conclusion that carba NMN (36) exists with rapid rotation between the *syn* and *anti* conformers does not imply that the same rotation occurs in the carbocyclic dinucleotide carba NAD^+ (28), but there is no reason to believe that this may not be the case.



^{1}H NOESY nmr spectrum of carbocyclic nicotinamide nucleoside (36)

Figure 3.28

3.3 Summary

The kinetic data for the carbocyclic NAD^+ (28) indicates that this analogue can act as a good substrate for both class A dehydrogenases (yeast alcohol dehydrogenase) and class B dehydrogenases (glycerol-3-phosphate dehydrogenase). The nmr spectroscopy and molecular modelling studies suggest that carba NAD^+ (28) can adopt the same conformation as NAD^+ (9) without creating any unfavourable interactions. In both cases the ribose or cyclopentane ring adopts a $C_{2'}$ *endo* conformation. The kinetic data is also consistent with this conclusion, as carba NAD^+ (28) acts as a good substrate for dehydrogenases the enzyme bound form of the cofactor analogue must be able to adopt the same conformation as NAD^+ (9) in order to make all of the intermolecular contacts which are necessary for molecular recognition. The stereospecificity of dehydrogenases is unchanged by the substitution of a methylene group for the ribose ring oxygen. The similarity of the kinetic isotope data for both carba NAD^+ (28) and NAD^+ (9) suggests that in both cases the reaction mechanism is the same, involving the cleavage of a C-H bond during the rate limiting step. All of the evidence points to the fact that the carbocyclic NAD^+ (28) is a good substrate for dehydrogenases and behaves in a very similar manner to the natural cofactor NAD^+ (9). The replacement of the ribose ring oxygen with a methylene unit does not alter by a large amount the ability of the cofactor to act as a substrate for dehydrogenases. The *ab initio* calculations and nmr spectroscopy evidence[234] contradict Benners second postulate[164] which states that *anti* NADH is a weaker reducing agent than *syn* NADH. There is also no significant change in the conformation of the dihydronicotinamide ring between the carbocyclic nucleoside and the natural nucleoside, suggesting that the reverse anomeric effect is not playing a very significant role. This leads to the conclusion that there are some flaws in the basic principles behind the hypothesis and therefore in the hypothesis itself.

CHAPTER 4

APPROACHES TO A SYNTHESIS OF A CARBOCYCLIC ANALOGUE OF cADPR

Chapter 4

Approaches to a Synthesis of a Carbocyclic Analogue of cADPR

4.0 Introduction

Cyclic ADP ribose (cADPR, 23), a metabolite of NAD^+ (9) is a naturally occurring cyclic nucleotide and a potent mediator of calcium mobilisation in sea urchin eggs[209,210] as well as mammalian tissues.[211] Studies have suggested that cADPR (23) is an important second messenger in Ca^{2+} signalling and is as active as inositol triphosphate (IP_3),[212] but targets a different intracellular Ca^{2+} channel, the ryanodine receptor.[213,214,215] It has also been proposed that cADPR (23) has a role in insulin release as a possible second messenger of glucose.[216] cADPR (23) was first discovered in 1987 from sea urchin egg microsomes,[209] and a cyclic structure was proposed in 1989.[217] This proposed structure involved a glycosyl linkage between the anomeric carbon of the terminal ribose unit and the N^6 amino group of the adenine moiety (figure 4.1). However, in 1993 the UV absorption properties of cADPR (23)

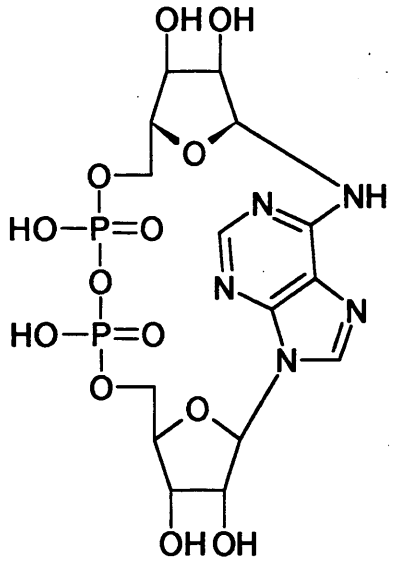
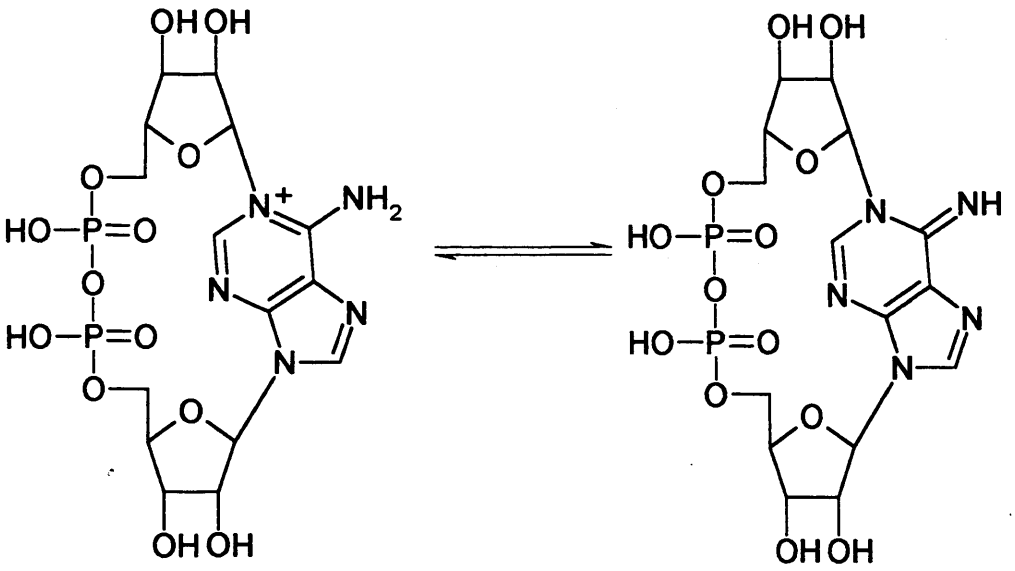


Figure 4.1

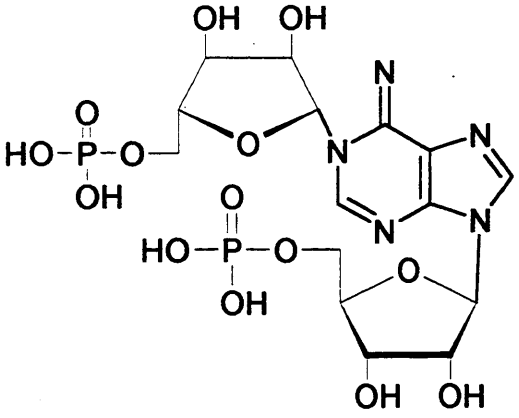
were examined and compared to those of related N substituted adenine derivatives, these results supported a cyclic structure but with the linkage at position 1 of the adenine rather than at N^6.[218] This is now the commonly accepted structure of cADPR (23) (figure 4.2).



cADPR (23)

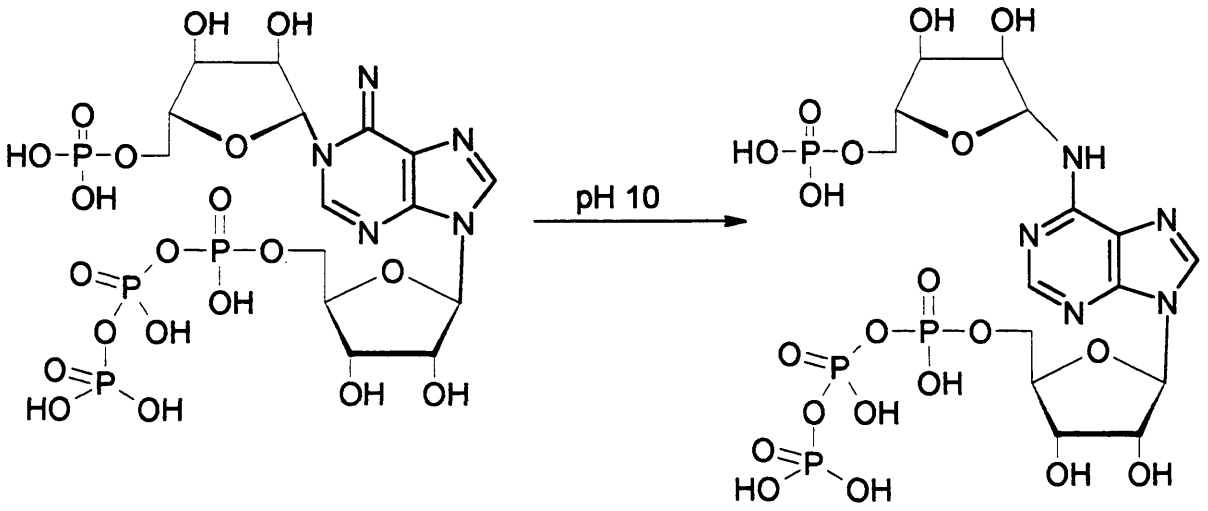
Figure 4.2

The position and stereochemistry of the N-glycosyl linkage was established by correlating cADPR (23) to N^1-(5'-phosphoribosyl) AMP (70) and N^1-(5'-phosphoribosyl) ATP (71), known intermediates of histidine biosynthesis. This was achieved by cleaving the pyrophosphate bond of cADPR (23) without perturbing the integrity of the glycosidic C-N bond.



(70)

N^1-(5'-phosphoribosyl) ATP (71) undergoes a base catalysed transformation to N^6-(5'-phosphoribosyl) ATP (72) (figure 4.3). The UV absorption spectra of cADPR (23) and N^1-(5'-phosphoribosyl) ATP (71) were compared when the pH was varied from basic to acidic, a



(71) (72)

Figure 4.3

similar pattern was observed. Protonation of (71) and cADPR (23) under acidic conditions resulted in a decrease in absorbance at 290nm and therefore resulted in an increase in the ratio A_{260}/ A_{290}. This can be explained by the transformation of the imino structure which has a larger extinction coefficient at 290nm to the cationic form which predominates under acidic conditions (figure 4.4). However, the A_{260}/ A_{290} of N^6-(5'-phosphoribosyl) ATP (72) was

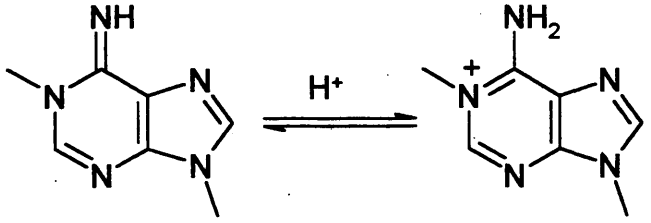


Figure 4.4

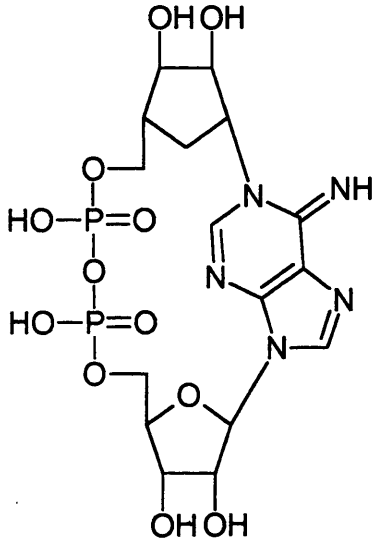
unaffected by pH changes. This is an indication that the N glycosyl linkage is attached to the N^1 nitrogen of the adenine ring rather than the N^6 amino group.

A direct correlation was made between cADPR (23) and N^1-(5'-phosphoribosyl) AMP (70) after cleavage of the pyrophosphate bond in cADPR (23). The retention time of this product on reverse phase HPLC was identical to a known sample of N^1-(5'-phosphoribosyl) AMP (70). The A_{260}/ A_{290} ratio under acidic and basic conditions were also identical, indicating that the N^1 glycosyl linkage is β.

The synthesis of cADPR (23) from NAD^+ (9) involves cleavage of the ribose-nicotinamide bond and then cyclisation of the ribose to the adenine ring, the enzyme which catalyses this reaction is ADP ribosyl cyclase.[219,220,221,22] The breakdown of cADPR (23) to ADP ribose (31)

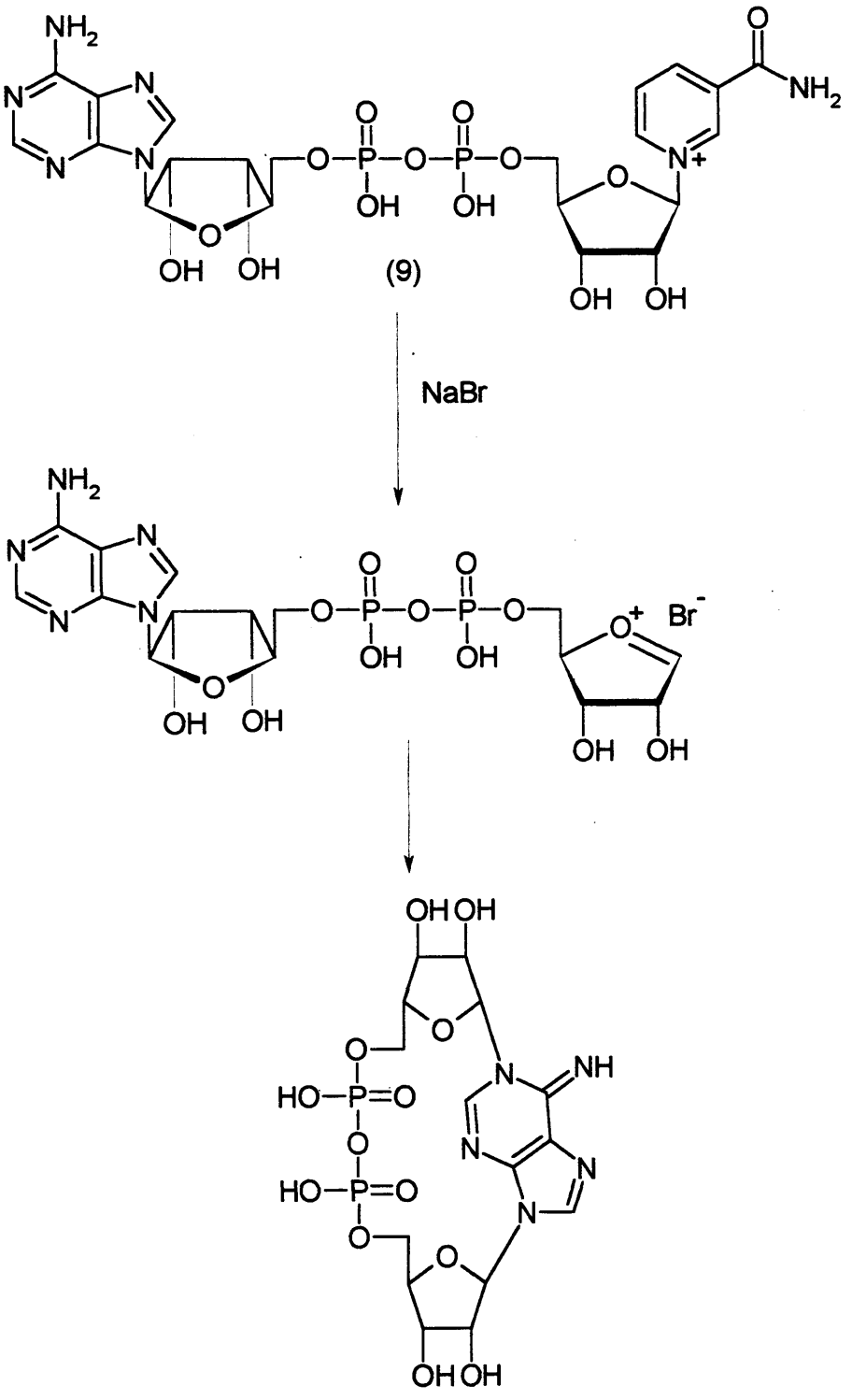
is catalysed by the cADP ribose hydrolase enzyme[223] (scheme 2.2). Cyclic ADPR (23) has been prepared from β-NAD^+ (9) by the use of ADP-ribosyl cyclase from *Aplysia Californica*, which has been purified[209,221] and cloned,[224] but this enzyme is not generally accessible.[221] It has also been prepared in small amounts using β-NAD^+ glycohydrolases,[225,226] which are enzymes that catalyse both cADPR (23) synthesis from β-NAD^+ (9) and hydrolysis of cADPR (23) to ADP ribose (31), another method involves cyclisation of N^1-(5'-phosphoribosyl)ATP catalysed by NAD^+ pyrophosphorylase.[225] A non-enzymatic synthesis has also been reported,[227] this involves the cyclization of β-NAD^+ (9) using NaBr to displace the nicotinamide group, this gives an oxo-carbenium intermediate which then cyclises stereoselectively to give cADPR (23) (scheme 4.1). The reaction gave only one isomer of cADPR (23) and comparison with the natural isomer showed that the N-glycosyl linkage had β stereochemistry, in other words the cyclisation was stereoselective with retention of configuration.

Cyclic ADPR (23) is a very topical molecule and analogues are currently needed as tools to thoroughly investigate its properties. They can be used as probes for the structure-activity relationship, or to provide fluorescent and radio labelled analogues, as well as receptor antagonists and non hydrolysable mimics. Several analogues of cADPR (26) have been prepared (figure 4.5) by a chemoenzymatic cyclisation of NAD^+ analogues[228] using ADP-ribosyl cyclase. The NAD^+ analogues were prepared from coupling reactions between modified nucleotides and β-NMN (13). This work demonstrated the broad substrate specificity of ADP ribosyl cyclase. A carbocyclic analogue of cADPR (73) would be an interesting non hydrolysable mimic of great potential in probing the physiological roles of



(73)

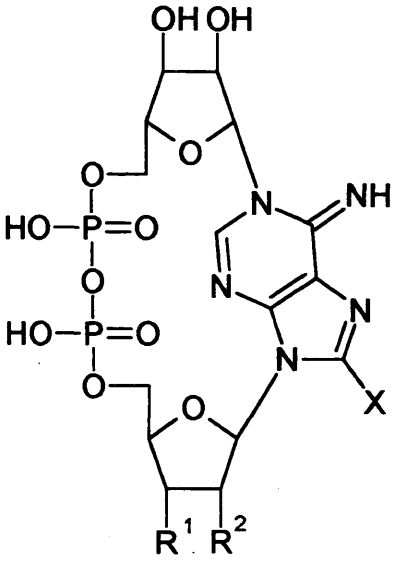
cADP ribose (23). Replacing the ring oxygen of ribose with a methylene group means that the linkage between the ribose ring and position 1 of the adenine would no longer be a glycosidic one. This means that the bond should be stronger and less labile to hydrolysis. A carbocyclic analogue of cADPR (73) may well act as an inhibitor of the cADP ribose hydrolase enzyme.

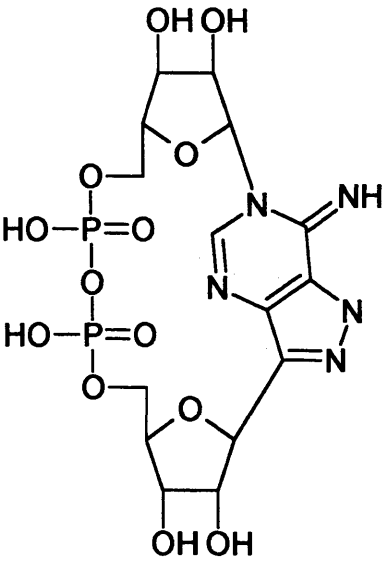


(9)

(23)

Scheme 4.1





cADPR (23) $R^1 = OH$ $R^2 = OH$ X = H

analogues (a) $R^1 = OH$ $R^2 = H$ X = H

(b) $R^1 = H$ $R^2 = OH$ X = H

(c) $R^1 = OH$ $R^2 = OH$ $X = NH_2$

(d) $R^1 = OH$ $R^2 = OH$ X = piperidyl

(e)

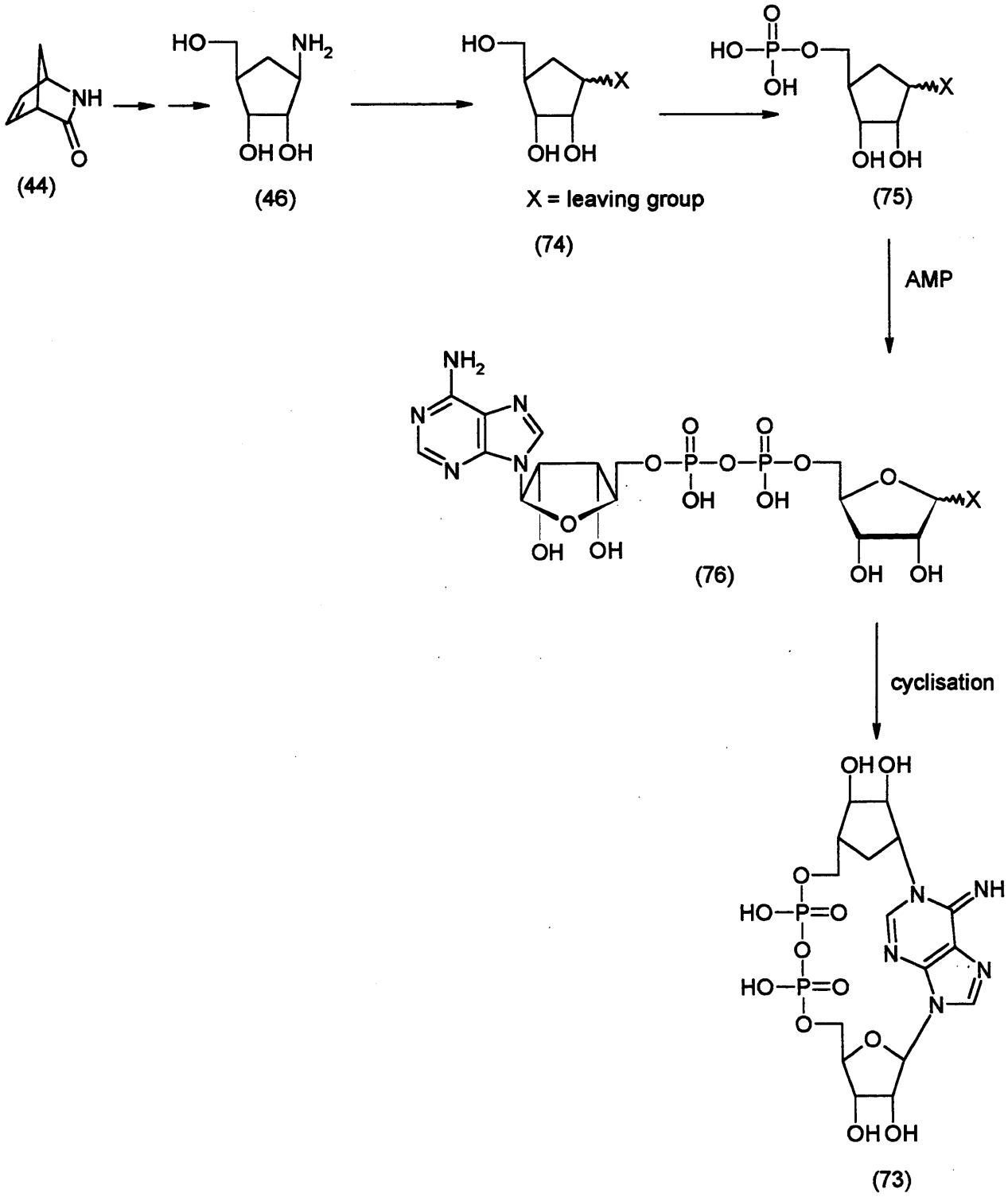
Figure 4.5

4.1 Aims

The aim of this project was to devise and develop a synthetic route to a carbocyclic analogue of cyclic ADP ribose (73) which could be used as a non hydrolysable mimic of cyclic ADP ribose and possible inhibitor of the cyclic ADPR hydrolase enzyme.

4.2 Results and Discussion

In order to make a carbocyclic analogue of cyclic ADP ribose (73) starting from the lactam 2-azabicyclo[2.2.1]hept-5-en-3-one (44) a deamination reaction would be required (scheme 4.2). However NH_2 is a very poor leaving group and any deamination reaction would involve



Proposed route to carbocyclic cADPR (73)

Scheme 4.2

conversion of the amine to a better leaving group, several methods are used to achieve this:

(1) Diazotization - The amine is treated with a nitrite, usually ^{t}butyl nitrite, and a metal halide, e.g. $TiCl_4$, (eqs 4.1 and 4.2).[229]

$$TiCl_4 + 4RONO \rightarrow Ti(OR)_4 + 4NOCl \quad (eq\ 4.1)$$

$$NOCl + RCH_2CH_2NH_2 \rightarrow RCH_2CH_2N_2^+Cl^- + H_2O \quad (eq\ 4.2)$$

Substitution can occur to give the alkyl halide product (eq 4.3).

$$RCH_2CH_2N_2^+Cl^- \rightarrow RCH_2CH_2Cl + N_2 \quad (eq\ 4.3)$$

However there are disadvantages, the yield of the substitution reaction (eq 4.3) is low because of competition by elimination (eq 4.4), rearrangement (eq 4.5), displacement by solvent (eq 4.6) and diazoalkane formation (eq 4.7) which arises because the reaction occurs via the carbocation intermediate.

$$RCH_2CH_2N_2^+Cl^- \rightarrow RCH{=}CH_2 + HCl + N_2 \quad (eq\ 4.4)$$

$$RCH_2CH_2N_2^+Cl^- \rightarrow RCHClCH_3 + N_2 \quad (eq\ 4.5)$$

$$RCH_2CH_2N_2^+Cl^- \rightarrow RCH_2CH_2\text{-}Sol^+Cl^- + N_2 \quad (eq\ 4.6)$$

$$RCH_2CH_2N_2^+Cl^- \rightarrow RCH_2CH{=}N_2 + HCl \quad (eq\ 4.7)$$

(2) Ditosylation - This is analogous to the conversion of an OH group to an OTs group in order to make it a better leaving group. The amine is converted to the ditosylate via two steps (eq 4.8).[230]

$$2TsCl + RNH_2 \rightarrow RNTs_2 + 2HCl \quad (eq\ 4.8)$$

Substitution goes well with nucleophiles such as Cl, Br and I (eq 4.9), but there is still some competition from the elimination reaction (eq 4.10).

$$RNTs_2 + X^- \rightarrow RX + {}^-NTs_2 \quad (eq\ 4.9)$$

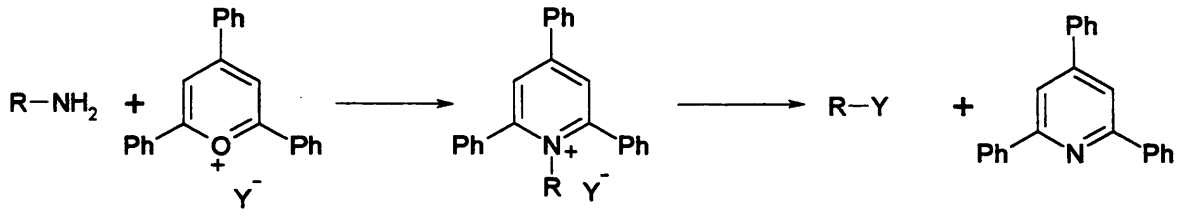
$$RCH_2CH_2NTs_2 \rightarrow RCH{=}CH_2 + Ts_2NH \quad (eq\ 4.10)$$

Another problem arises when very basic nucleophiles are used, e.g. ^-OH and ^-CN; cleavage of the sulphur nitrogen bond occurs preferentially to cleavage of the carbon nitrogen bond (eq 4.11).

$$(R'SO_2)_2NR + {}^-OH \rightarrow R'SO_3^- + RNHSO_2R' \quad (eq\ 4.11)$$

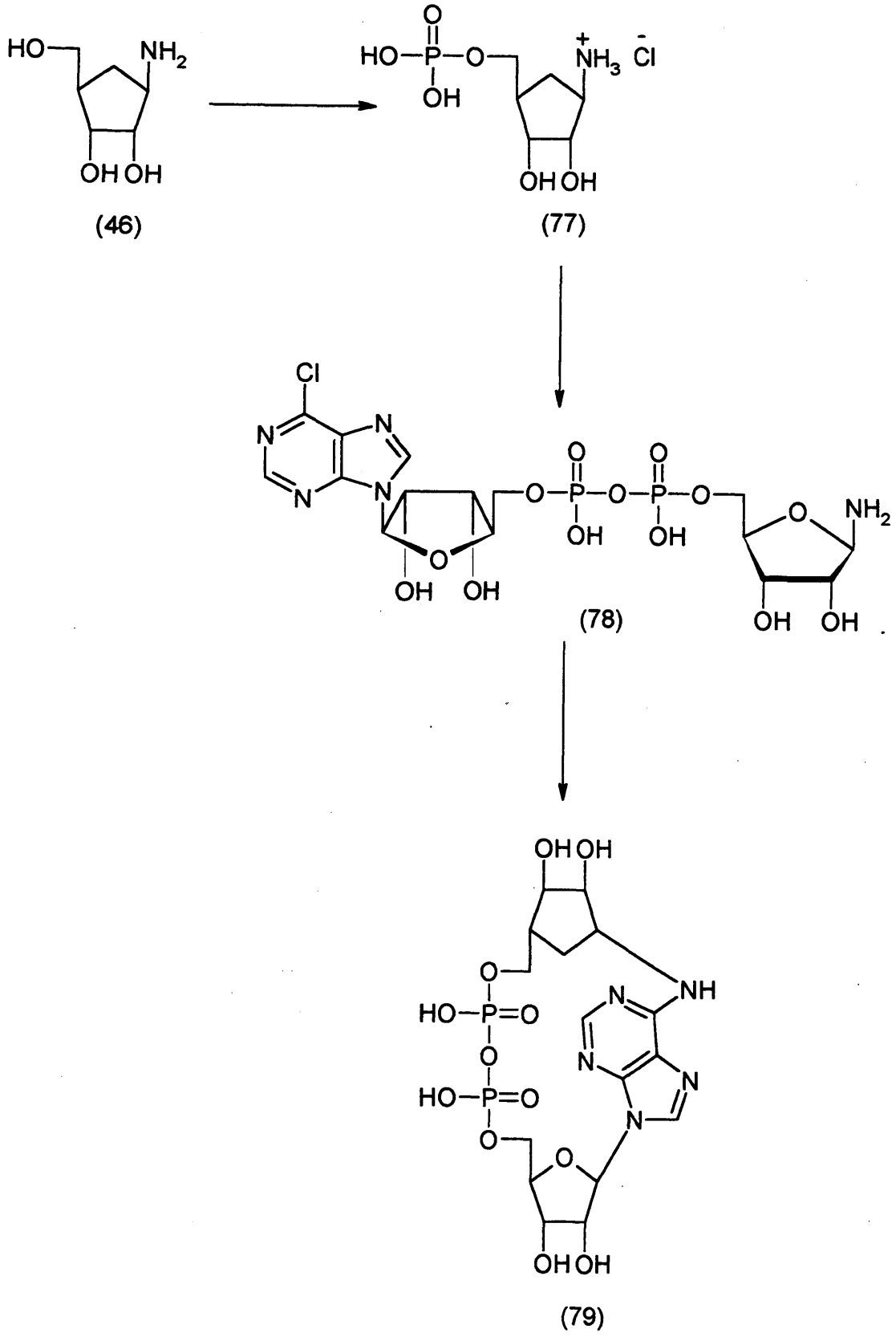
(3) Katritsky Method- The amine is converted to a pyridinium compound by treatment with a pyrilium salt. When the salt is heated the counterion acts as a nucleophile (scheme 4.3).[231] This reaction works well with a wide variety of nucleophiles including halide ions and the acetate

anion. The acetate anion is interesting because this would put an oxygen onto the ring that could lead to the production of a pseudo ribofuranose structure.



Scheme 4.3

However, as indicated above there are difficulties with each method, especially in a multifunctional molecule such as the amine (46). Model studies with cyclopentane derivatives demonstrated that this would not be a practical route to the analogue (73).[232] Alternatively a route to the macrocyclic molecule (79) is available without breaking the C-N bond (scheme 4.4). This would produce a large cyclic carbocyclic molecule which is analogous to the structure which was originally proposed for cyclic ADP ribose (figure 4.1). This molecule may still function as a non hydrolysable mimic of cyclic ADP ribose (23) and act as an inhibitor of the cADPR hydrolase enzyme.



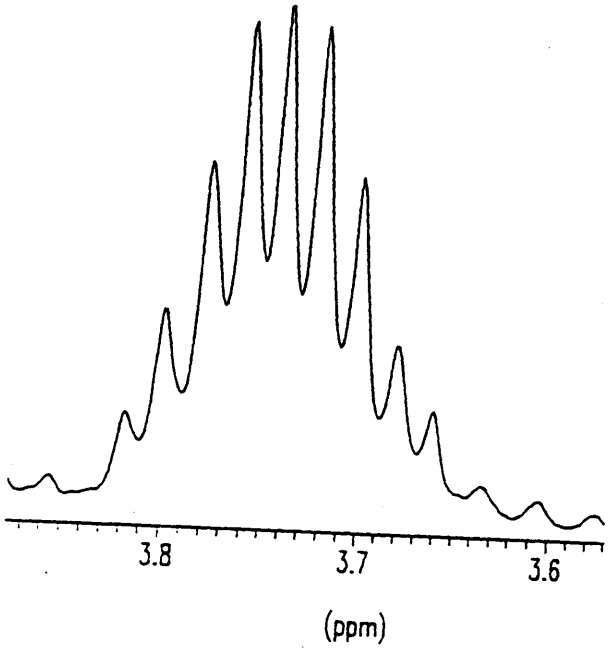
Proposed route to a carbocyclic analogue of cADPR (79)

Scheme 4.4

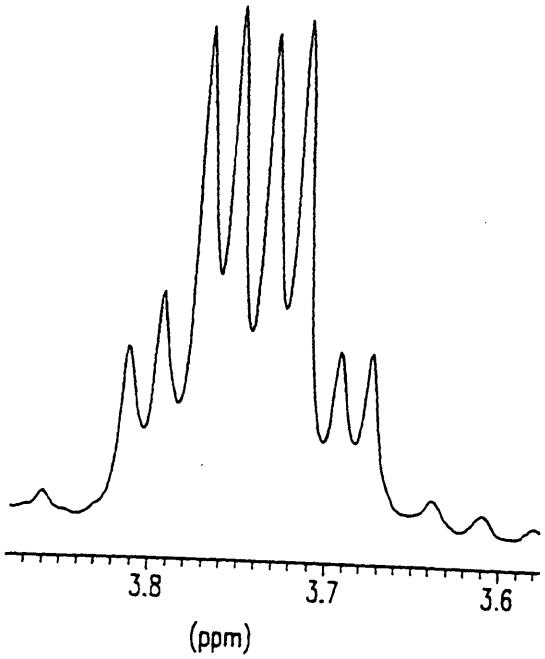
4.2.1 Preparation of the Phosphorylated Amine 4β-Amino-2α, 3α-dihydroxy-1β-(hydroxymethyl)cyclopentane-6-phosphate (77)

The first attempts at phosphorylation of the amine (46) involved using the same method which was employed to phosphorylate the carbocyclic nicotinamide nucleoside (35) (section 2.2.2.7), using *m*-cresol as the solvent and pyrophosphoryl chloride as the phosphorylating agent. ^{1}H and $^{1}H\{^{31}P\}$ nmr spectroscopy indicated that the amine was indeed being phosphorylated at the primary hydroxy group but unfortunately the material was contaminated with *m*-cresol. The *m*-cresol contaminant could not be removed either by ion exchange chromatography (DEAE sephadex) or continuous extraction with ether. The ratio of phosphorylated amine:ether was estimated as 1:0.8 from the ^{1}H nmr data, this suggests that the *m*-cresol was attached to the phosphorylated amine.

A second method was used to carry out the phosphorylation, this involved using trimethylphosphate as the solvent and phosphoryl chloride as the phosphorylating agent. Hydrolysis with ice water gave the required product (77) which could be purified by precipitation from acetone followed by ion exchange chromatography (DEAE Sephadex). It is conceivable that the nitrogen could be phosphorylated under the reaction conditions, however N-P bonds are very acid labile and the hydrolysis work up step makes the reaction mixture acidic (pH=1.5), therefore any N-P bonds which were formed during the reaction would be cleaved during hydrolysis. No phosphorylation of the nitrogen was observed in the final product. The position of phosphorylation was determined using ^{1}H and $^{1}H\{^{31}P\}$nmr spectra, the signal for the two protons 6-H and 6'-H collapsed after ^{31}P decoupling to an eight peak multiplet which is consistent with an ABX system (figure 4.6). This is similar to the decoupling shown by the carbocyclic nicotinamide mononucleotide (36) (section 2.2.2.7). Further evidence was obtained from the ^{13}C nmr spectra, the signal at 65.56 ppm for the 6 carbon atom was split into a doublet by the ^{31}P and shifted downfield from the 6-C signal in the amine (46) 63.95 ppm. The signal for the adjacent carbon atom 1-C at 43.63 ppm was also split into a doublet.



^{1}H nmr signal for 6-H and 6'-H in (77)

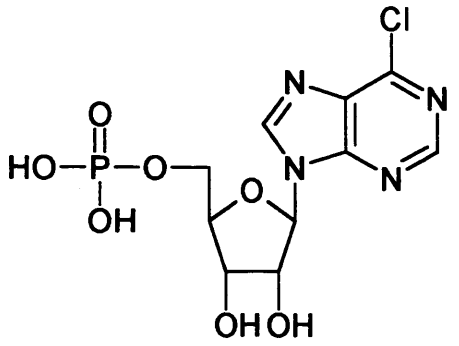


$^{1}H\{^{31}P\}$nmr signal for 6-H and 6'-H in (77)

Figure 4.6

4.2.2 Further Work

The phosphorylated amine (77) is an important building block for the synthesis of (79). Coupling of (77) to 6 chloropurine riboside 5'-monophosphate (80) either using DCC or a morpholidate reaction would yield the carbocyclic dinucleotide (79). Cyclisation of



(80)

this material would give the carbocylic cADP ribose analogue (79), this step could prove to be quite difficult as the reactive centres are separated by a large distance. However, a palladium catalysed reaction may be used to overcome this problem, recently the palladium catalyzed amination of aryl halides has been examined (figure 4.7).[233] In this study an intramolecular palladium catalysed amination of the aryl chloride would lead to cyclization to give the desired cyclic dinucleotide analogue (79).

$$ArBr + \text{“}R_2N\text{”} \rightarrow ArNR_2$$

Figure 4.7

4.2.3 Summary

The phosphorylated amine (77) has been prepared and isolated. This is the first step towards the synthesis of a non hydrolysable carbocyclic analogue of cyclic ADP ribose.

CHAPTER 5

EXPERIMENTAL

Chapter 5

Experimental

5.0 General Comments

(i) Materials

Unless otherwise stated all chemicals and enzymes were obtained from: Aldrich Chemical Company Ltd., Sigma Chemical Ltd. or Fluka Chemicals Ltd.. The (-)2-azabicyclo[2.2.1] hept-5-en-3-one (44) was a gift from Chiros Ltd..

(ii) Solvents

Solvents were purified following the methods of Perrin and Armarego,[235] as follows: methanol and ethanol were dried and distilled from their respective magnesium alkoxides prepared *in situ*. Tetrahydrofuran (THF) was distilled from sodium and benzophenone. Tert-butanol, dichloromethane, chloroform and ethyl acetate were distilled from calcium hydride and then stored over 4Å molecular sieves. Diethyl ether (ether) was dried over sodium wire and then purified by reflux followed by distillation from lithium aluminium hydride.

(iii) Methods

Flash chromatography was carried out according to the method of Still *et al.*[236] using silica (Merck and Co., Kiesel 60, 230-400 mash). Thin layer chromatography (tlc) was conducted on precoated aluminium sheets (60F-254) with a 0.2mm layer thickness manufactured by Merck and Co.. Triethylammonium bicarbonate was prepared by bubbling CO_2 through a 1 molar solution of triethylamine in water until the pH was 7.0-7.5.

(iv) Instrumentation

Melting points were determined using a Kofler hot stage apparatus are reported uncorrected. The pH's of aqueous solutions were measured with a single glass electrode. Infra-red IR spectra were recorded with a Perkin-Elmer 298 spectrometer. Ultraviolet and visible spectrophotomeric measurements were performed on a Hewlett Packard 8452A diode array spectrophotometer. Chemical ionisation (CI), electronic ionisation (EI) and fast atom bombardment (FAB) mass spectrometry was carried out on a Kratos Concept mass spectrometer. Routine ^{1}H nmr spectra were recorded with a Varian EM-390 (90MHz) spectrometer. Routine ^{31}P nmr spectra (^{1}H decoupled) were recorded on a Joel FX90Q spectrometer. Highfield nmr spectra were recorded with either a Bruker ARX 250 nmr

spectrometer, a Bruker AM 300 nmr spectrometer or a Bruker DRX 400 nmr spectrometer. Chemical shifts are reported as positive when downfield of the reference, and negative when upfield. All J and δ values are reported as observed values.

(v) New Compounds

All new compounds were characterised by highfield ^{1}H, ^{13}C and ^{31}P (where appropriate) nmr spectroscopy and by high resolution chemical ionisation, electronic ionisation, fast atom bombardment or electrospray mass spectrometry.

5.1 Chapter 2, Synthesis

5.1.1 Prins Reaction[111,112,113]

Freshly cracked and dried ($MgSO_4$) cyclopentadiene (83 ml, 1 mol) was added to a cooled solution of acetic acid (1000 ml), paraformaldehyde (90 g, 3 mol) and *p*-toluenesulphonic acid (0.076 g, 0.4×10^{-3} mol). The solution was left stirring at 10°C for 23 hours. The solution was rotary evaporated to dryness to leave a yellow oil. The oil was washed with sodium bicarbonate solution and then extracted with ethyl acetate. The solvent was removed under vacuum to leave a yellow oil which was a mixture of the four isomeric products (40a), (41a), (42a) and (43a), (15.5 g,10%).

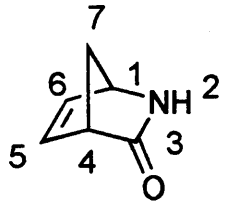
5.1.2 Hydrolysis of Prins Reaction Products[111]

10 g (64 mmol) of the Prins reaction products (40a), (41a), (42a) and (43a) were added to a mixture of methanol:water:triethylamine (5:4:1) (420 ml). The mixture was left stirring at room temperature and pressure for 48 hours. The solution was rotary evaporated to dryness to give a mixture of the diols (40b), (41b), (42b) and (43b) as a brown oil. The oil was purified by column chromatography using 9:1 dichloromethane:methanol on silica gel 60 to give two mixtures (A) and (B). δ_H(300 MHz, $CDCl_3$) for (A) 5.7-6.1 (18H, m), 5.1 (2H, br), 4.9 (1H, m), 4.5-4.7 (4H, m), 4.3 (14H, m), 3.3-3.8 (19H, m), 3.0-3.2 (2H, m), 2.85 (3H, m), 2.6 (1H, m), 2.3 (5H, m), 2.05 (10H, m), 1.9 (6H, m), 1.6 (3H, m); δ_H(300 MHz, $CDCl_3$) for (B) 5.7-6.1 (23H, m), 5.1 (1H, m), 4.9 (2H, m), 4.5-4.8 (6H, m), 4.3-4.5 (1H, m), 4.1 (1H, q, J 6), 3.4-3.8 (1H, m), 2.8-3.3 (22H, m), 2.55-2.7 (2H, m), 2.2-2.4 (8H, m), 2.05 (11H, m), 1.8-2.0 (10H, m), 1.6 (3H, m), 1.25 (1H, t, J 6).

5.1.3 Silylation of the Diol Mixture[111]

tert-Butyldimethylsilyl chloride (1.32 g, 9 mmol), imidazole (1.34 g, 20 mmol) and DMAP (38.9 mg, 0.32 mmol) were added to a cooled solution of the mixed diols (40b), (41b), (42b) and (43b) (1 g, 0.01 mol) in dry dichloromethane (55 ml) under argon. After being stirred for 3 hours at 0°C, the solids were removed by filtration and washed with dichloromethane. The combined filtrate and washings were concentrated under reduced pressure. The residue was purified by column chromatography using (9:1) light petroleum:ethyl acetate on silica gel 60 to give a mixture of (40c), (41c), (42c) and (43c) as an oil (685 mg, 35%). After silylation (A) gave (C) and (D), (B) gave (E) and (F). δ_H(300 MHz, $CDCl_3$) for (C) 5.9 (1H, m), 4.9 (1H, br), 4.15 (1H, q, J 7), 3.85 (1H, m), 3.3 (1H, br), 2.8-3.0 (17H, m), 2.05 (1H, s), 1.15-1.5 (16H, m), 0.87 (120H, m), 0.1 (57H, m); δ_C(75 MHz, $CDCl_3$) for (C)133.6 (CH), 132.7 (CH), 77.6 (CH), 63.1 (CH_2), 60.3 (C), 42.0 (CH), 33.8 (CH_2), 31.4 (CH_2), 25.5 (CH_3), 22.5 (C), 17.8 (C), 13.9 (CH_3), -0.2 (CH_3), -3.4 (CH_3), -3.7 (CH_3), -4.1 (CH_3), -5.6 (CH_3), -5.7 (CH_3); δ_H(300 MHz, $CDCl_3$) for (D) 5.9 (5H, m), 4.6 (5H, br), 4.15 (1H, q, J 7), 3.66 (8H, m), 2.8 (8H, br), 2.12 (5H, m), 2.08 (1H, s), 1.83-2.0 (2H, m), 1.5-1.8 (24H, m), 1.47 (2H, s), 1.3 (75H, m), 0.9 (150H, m), 0.05 (50H, m); δ_C(75 MHz, $CDCl_3$) for (D) 135.1 (CH), 134.6 (CH), 75.4 (CH), 64.3 (CH_2), 46.2 (CH), 36.9 (CH_2), 34.5 (CH_2), 31.7 (CH_2), 31.4 (CH_2), 28.9 (CH_2), 26.7 (CH_2), 26.2 (CH_2), 26.1 (CH_2), 25.8 (CH_3), 25.5 (CH_3), 25.1 (CH_2), 22.5 (CH_2), 20.5 (CH_3), 19.9 (C), 18.9 (CH_3), 18.5 (CH_3), 18.3 (C), 13.9 (CH_3), 11.2 (CH_3), -0.235 (CH_3), -.038 (CH_3), -5.7 (CH_3); δ_H(300 MHz, $CDCl_3$) for (E) 4.1 (1H, q, J 7), 2.6 (9H, s), 2.05 (1H, s), 1.45 (1H, s), 1.2-1.4 (16H, m), 0.8-1.1 (119H, m), 0.05-0.2 (58H, m); δ_C(75 MHz, $CDCl_3$) for (E) 34.7 (CH_2), 31.9 (CH_2), 31.6 (CH_2), 25.7 (CH_3), 25.3 (CH_2), 22.7 (CH_2), 18.0 (C), 14.1 (CH_3), -0.02 (CH_3), -032 (CH_3), -3.6 (CH_3); δ_H(300 MHz, $CDCl_3$) for (F) 4.1 (1H, q, J 7), 2.1 (1H, s), 1.45-1.95 (12H, m), 1.43 (1H, s), 1.2-1.4 (45H, m), 0.8-1.0 (60H, m); δ_C(75 MHz, $CDCl_3$) for (F) 41.4 (C), 39.0 (C), 36.1 (CH), 35.5 (C), 34.7 (CH_2), 34.6 (CH), 31.9 (CH_2), 31.7 (CH_2), 26.9 (CH_2), 25.9 (CH_3), 25.5 (CH_2), 22.7 (CH_2), 20.7 (CH_3), 20.5 (CH_2), 20.3 (CH_2), 19.2 (CH_3), 18.7 (CH_3), 14.1 (CH_3), 11.4 (CH_3).

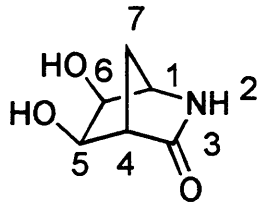
5.1.4 Preparation of Racemic 2-Azabicyclo[2.2.1]hept-5-en-3-one (44)[114,115]



(44)

A solution of tosyl cyanide (5 g, 27.6 mmol) in freshly prepared and dried ($MgSO_4$) cyclopentadiene (75 ml) was, after standing at room temperature for 45 minutes, evaporated to dryness *in vacuo* without heating. The white solid residue was washed with cold anhydrous ether and dissolved in acetic acid (25 ml). A white solid which formed immediately, was separated after addition of the mixture to ice water (125 ml). The mother liquor was neutralised with aq. NaOH, then extracted with dichloromethane (8×100 ml). The dried ($MgSO_4$) extract was rotary evaporated to dryness to give the lactam (44) as a yellow solid which was purified by resublimation to give an off white solid (1.5 g, 50%), mp 54-56^{0}C (lit. 54-55^{0}C[115]), R_f 0.67 (9:1, CH_2Cl_2:MeOH). ^{1}H-nmr and IR data matched the literature,[115] the ^{13}C-nmr data also corresponded to the predicated values for the lactam (44). υ_{max}/cm^{-1} (nujol) 3200(NH), 1710 (CO); δ_H(300 MHz, $CDCl_3$) 6.6-6.9 (2H, m, 5-H and 6-H), 6.1 (1H, br, NH), 4.35 (1H, m, 1-H), 3.22 (1H, m, 4-H), 2.40 (1H, dt, J 1.62, 7.62, 7-H), 2.22 (1H, dq, J 1.62, 7.62, 7'-H); δ_C(75 MHz, $CDCl_3$) 185.37 (3-C), 141.11 (5-C), 138.10 (6-C), 60.33 (7-C), 59.31 (1-C), 53.21 (4-C); *m/z* (NH_3; CI) 110 ([MH^+], 3%), 80 (5.3), 66 (100).

5.1.5 Preparation of Racemic *exo-cis*-5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (47)[124]



(47)

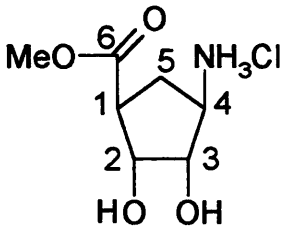
The diol (47) was prepared according to the method of Cermak and Vince.[124] A solution of 4-methylmorpholine N-oxide monohydrate (6.27 g, 46.4 mmol) in water (20 ml) was added to a

stirred solution of racemic 2-azabicyclo[2.2.1] hept-5-en-3-one (44) (5.0 g, 45.8 mmol) in t-butyl alcohol (60 ml). A 2.5% (wt/v) solution of osmium tetroxide in *t*-butyl alcohol (2.1 ml, 0.33 mmol) was added and the mixture was stirred for 2 hours at room temperature in a water bath. The mixture was warmed to 50^{0}C for 15 minutes on a steam bath, then cooled to room temperature before sodium metabisulphite (22 g, 2.5 mol equiv) was added. The mixture was stirred for 15 minutes at room temperature, then diluted with acetone (100 ml) and stirred for a further 15 minutes. The mixture was then filtered, the residue was washed with acetone (3×30 ml), the filtrate and washings were combined and evaporated to give a syrup which was azeotroped with water (3×100 ml) to remove 4-methylmorpholine. The residual syrup was dried by the addition and evaporation of isopropyl alcohol (3×100 ml) to give (47) (5.5 g, 84%) as an off white solid crystallised from MeOH/ether, mp 167-168^{0}C (lit. 173-180^{0}C[124]), R_f 0.27 (9:1, CH_2Cl_2:MeOH). υ_{max}/cm^{-1} (nujol) 3300 (OH, NH), 1690 (CO), 1590 (lactam 2); δ_H(250 MHz, D_2O) 4.1 (1H, d, J 5.8, 5-H), 4.05 (1H, d, J 5.8, 6-H), 3.8 (1H, brs, 1-H), 2.48 (1H, m, 4-H), 1.9 (2H, brs, 7-H and 7'-H); δ_C(63 MHz, CD_3OD) 181.7 (3-C), 73.1 (5-C), 69.4 (6-C), 60.5 (1-C), 53.5 (4-C), 36.7 (7-C); *m/z* (EI) 143 ([M$^+$], 3.5%), 83 (35), 66 (100), 55 (41).

5.1.6 Preparation of (-) *exo-cis*-5,6-Dihydroxy-2-azabicyclo[2.2.1]heptan-3-one (47)[124]

(-)(47) was prepared from (-)(44) using the method described in section 5.1.5. $[\alpha]_D$ -93 (c 1 g in 100 ml water, 20^{0}C, l 10 mm); δ_H(250 MHz, D_2O) 3.94 (1H, d, J 5.9, 5-H), 3.89 (1H, d, J 5.9, 6-H), 3.66 (1H, brs, 1-H), 2.50 (1H, m, 4-H), 1.95 (2H, brs, 7-H and 7'-H); δ_C(63 MHz, D_2O) 181.7 (3-C), 71.5 (5-C), 68.1 (6-C), 59.0 (1-C), 51.5 (4-C), 36.0 (7-C); *m/z* (NH_3, CI) 161 (MNH_4^+, 32%), 144(MH^+,100), 110(52).

5.1.7 Preparation of Racemic Methyl 4β-Amino-2α, 3α-dihydroxy-1-β-cyclopentanecarboxylate Hydrochloride (48)[124]



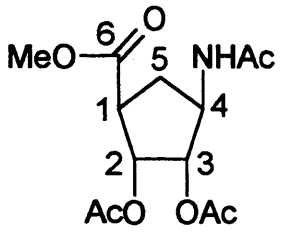
(48)

Diol (48) was prepared according to the method of Vince and Cermak.[124] Lactam (47) (5.5 g, 26 mmoles) was dissolved in methanol (100 ml), hydrogen chloride was bubbled through the mixture for 5 minutes. The resulting solution was refluxed for 2 hours and then evaporated *in vacuo* to a syrup that was crystallised from THF/ MeOH. Diol (48) (8.1 g, 100%) was obtained as an off white solid, mp 143-146^{0}C (lit. 151-153^{0}C[124]), R_f 0.52 (5:2:3, n-butanol:acetic acid:water). υ_{max}/cm^{-1} (nujol) 3100-3500 (OH, NH), 1735 (CO), 1625 (amine 2); δ_H(250 MHz, D_2O) 4.3 (1H, m, 2-H), 4.08 (1H, m, 3-H), 3.75 (3H, s, MeO), 3.6 (1H, m, 4-H), 2.98 (1H, m, 1-H), 2.55 (1H, dt, J 13.8, 8.6, 5-H), 1.86 (1H, dt, J 13.8, 9.2, 5'-H); δ_C(63 MHz, CD_3OD): 175.8 (6-C), 76.0 (2-C), 74.9 (3-C), 56.7 (MeO), 53.3 (4-C), 49.7 (1-C), 29.4 (5-C); *m/z* (EI) 176 ([M-Cl]$^+$, 4%), 144 (32), 115 (53), 98 (73), 83 (75), 56 (100).

5.1.8 Preparation of (+) Methyl 4β-Amino-2α, 3α-dihydroxy-1β-cyclopentanecarboxylate Hydrochloride (48)[124]

(+)(48) was prepared from (-)(46) using the method described in section 5.1.7. $[\alpha]_D$ +15 (c 1.8 g in 100 ml water, 20^{0}C, l 10 mm); δ_H(250 MHz, D_2O) 4.3 (1H, m, 2-H), 4.08 (1H, m, 3-H), 3.75 (3H, s, MeO), 3.54 (1H, m, 4-H), 2.92 (1H, m, 1-H), 2.49 (1H, dt, J 13.8, 8.6, 5-H), 1.80 (1H, dt, J 13.8, 9.2, 5'-H); δ_C(63 MHz, D_2O): 175.8 (6-C), 74.6 (2-C), 73.0 (3-C), 54.8 (MeO), 53.2 (4-C), 47.9 (1-C), 27.6 (5-C); *m/z* (+FAB) 176 ([M-Cl]$^+$, 100%), 162 (11), 144 (10).

5.1.9 Preparation of Racemic Methyl 4β-Acetamido-2α, 3α-diacetoxy-1β-cyclopentanecarboxylate (49)[124]



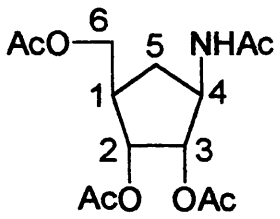
(49)

Triacetate (49) was prepared according to the method of Vince and Cermak.[124] Diol (48) (7.9 g, 37 mmol) was dissolved in pyridine (18.5 ml). The solution was cooled in an ice bath, then acetic anhydride (21.2 ml, 223 mmol) was added. After 5 minutes the mixture was removed from the ice bath and left stirring at room temperature for 2 hours. Ice water (93 ml) was added and the mixture was stirred for 30 minutes. The solution was extracted with dichloromethane (3×93 ml), the combined extracts were washed with water (93 ml), 1M $NaHCO_3$ (186 ml) and water (93 ml) respectively. The dichloromethane solution was dried ($MgSO_4$) and evaporated to give a syrup which was azeotroped with toluene (2×100 ml) to give (46) (8.5 g, 75%) as an off white solid crystallised from EtOAc/Hexane mp 114-116^{0}C(lit. 116-117^{0}C[124]), R_f 0.56 (9:1, CH_2Cl_2:MeOH). δ_H(250 MHz, $CDCl_3$) 6.45(1H, broad d, J 7.0, NH), 5.4 (1H, t, J 5.1, 2-H), 5.1 (1H, t, J 5.7, 3-H), 4.4 (1H, m, 4-H), 3.65 (3H, s, MeO), 2.9 (1H, m, 1-H), 2.6 (1H, m, 5-H), 2.0 (6H, s, 2×AcO), 1.95 (3H, s, AcN), 1.65 (1H, m, 5'-H); δ_C(63 MHz, $CDCl_3$) 174.5 (6-C), 170.8, 170.6, 170.3 (3×$CH_3\underline{C}O$), 76.1 (2-C), 73.9 (3-C), 53.0 (OMe), 52.7 (4-C), 46.0 (1-C), 31.5 (5-C), 23.6, 21.1, 21.1 (3×$\underline{C}H_3CO$); *m/z* (EI) 301 ([M^+], 9%), 241 (42), 198 (53), 182 (67), 156 (51), 140 (100).

5.1.10 Preparation of (+) Methyl 4β-Acetamido-2α, 3α-diacetoxy-1β-cyclopentanecarboxylate (49)[124]

(+)(49) was prepared from (+)(48) using the method described in section 5.1.9. $[\alpha]_D$ +54 (c 1 g in 100 ml water, 20^{0}C, l 10 mm); δ_H(250 MHz, $CDCl_3$) 6.82(1H, broad d, J 7.0, NH), 5.42 (1H, t, J 5.1, 2-H), 5.15 (1H, t, J 5.7, 3-H), 4.45 (1H, m, 4-H), 3.73 (3H, s, MeO), 3.00 (1H, m, 1-H), 2.60 (1H, m, 5-H), 2.07 (6H, s, 2×AcO), 1.98 (3H, s, AcN), 1.71 (1H, m, 5'-H); δ_C(63 MHz, $CDCl_3$): 174.3 (6-C), 170.7, 170.7, 170.3 (3×$CH_3\underline{C}O$), 76.0 (2-C), 73.7 (3-C), 52.9 (OMe), 52.5 (4-C), 45.9 (1-C), 31.3 (5-C), 23.5, 21.1, 21.0 (3×$\underline{C}H_3CO$).

5.1.11 Preparation of Racemic 4β-Acetamido-2α, 3α-diacetoxy-1β-(acetoxymethyl)cyclopentane (50)[124]



(50)

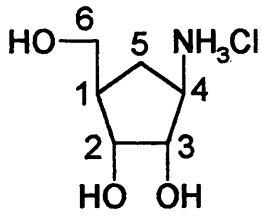
Tetracetate (50) was prepared according to the method of Vince and Cermak.[124] Finely ground anhydrous calcium chloride (4.2 g, 37 mmol) and sodium borohydride (2.8 g, 74 mmol) were suspended in THF (80 ml), the mixture was stirred rapidly at room temperature for 8 hours. A solution of (49) (3.78 g, 12.5 mmol) in THF (48 ml) was added to the borohydride mixture, the mixture was cooled in an ice bath. It was stirred overnight and allowed to come to room temperature. Water was added slowly until effervescence subsided, then some more water (10 ml) was added. 6M HCl was added until the pH was 1.5. The solution was evaporated *in vacuo*, the residue was coevaporated with methanol (2×200 ml) to remove the borates. The residue was then dried by the addition and evaporation of pyridine (2×75 ml). The residue was dissolved in pyridine (32 ml) and then filtered to remove the inorganic matter. The residue was washed with pyridine (16 ml), then the combined filtrate and washings were cooled in an ice bath and treated with acetic anhydride (32 ml, 340 mmol). The mixture was stirred overnight at room temperature. The mixture was evaporated then methanol (50 ml) was added and the solution was refluxed for 10 minutes, after cooling to room temperature it was evaporated. The residue was partitioned between dichloromethane (100 ml) and 1M $NaHCO_3$ (40 ml). The aqueous layer was extracted with dichloromethane (3×25 ml). The combined organic layers were washed with saturated sodium chloride solution (70 ml), dried ($MgSO_4$) and evaporated. The yellow oily residue was azeotroped with toluene (2×100 ml) to give crude (50). (50) was purified using flash chromatography on silica gel 60 eluted with gradually increasing strength solutions of methanol in dichloromethane (from 0% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2), this gave (50) as a yellow oil (2.4 g, 60%), R_f 0.57 (9:1, CH_2Cl_2:MeOH). δ_H(250 MHz, $CDCl_3$) 6.15(1H, broad, NH), 5.08 (2H, m, 2-H and 3-H), 4.4 (1H, m, 4-H), 4.1 (2H, m, 6-H and 6'-H), 2.5 (2H, m, 1-H and 5-H), 2.05 (9H, s, 3×AcO), 1.96 (3H, s, AcN), 1.2 (1H, m, 5'-H); δ_C(63 MHz, $CDCl_3$) 170.0, 169.9, 169.5,

169.2 (4×$CH_3\underline{C}O$), 74.1 (2-C), 71.5 (3-C), 63.6 (6-C), 51.6 (4-C), 39.0 (1-C), 29.1 (5-C), 22.2, 19.8, 19.8 (3×$\underline{C}H_3CO$); *m/z* (+FAB) 316 ([MH^+], 100%), 274 (14), 256(64), 214 (23).

5.1.12 Preparation of (+) 4β-Acetamido-2α, 3α-diacetoxy-1β-(acetoxymethyl)cyclopentane (50)[124]

(+)(50) was prepared from (+)(49) using the method described in section 5.1.11. $[\alpha]_D$ +38 (c 2.2 g in 100 ml water, 20°C, l 10 mm); δ_H(250 MHz, $CDCl_3$) 6.6(1H, broad d, NH), 5.08 (2H, m, 2-H and 3-H), 4.4 (1H, m, 4-H), 4.1 (2H, m, 6-H and 6'-H), 2.5 (2H, m, 1-H and 5-H), 2.05 (9H, s, 3×AcO), 1.96 (3H, s, AcN), 1.2 (1H, m, 5'-H); *m/z* (EI) 315 ([M^+], 1%), 212 (6), 153 (24), 80 (100), 51 (89).

5.1.13 Preparation of Racemic 4β-Amino-2α, 3α-dihydroxy-1β-(hydroxymethyl)cyclopentane Hydrochloride (51)[124]



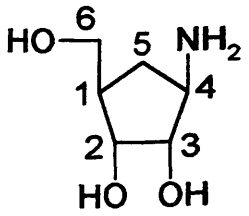
(51)

Triol (51) was prepared according to the method of Cermak and Vince.[124] (50) (5.1 g, 16.2 mmol) was dissolved in 6M HCl (150 ml) and refluxed for 4 hours. The solution was evaporated *in vacuo* to give (51) as a yellow oil (2.9 g, 100%), R_f 0.45 (5:2:3, n-butanol:acetic acid: water). υ_{max}/cm^{-1} (neat) 3300 (OH, NH), 1635 (amine 2); δ_H(250 MHz, D_2O) 3.85 (1H, dd, J 5.7, 7.9, 2-H), 3.77 (1H, dd, J 4.1, 5.7, 3-H), 3.3-3.5 (3H, m, 4-H, 6-H and 6'-H), 2.19 (1H, dt, J 8.2, 13.2, 5-H), 2.02 (1H, m, 1-H), 1.25 (1H, dt, J 9.6, 13.2, 5'-H); δ_C(63 MHz, D_2O) 74.82 (2-C), 72.24 (3-C), 62.98 (6-C), 55.03 (4-C), 44.96 (1-C), 27.48 (5-C); *m/z* (+FAB) 148 ([M-Cl]$^+$, 100%), 132 (5).

5.1.14 Preparation of (-) 4β-Amino-2α, 3α-dihydroxy-1β-(hydroxymethyl)cyclopentane Hydrochloride (51)[124]

(-)(51) was prepared from (+)(50) using the method described in section 5.1.13. $[\alpha]_D$ -8.5 (2 g in 100 ml water, 20°C, l 10 mm); δ_H(250 MHz, D_2O) 3.85 (1H, dd, J 5.7, 7.9, 2-H), 3.77 (1H, dd, J 4.1, 5.7, 3-H), 3.3-3.5 (3H, m, 4-H, 6-H and 6'-H), 2.19 (1H, dt, J 8.2, 13.2, 5-H), 2.02 (1H, m, 1-H), 1.25 (1H, dt, J 9.6, 13.2, 5'-H); δ_C(63 MHz, D_2O): 74.82 (2-C), 72.24 (3-C), 62.98 (6-C), 55.03 (4-C), 44.96 (1-C), 27.48 (5-C); *m/z* (+FAB) 148 ($[M-Cl]^+$, 100%), 80 (28).

5.1.15 Preparation of Racemic 4β-Amino-2α, 3α-dihydroxy-1β-(hydroxymethyl)cyclopentane (46)[85]



(46)

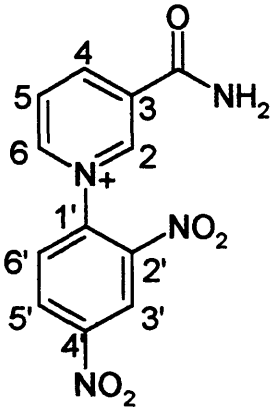
Triol (51) (2.9 g, 16 mmol) was dissolved in methanol (20 ml) and passed through a column of Dowex 1 (OH^- form) (70 ml). The column was washed with methanol (200 ml) and the combined methanol solutions were evaporated to give amine (46) as a yellow oil (1.9 g, 81%), R_f 0.47 (5:2:3, n-butanol:acetic acid: water). δ_H(250 MHz, D_2O) 3.6 (1H, t, J 5.0, 2-H), 3.3-3.4 (3H, m, 3-H, 6-H and 6'-H), 2.9 (1H, m, 4-H), 1.8-2.0 (2H, m, 1-H and 5-H), 0.8 (1H, dt, J 8.5, 12.5, 5'-H); δ_C(63 MHz, D_2O) 79.17 (2-C), 73.30 (3-C), 63.95 (6-C), 55.10 (4-C), 44.97 (1-C), 31.64 (5-C); *m/z* (EI) 147 ($[M^+]$, 7%), 98 (100), 82 (36), 68 (95), 56 (94).

5.1.16 Preparation of (-) 4β-Amino-2α, 3α-dihydroxy-1β-(hydroxymethyl)cyclopentane (46)[85]

(-)(46) was prepared from (-)(51) using the method described in section 5.1.15. $[\alpha]_D$ -7.5 (c 5.3 g in 100 ml MeOH, 20°C, l 10 mm), (lit. -10.0 (c 1 g in 100 ml MeOH))[85]; δ_H(250 MHz, D_2O): 3.6 (1H, t, J 5.0, 2-H), 3.3-3.4 (3H, m, 3-H, 6-H and 6'-H), 2.9 (1H, m, 4-H),

1.8-2.0 (2H, m, 1-H and 5-H), 0.8 (1H, dt, J 8.5, 12.5, 5'-H); *m/z* (+FAB) 148 (MH^+, 100%), 89 (24), 77 (23).

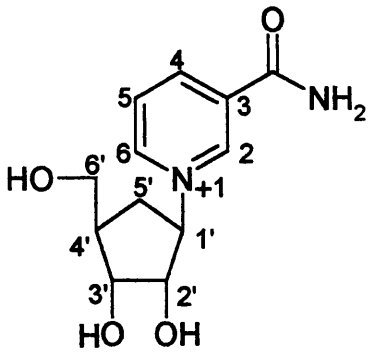
5.1.17 Preparation of N1 (2,4-dinitrophenyl) 3 carbamoyl-pyridinium chloride (55)[147]



(55)

Nicotinamide (12) (2 g, 16 mmol) and 2,4 dinitrochlorobenzene (56) (10 g, 50 mmol) were ground up together to give a fine powder. This powder was heated to 100°C for 1 hour, then dissolved in methanol (15 ml) and precipitated with hot ether (150 ml) this precipitation step was repeated twice to give a yellow residue which was dissolved in water (50 ml) and treated with activated charcoal. The solution was evaporated to give (55) (1.9 g, 36%) as a pale yellow solid, mp 74-76°C (lit. 76°C[147]). δ_H(250 MHz, D_2O) 9.36 (1H, s, 2-H), 9.05 (1H, d, J 2.5, 3'-H), 9.03 (1H, s, 6-H), 8.96 (1H, d, J 8.1, 4-H), 8.63 (1H, dd, J 8.8, 2.5, 5'-H), 8.19 (1H,dd, J 8.1, 6.4, 5-H), 7.97 (1H, d, J 8.8, 6'-H); δ_C(63 MHz, D_2O) 165.3 (C=O), 150.1 (1'-C), 147.9 (2-C), 147.7 (3'-C), 146.0 (6-C), 143.0 (2'-C or 4'-C), 138.7 (2'-C or 4'-C), 134.4 (3-C), 131.6 (4-C), 131.1 (5'-C), 129.0 (5-C), 123.1 (6'-C); λ_{max} 226 nm, ε=26088 $M^{-1}cm^{-1}$; *m/z* (+FAB) 289 ($[M-Cl]^+$, 100%), 273 (13).

5.1.18 Preparation of Racemic Carbocyclic Nicotinamide Nucleoside (35)[148]



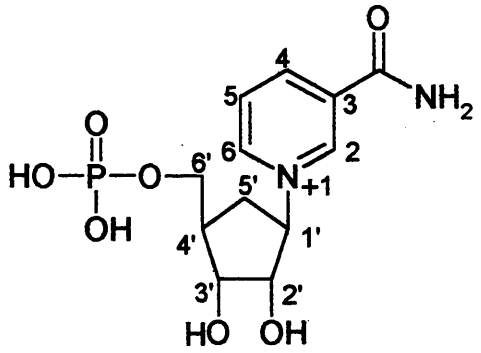
(35)

The carbocyclic nucleoside (35) was prepared according to the method of Sicsic et al..[148] Amine (46) (1.59 g, 10.8 mmol) was dissolved in methanol (32 ml), (55) (2.3 g, 7.1 mmol) was added. The mixture was stirred at 25°C for 48 hours, a yellow precipitate of 2,4-dinitroaniline (57) separated out. The mixture was evaporated and then dissolved in water (30 ml). The aqueous solution was extracted with ether (12×30 ml) to remove the precipitate. The aqueous solution was concentrated and treated with charcoal. The solution was applied to an ion-exchange column of Dowex 50W×8-200 resin (H^+ form) (100 ml). The column was eluted with a linear gradient (0 mM-0.8 mM) HCl (total volume used 1.4 l). The eluent from the column was monitored at λ 266 nm. The appropriate fractions were combined and concentrated to give (35) (704 mg, 35%) as a white solid. δ_H(250 MHz, D_2O) 9.49 (1H, s, 2-H), 9.24 (1H, d, J 6.0, 6-H), 9.03 (1H, d, J 8.0, 4-H), 8.33 (1H, dd, J 8.0, 6.0, 5-H), 5.17 (1H, m, 1'-H), 4.53 (1H, dd, J 8.9, 5.9, 2'-H), 4.2 (1H, dd, J 5.9, 3.1, 3'-H), 3.83 (2H, d, J 5.9, 6'-H and 6"-H), 2.78 (1H, m, 5'-H), 2.42 (1H, m, 4'-H), 2.07 (1H, m, 5"-H); δ_C(63 MHz, D_2O) 166.1 (C=O), 145.7 (2-C), 145.1 (6-C), 143.4 (4-C), 134.4 (3-C), 128.9 (5-C), 77.0 (1'-C), 75.9 (2'-C), 71.9 (3'-C), 62.9 (6'-C), 45.0 (4'-C), 29.6 (5'-C); λ_{max} 266 nm, ε=5400 $M^{-1}cm^{-1}$ in water, (lit. λ_{max} 266 nm, ε=5450 $M^{-1}cm^{-1}$ in water)[131]; *m/z* (+FAB) 254 ($[M\text{-}Cl+H]^+$, 100%), 253 ($[M\text{-}Cl]^+$, 56), 154 (77), 136(67) (Found: $[M\text{-}Cl]^+$, 253.11883. $C_{12}H_{17}N_2O_4$ requires 253.11884).

5.1.19 Preparation of (-) Carbocyclic Nicotinamide Nucleoside (35)[148]

(-)(35) was prepared from (-)(46) using the method described in section 5.1.18. $[\alpha]_D$ -9 (c 5 g in 100 ml water, 20°C, l 10 mm), (lit. -9 (c 5 g in 100 ml water, 20°C, l 10 mm)).[148] δ_H(250 MHz, D_2O) 9.49 (1H, s, 2-H), 9.24 (1H, d, J 6.0, 6-H), 9.03 (1H, d, J 8.0, 4-H), 8.33 (1H, dd, J 8.0, 6.0, 5-H), 5.17 (1H, m, 1'-H), 4.53 (1H, dd, J 9.0, 5.9, 2'-H), 4.2 (1H, dd, J 5.9, 3.1, 3'-H), 3.83 (2H, d, J 5.9, 6'-H and 6"-H), 2.78 (1h, m, 5'-H), 2.42 (1H, m, 4'-H), 2.07 (1H, m, 5"-H).

5.1.20 Preparation of Racemic Carbocyclic Nicotinamide Nucleotide (36)[55]



(36)

Carba nucleoside (35) (163 mg, 565 μmol) was dissolved in *m*-cresol (8.5 ml), then cooled in an ice bath. Pyrophosphoryl tetrachloride (282 μl, 2 mmol) was added and the mixture was stirred in an ice bath for 4 hours. Ice water (28 ml) was added and the mixture was extracted with ether (11 ml). The pH of the solution was adjusted to 5 by the addition of 10% aq NH_4OH. The solution was concentrated and passed through an anion exchange column of Dowex-2 resin (acetate form) (20 ml) to remove the chloride and phosphate ions, the column was washed with 0.001M acetic acid (50 ml) to remove the last traces of nucleotide. The solution was concentrated and the passed through a cation exchange column of Dowex50W×8-200 resin (H^+ form) (20 ml). The column was washed with water (100 ml) and the solution which was now free from nicotinamide and carbocyclic nucleoside was concentrated and applied to an anion exchange column of DEAE-Sephadex resin (70 ml). The column was eluted with a linear gradient (25mM-200 mM) TEAB (total volume used 1000 ml). The eluent from the column was monitored at λ 266 nm. The appropriate fractions were

combined and concentrated. TEAB was removed by coevaporation with methanol. The carbocyclic nucleotide (36) (80 mg, 26%) was obtained as a white solid. δ_H(250 MHz, D_2O) 9.37 (1H, s, 2-H), 9.12 (1H, d, J 6.3, 6-H), 8.82 (1H, d, J 8.0, 4-H), 8.12 (1H, dd, J 8.0, 6.3, 5-H), 4.96 (1H, q, J 9.4, 1'-H), 4.51 (1H, dd, J 9.6, 5.0, 2'-H), 4.08 (1H, d, J 5.0, 3'-H), 3.76 (2H, m, 6'-H and 6"-H), 2.66 (1H, m, 5'-H), 2.29 (1H, m, 4'-H), 2.07 (1H, m, 5"-H); δ_C(75 MHz, D_2O) 166.4 (C=O), 148.3 (2-C), 147.6 (6-C), 145.7 (4-C), 136.7 (3-C), 131.3 (5-C), 80.2 (1'-C), 78.8 (2'-C), 75.6 (3'-C), 68.0 (d) (6'-C), 49.3 ($\underline{C}H_2CH_3$ from $^+NHEt_3$ counter ions), 46.0 (d) (4'-C), 31.9 (5'-C), 10.9 ($CH_2\underline{C}H_3$ from $^+NHEt_3$ counter ions); δ_P(101 MHz, D_2O, MeOH, NEt_3): 3.56 (s); *m/z* (+FAB) 434([Et_3NHM^+], 5%), 333 (MH^+, 15), 239 (100) (Found: $[MH]^+$, 333.08516. $C_{12}H_{18}N_2O_7P$ requires 333.08518); λ_{max} 266 nm, ε=4.5×10^3 $M^{-1}cm^{-1}$ in water, (lit. λ_{max} 266 nm, ε=4.92×10^3 $M^{-1}cm^{-1}$ in water).[133]

5.1.21 Preparation of (-) Carbocyclic Nicotinamide Nucleotide (36)[55]

(-)(36) was prepared from (-)(35) using the method described in section 5.1.20. $[\alpha]_D$ -39 (c 5.9 g in 100 ml water, 28.6^{0}C, l 10 mm); δ_H(250 MHz, D_2O) 9.45 (1H, s, 2-H), 9.25 (1H, d, J 6.3, 6-H), 8.95 (1H, d, J 8.0, 4-H), 8.30 (1H, dd, J 8.0, 6.3, 5-H), 5.12 (1H, q, J 9.4, 1'-H), 4.20 (1H, dd, J 9.6, 5.3, 2'-H), 4.20 (1H, m, 3'-H), 4.00 (2H, m, 6'-H and 6"-H), 2.80 (1H, m, 5'-H), 2.50 (1H, m, 4'-H), 2.20 (1H, m, 5"-H); *m/z* (+FAB) 434 ([Et_3NHM^+], 15%), 333 (MH^+, 60), 239 (100).

5.1.22 Preparation of Carbocyclic NAD^+ (28) and Pseudo Carbocyclic NAD^+ (29)

Carba NMN (36) (389 mg, 6.52×10^{-4}mol) and AMP (1) (436 mg, 1.3 mmol) were dissolved in water (6.5 ml), pyridine (45.7 ml) was added and the mixture was stirred at room temperature. Hot dicyclohexylcarbodiimide (DCC) (20.7 g) was added and the mixture was left stirring overnight. More hot DCC (7.5 g) was added and the mixture was again stirred overnight at room temperature. The reaction mixture was poured into water (450 ml) and allowed to stand for 2 hours, it was then concentrated *in vacuo*. The solution was applied to a column of DEAE sephadex resin (70 ml). The column was eluted with a linear gradient (25

mM-200 mM) TEAB (total volume used 1600 ml). The elutent from the column was monitored a λ 260 nm. Unreacted (36) was eluted from the column first, this was recovered and recycled. Fractions containing (28) and (29) were combined and concentrated. TEAB was removed by coevaporation with methanol. The mixture of (28) and (29) was obtained as a white solid. δ_H(250 MHz, D_2O) signals attributed to both cyclopentane rings 2.03-2.16 (2H, m, 5'-H), 2.39-2.41 (2H, m, 4-H), 2.55-2.67 (2H, m, 5-H), 4.00-4.10 (8H, m, 2-H, 3-H, 6-H, 6'-H), 4.91-5.16 (2H, m, 1-H), signals attributed to both ribosyl rings 4,16-4.21 (4H, m), 4.35-4.46 (6H,m), 6.03 (1H, d, J 6.3, carba NAD^+ anomeric proton), 6.07 (1H, d, J 5.9, pseudo carba NAD^+ anomeric proton), signals attributed to the aromatic rings 8.11-8.28 (4H, m, A2-H(carba NAD^+ and pseudo carba NAD^+), N5-H (carba NAD^+ and pseudo carba NAD^+)), 8.45 (2H, brs, A8-H (carba NAD^+ and pseudo carba NAD^+)), 8.82 (2H, d, J 7.9, N4-H (carba NAD^+ and pseudo carba NAD^+)), 9.08 (1H, d, J 6.3, N6-H (carba NAD^+)), 9.12 (1H, d, J6.0, N6-H (pseudo carba NAD^+)), 9.31 (1H, s, N2-H (carba NAD^+)), 9.35 (1H, s, N2-H (pseudo carba NAD^+)); λ_{max} 260 nm, $\varepsilon=15.0\times10^3$ $M^{-1}cm^{-1}$ in TRIS.EDTA, pH 8 (lit. 260 nm, $\varepsilon=15.4\times10^3$ $M^{-1}cm^{-1}$ in TRIS.HCl, 0.5M EtOH, pH 10).[133]

5.1.23 Preparation of Carbocyclic NAD^+ (28)

(28) was prepared as a single diastereoisomer from (-)(36) using the method described in section 5.1.22. δ_H(250 MHz, D_2O) signals attributed to the cyclopentane ring 2.03-2.16 (1H, m, 5'-H), 2.39-2.41 (1H, m, 4-H), 2.55-2.67 (1H, m, 5-H), 4.00-4.10 (4H, m, 2-H, 3-H, 6-H, 6'-H), 5.01 (1H, q, J 9.6, 1-H), signals attributed to the ribosyl ring 4,16-4.21 (2H, m), 4.35-4.46 (3H,m), 6.00 (1H, d, J 5.7, anomeric proton), signals attributed to the nicotinamide ring 8.15 (1H, dd, J 7.5, 6.7, 5-H), 8.81 (1H, d, J 7.8, 4-H), 9.08 (1H, d, J 5.7, 6-H), 9.31 (1H, s, 2-H), signals attributed to the adenine ring 8.45 (1H, s, 8-H), 8.21 (1H, s, 2-H); δ_C(100 MHz, D_2O) signals attributed to the cyclopentane ring 77.5 (CH, 1-C), 76.3 (CH, 2-C), 72.6 (CH, 3-C), 65.8 (CH_2, 6-C), 43.5 (d, CH, 4-C), 29.2 (CH_2, 5-C), signals attributed to the ribosyl ring 87.2 (CH), 84.3 (d, CH), 74.6 (CH), 70.7 (CH), 67.1 (CH_2), signals attributed to the nicotinamide ring 165.5 (C=O), 145.8 (CH, 2-C), 145.2 (CH, 6-C), 143.1 (CH, 4-C), 134.1 (C, 3-C), 129.1 (CH, 5-C), signals attributed to the adenine ring 155.5 (C), 152.9 (CH), 149.3 (C), 140.3 (C), 118.7 (CH); δ_P(63 MHz, D_2O, TRIS EDTA buffer pH 8): -10.9 (d, J 21.1), -

11.4 (d, 21.1), *m/z* (Electrospray) 662 (M^+, 7%), 72(100) (Found: M^+, 662.13767. $C_{22}H_{30}N_7O_{13}P_2$ requires 662.13768); λ_{max} 260 nm, ε=15.0×10^3 $M^{-1}cm^{-1}$ in TRIS.EDTA pH 8; $[\alpha]_D$ -31 (c 0.6 g in 100 ml water, 26.5^{0}C, l 10 mm).

5.1.24 Separation of Carbocyclic NAD$^+$ (28) and Pseudo Carbocyclic NAD$^+$ (29)[87]

The two diastereoisomers (28) and (29) were separated by reversed phase HPLC using a PhaseSep Spherisorb S5ODS1 column (20 mm diameter×250 mm length). The column was equilibrated with 90% buffer (20 mM sodium dihydrogen phosphate and 2mM tetrabutyl ammonium dihydrogen phosphate adjusted to pH6 with tetrabutyl ammonium hydroxide) and 10% methanol. The mixture of dinucleotides (15 mg) was applied to the column and the chromatography developed with a gradient from 10% methanol to 30% methanol over a period of 40 minutes with a flow rate of 8 ml/ minute. The carba NAD$^+$ (28) eluted first with a retention time = 24.3 minutes followed immediately by pseudo carba NAD$^+$ (29) with a retention time = 25.0 minutes. The dinucleotides were desalted by ion exchange chromatography. A solution of the crude dinucleotide (28 or 29) (100 mg) in distilled water was applied to a column of Dowex 1×2-200 (formate form) (10 ml). The resin was washed water (100 ml) to remove the salts. The dinucleotide was eluted with 0.2M formic acid until all of the dinucleotide was eluted from the column (monitored at λ=260 nm). The elutant was concentrated to give the dinucleotide. δ_H for carba NAD$^+$ (28) (250 MHz, D_2O) signals attributed to the cyclopentane ring 2.11-2.24 (1H, m, 5'-H), 2.42 (1H, m, 4-H), 2.62-2.74 (1H, m, 5-H), 4.10-4.16 (4H, m, 2-H, 3-H, 6-H, 6'-H), 5.10 (1H, q, J 9.4, 1-H), signals attributed to the ribosyl ring 4.22-4.90 (5H, m) (region includes HOD signal), 6.17 (1H, d, J 5.4, anomeric proton), signals attributed to the nicotinamide ring 8.25 (1H, dd, J 7.6, 6.3, 5-H), 8.93 (1H, d, J 8.2, 4-H), 9.18 (1H, d, J 6.0, 6-H), 9.41 (1H, s, 2-H); signals attributed to the adenine ring 8.64 (1H, s, 8-H), 8.45 (1H, s, 2-H); *m/z* (Electrospray) 662 (M^+, 7%), 72 (100), (Found: M^+, 662.13767. $C_{22}H_{30}N_7O_{13}P_2$ requires 662.13768); $[\alpha]_D$ -30.7 (c 0.65 g in 100ml water, 26.5^{0}C, l 10 mm); δ_H for pseudo carba NAD$^+$ (29) (250 MHz, D_2O) signals attributed to the cyclopentane ring 2.17-2.29 (1H, m, 5'-H), 2.48 (1H, m, 4-H), 2.69-2.81 (1H, m, 5-H), 4.09-4.16 (4H, m, 2-H, 3-H, 6-H, 6'-H), 5.10 (1H, q, J 9.4, 1-H), signals attributed to the ribosyl ring 4.22-4.9 (5H, m)(region includes HOD signal) 6.18 (1H, d, J 5.3, anomeric proton), signals attributed to the nicotinamide ring 8.24 (1H, dd, J 7.5, 6.7, 5-H),

8.93 (1H, d, J 7.8, 4-H), 9.19 (1H, d, J 56.3, 6-H), 9.41 (1H, s, 2-H), signals attributed to the adenine ring 8.65 (1H, s, 8-H), 8.45 (1H, s, 2-H); *m/z* (Electrospray) 662 (M^+, 42%), 243 (100), (Found: M^+, 662.13777. $C_{22}H_{30}N_7O_{13}P_2$ requires 662.13768); $[\alpha]_D$ +7.8 (c 0.57 g in 100 ml water, 23.8^{0}C, l 10 mm).

5.2 Chapter 3 Experimental

Yeast alcohol dehydrogenase was purchaesd from Sigma (340 units per mg) as a lyophillised powder. Glycerol-3-phosphate dehydrogenase was also purchaesd from Sigma (130 units per mg) as a sulphate free lyophillised powder.

5.2.1 Measurement of K_M and V_{max} for NAD^+ (9) with YADH and EtOH

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 30°C in 3.0 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.33 M ethanol. The NAD^+ (9) concentration was varied from 2.39×10^{-4} M to 7.19×10^{-4} M per assay. The reaction was initiated by addition of 0.04 units of enzyme and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots.

5.2.2 Measurement of K_M and V_{max} for Carba NAD^+ (28) with YADH and EtOH

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 30°C in 3.0ml of 0.025M sodium pyrophosphate pH 8.8 containing 0.33 M ethanol. The carba NAD^+ (28) concentration was varied from 3.47×10^{-4} M to 9.90×10^{-4} M per assay. The reaction was initiated by addition of 0.13 units of enzyme and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots.

5.2.3 Measurement of K_M and V_{max} for NAD^+ (9) with GPDH and Glycerol-3-Phosphate

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 30°C in 3.0 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.042 M glycerol-3-phosphate. The NAD^+ (9) concentration was varied from 2.39×10^{-4} M to 7.16×10^{-4} M per assay. The reaction was initiated by addition of 0.04 units of enzyme and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots.

5.2.4 Measurement of K_M and V_{max} for Carba NAD^+ (28) with GPDH and Glycerol-3-Phosphate

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 30°C in 1.5 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.084 M glycerol-3-phosphate. The carba NAD^+ (28) concentration was varied from 2.46×10^{-4} M to 12.3×10^{-4} M per assay. The reaction was initiated by addition of 0.26 units of enzyme and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots.

5.2.5 Measurement of K_{eq} for NAD^+ (9) with YADH and EtOH

K_{eq} was measured according to the method of Racker.[198] Assay was conducted at 25°C in 3.0 ml of 0.025 M sodium pyrophosphate pH 7.4 containing 0.0162 M ethanol. The NAD^+ (9) concentration was 3.35×10^{-4} M. The reaction was initiated by addition of 45 units of YADH and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm, and from the stoichiometry of the reaction it was assumed that the concentration of acetaldehyde was equal to the concentration of NADH (30).

5.2.6 Measurement of K_{eq} for Carba NAD^+ (28) with YADH and EtOH

K_{eq} was measured according to the method of Racker.[198] Assay was conducted at 25°C in 3.0ml of 0.025 M sodium pyrophosphate pH 8.06 containing 0.0162 M ethanol. The carba NAD^+ (28) concentration was 5.0×10^{-4} M. The reaction was initiated by addition of 45 units of YADH and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm, and from the stoichiometry of the reaction it was assumed that the concentration of acetaldehyde was equal to the concentration of carba NADH (65).

5.2.7 Measurement of K_{eq} for NAD^+ (9) with GPDH and Glycerol-3-Phosphate

Assay was conducted at 25°C in 1.0 ml of 0.25 M Tris.HCl pH 7.97 containing 0.025 M EDTA and 55.9 mM glycerol-3-phosphate. The initial NAD^+ (9) concentration was 1.76×10^{-4} M. The reaction was initiated by addition of 65 units of GPDH and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm, and from the stoichiometry of the reaction it was assumed that the concentration of dihydroxyacetone was equal to the concentration of NADH (30).

5.2.8 Measurement of K_{eq} for Carba NAD^+ (28) with GPDH and Glycerol-3-Phosphate

Assay was conducted at 25°C in 1.0 ml of 0.25 M Tris.HCl pH 7.97 containing 0.025 M EDTA and 79.5 mM glycerol-3-phosphate. The initial carba NAD^+ (28) concentration was 6.69×10^{-4} M. The reaction was initiated by addition of 70 units of GPDH and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically until the reaction was at equilibrium.. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm, and from the stoichiometry of the reaction it was assumed that the concentration of dihydroxyacetone was equal to the concentration of carba NADH (65).

5.2.9 Reduction of NAD^+ (9) to NADH (30) using YADH and EtOH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 1.08×10^{-3} M NAD^+ (9) and 0.33 M EtOH. The reaction was initiated by addition of 817 units of YADH and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260nm and 340 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.10 Reduction of NAD^+ (9) to $NADD_R$ (62a) using YADH and CH_3CD_2OH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 1.1×10^{-3} M NAD^+ (9) and 0.33 M CH_3CD_2OH. The reaction was initiated by addition of 559 units of YADH and the resulting increase in absorbance at 340 nm due to the production of $NADD_R$ (62a) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of $NADD_R$ (62a) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3$ $M^{-1}cm^{-1}$ at 340 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 340 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.11 Oxidation of $NADD_R$ (62a) to $NAD(D)^+$ (63) using GPDH and Dihydroxyacetone

The oxidation was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 3.04×10^{-4} M $NADD_R$ (62a) and 0.049 M dihydroxyacetone phosphate. The reaction was initiated by addition of 117 units of GPDH and the resulting decrease in absorbance at 340 nm due to the loss of $NADD_R$ (62a) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of $NADD_R$ (62a) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3$ $M^{-1}cm^{-1}$ at 340 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 340 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.12 Reduction of NAD(D)$^+$ (63) to NADD$_S$ (62b) using YADH and EtOH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 2.99×10^{-4} M NAD(D)$^+$ (63) and 0.33 M EtOH. The reaction was initiated by addition of 559 units of YADH and the resulting increase in absorbance at 340 nm due to the production of NADD$_S$ (62b) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADD$_S$ (62b) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3$ $M^{-1}cm^{-1}$ at 340 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 340 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.13 Oxidation of NADD$_S$ (62b) to NAD$^+$ (9) using GPDH and Dihydroxyacetone

The oxidation was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 2.60×10^{-4} M NADD$_S$ (62b) and 0.048 M dihydroxyacetone phosphate. The reaction was initiated by addition of 120 units of GPDH and the resulting decrease in absorbance at 340 nm due to the loss of NADD$_S$ (62b)was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADD$_S$ (62b) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3$ $M^{-1}cm^{-1}$ at 340 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 340 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.14 Reduction of Carba NAD^+ (28) to Carba NADH (65) using YADH and EtOH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 6.8×10^{-4} M carba NAD^+ (28), 0.01 M semicarbazide and 0.33 M EtOH. The reaction was initiated by addition of 860 units of YADH and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 360 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.15 Reduction of Carba NAD^+ (28) to Carba $NADD_R$ (64a) using YADH and CH_3CD_2OH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 1.03×10^{-3} M NAD^+ (28), 0.01 M semicarbazide and 0.33 M CH_3CD_2OH. The reaction was initiated by addition of 645 units of YADH and the resulting increase in absorbance at 360 nm due to the production of carba $NADD_R$ (64a) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba $NADD_R$ (64a)was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260nm and 360 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.16 Oxidation of Carba $NADD_R$ (64a) to Carba $NAD(D)^+$ (66) using GPDH and Dihydroxyacetone

The oxidation was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 9.39×10^{-4} M $NADD_R$ (64a) and 0.047 M dihydroxyacetone phosphate. The reaction was initiated by addition of 130 units of GPDH and the resulting decrease in absorbance at 360 nm due to the loss of carba $NADD_R$ (64a) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba $NADD_R$ (64a) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 360 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.17 Reduction of Carba $NAD(D)^+$ (66) to Carba $NADD_S$ (64b) using YADH and EtOH

The reduction was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 1.65×10^{-4} M carba $NAD(D)^+$ (66), 0.01 M semicarbazide and 0.33 M EtOH. The reaction was initiated by addition of 860 units of YADH and the resulting increase in absorbance at 360 nm due to the production of carba $NADD_S$ (64b)was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba $NADD_S$ (64b) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 360 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.18 Oxidation of Carba $NADD_S$ (64b) to Carba NAD^+ (28) using GPDH and Dihydroxyacetone

The oxidation was carried out in 25 ml sodium pyrophosphate buffer at pH 8.8 containing 1.0×10^{-4} M $NADD_S$ (64b) and 0.048 M dihydroxyacetone phosphate. The reaction was initiated by addition of 65 units of GPDH and the resulting decrease in absorbance at 360 nm due to the loss of carba $NADD_S$ (64b) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba $NADD_S$ (64b) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360nm. The enzyme was destroyed by immersing the reaction flask in boiling water for 30 seconds. The products were separated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm and 360 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH.

5.2.19 Chemical Conversion of NAD^+ (9) to $NAD(D)^+$ (63)

This was done according to the method of San Pietro.[205] NAD^+ (9) (14.8 mg) was dissolved in 1 M KCN in D_2O (213 µl), 5 M KOH in D_2O (4.25 µl) was added. The bright orange solution was stirred at room temperature for 4 hours then KH_2PO_4 in D_2O (1.8 ml, 11 mole equivalents) was added. The HCN was removed by bubbling nitrogen through the solution. The $NAD(D)^+$ (63) was isolated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-200 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH. The $NAD(D)^+$ (63) (16 mg, 80%) was obtained as a white solid. δ_H(250 MHz, D_2O) signals attributed to the ribose rings 4.2-4.8 (10H, m), 6.0 (1H, d, J 6.0, anomeric proton), 6.1 (1H, d, J 6.0, anomeric proton), signals attributed to the nicotinamide ring 8.2 (1H, d, J 6.0, 5-H), 9.15 (1H, d, J 6.0, 6-H), 9.32 (1H, s, 2-H), signals attributed to the adenine ring 8.19 (1H, s, 2-H), 8.42 (1H, s, 8-H).

5.2.20 Chemical Conversion of Carba NAD^+ (28) to Carba $NAD(D)(D)^+$ (67)

This was done according to the method of San Pietro.[205] Carba NAD^+ (28) (4.6×10^{-5} moles) was dissolved in 1M KCN in D_2O (547 µl), 5M KOH in D_2O (10.9 µl) was added. The bright orange solution was stirred at room temperature for 4 hours then KH_2PO_4 in D_2O (4.65 ml, 11 mole equivalents) was added. The HCN was removed by bubbling nitrogen through the solution. The carba $NAD(D)(D)^+$ (67) was isolated using ion exchange chromatography on a column of DEAE Sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-200 mM) TEAB (total volume used 800 ml). The eluent from the column was monitored at λ 260 nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with MeOH. The carba $NAD(D)(D)^+$ (67) (30 mg, 70%) was obtained as a white solid. . δ_H(250 MHz, D_2O) signals attributed to the cyclopentane ring 2.03-2.16 (1H, m, 5'-H), 2.39-2.41 (1H, m, 4-H), 2.55-2.67 (1H, m, 5-H), 4.00-4.10 (4H, m, 2-H, 3-H, 6-H, 6'-H), 5.01 (1H, q, J 9.6, 1-H), signals attributed to the ribosyl ring 4,16-4.21 (2H, m), 4.35-4.46 (3H,m), 6.00 (1H, d, J 5.7, anomeric proton), signals attributed to the nicotinamide ring 8.15 (1H, dd, J 7.5, 6.7, 5-H), 9.08 (1H, d, J 5.7, 6-H), signals attributed to the adenine ring 8.45 (1H, s, 8-H), 8.21 (1H, s, 2-H).

5.2.21 Kinetic Isotope Effects on the Reaction of NAD^+ (9) with YADH and Alcohol

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 25°C in 1.0 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.33 M ethanol. The NAD^+ (9) concentration was varied from 1.03×10^{-4} M to 3.10×10^{-4} M per assay. The reaction was initiated by addition of 0.04 units of YADH and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots. The above reaction was then repeated using 0.33 M CH_3CD_2OH instead of 0.33 M CH_3CH_2OH, all other conditions were the same. $K_{M,H}/K_{M,D}$ = 1.50 and $V_{max,H}/V_{max,D}$ = 2.0.

5.2.22 Kinetic Isotope Effects on the Reaction of Carba NAD^+ (28) with YADH and Alcohol

K_M and V_{max} were measured according to the method of Slama and Simmons.[87] Assay was conducted at 25°C in 1.0 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.33 M ethanol. The carba NAD^+ (28) concentration was varied from 1.34×10^{-4} M to 4.03×10^{-4} M per assay. The reaction was initiated by addition of 0.127 units of YADH and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots. The above reaction was then repeated using 0.33 M CH_3CD_2OH instead of 0.33 M CH_3CH_2OH, all other conditions were the same. $K_{M,H}/K_{M,D}$ = 1.23 and $V_{max,H}/V_{max,D}$ = 2.6.

5.2.23 Kinetic Isotope Effects on the Reaction of NAD^+ (9) with YADH and Benzyl Alcohol

Assay was conducted at 25°C in 1.0 ml of 0.025 M sodium pyrophosphate pH 8.8 containing 0.33 M benzyl alcohol. The NAD^+ (9) concentration was varied from 2.42×10^{-4} M to 7.25×10^{-4} M per assay. The reaction was initiated by addition of 813 units of YADH and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically for 5 minutes. The linear portion of this increase was used to determine the initial rate of reaction. The concentration of NADH (30) was calculated using the extinction coeficient $\varepsilon=6.2\times10^3M^{-1}cm^{-1}$ at 340 nm. K_M and V_{max} values were calculated from the Lineweaver Burke plots. The above reaction was then repeated using 50 mM $C_6D_5CD_2OH$ instead of 50 mM $C_6H_5CH_2OH$, all other conditions were the same. $K_{M,H}/K_{M,D}$ = 1.8 and $V_{max,H}/V_{max,D}$ = 3.3.

5.2.24 Solution Conformation of Carba NAD^+ (28) and Carba NADH (65)

All proton nmr spectroscopy was carried out at 250 MHz in D_2O at 25°C and referenced to an internal reference of TSP. The destacking experiments were carried out at 250 MHz, in 7:3 D_2O:CD_3OD at 25°C and referenced to an internal reference of TSP. All NAD^+ (9), NMN (13), NADH (30) and AMP (1) samples were 60 mM and all carba NAD (28), carba NMN (36) and carba NADH (65) samples were 30 mM.

5.2.25 Measurement of Internal K_{eq} for NAD^+ (9) with YADH and EtOH

K_{eq} was measured according to the method of Benner.[167] Assay was conducted at 25°C in 1.0ml of 50 mM potassium phosphate pH 7.25. The YADH concentration was 1.58 mM and the initial NAD^+ (9) concentration was 6×10^{-4} M. Ethanol was added until the ethanol concentration was 0.24 M and the resulting increase in absorbance at 340 nm due to the production of NADH (30) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of NADH (30) was calculated using the extinction coefficient $\varepsilon=6.2\times10^3\ M^{-1}cm^{-1}$ at 340 nm.

5.2.26 Measurement of Internal K_{eq} for Carba NAD^+ (28) with YADH and EtOH

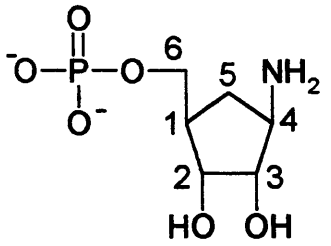
K_{eq} was measured according to the method of Benner.[167] Assay was conducted at 25°C in 1.0ml of 50 mM potassium phosphate pH 7.25. The YADH concentration was 1.58 mM and the initial carba NAD^+ (28) concentration was 6×10^{-4} M. Ethanol was added until the ethanol concentration was 0.24 M and the resulting increase in absorbance at 360 nm due to the production of carba NADH (65) was measured spectrophotometrically until the reaction was at equilibrium. The concentration of carba NADH (65) was calculated using the extinction coefficient $\varepsilon=3.1\times10^3\ M^{-1}cm^{-1}$ at 360 nm.

5.2.27 Reclamation of Carba NAD^+ (28) from Buffer Solutions

Activated charcoal was added to an aqueous solution of crude carba NAD^+ (28) from the kinetics experiments. The mixture was centrifuged and the supernatant liquid examined by UV at λ=260 nm. Charcoal was added until all of the carba NAD^+ (28) was adsorbed. The charcoal was washed with water (3 volumes), then the carba NAD^+ (28) was eluted using ethanol:ammonia:water (50:2:48). The eluent was monitored at λ 260 nm. The solution was concentrated and then applied to an anion exchange column of DEAE-Sephadex resin (60 ml). The column was eluted with a linear gradient (25 mM-200 mM) TEAB (total volume used 1000 ml). The eluent was monitored at λ 260nm. The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with methanol.

5.3 Chapter 4 Experimental

5.3.1 Preparation of the Phosphorylated Amine 4β-Amino-2α,3α-dihydroxy-1β-(hydroxymethyl)cyclopentane-6-phosphate (77)



(77)

The amine (46) (143 mg, 0.97 mmoles) was dissolved in trimethylphosphate (1.2 ml). A mixture of trimethylphosphate (0.33 ml) and phosphoryl chloride (0.33 ml, 3.5 mmoles) was added to the amine solution and the reaction mixture was stirred in an ice bath for 3 hours. Ice water (1 ml) was added and the mixture stirred for a further 30 minutes then acetone (40 ml) was added. The crude product was collected by centrifugation and dissolved in water (20 ml). The solution was applied to a column of DEAE sephadex resin (20 ml). The column was eluted with a linear gradient (25 mM-250 mM) TEAB (total volume used 800 ml). The elutent from the column was monitored using the Briggs test for phosphate (section 5.3.2).[236] The appropriate fractions were combined and concentrated. TEAB was removed by coevaporation with methanol. Amine (77) was obtained as a white solid (63 mg, 20% yield). δ_H(250 MHz, D_2O) 3.96 (2H, m, 2-H and 3-H), 3.74 (2H, m, 6-H and 6'-H), 3.46 (1H, q, J 7.8, 4-H), 3.08 (5H, q, J 6.8, $CH_3\underline{CH_2}$ from $^+NHEt_3$ counterion), 2.16-2.34 (2H, m, 1-H and 5-H), 1.42 (1H, m, 5'-H), 1.16 (5H, t, J 6.8, $\underline{CH_3}CH_2$ from $^+NHEt_3$ counterion); δ_C(63 MHz, D_2O) 75.12 (2-C), 72.91 (3-C), 65.56 (d)(6-C), 55.19 (4-C), 46.97 ($CH_3\underline{CH_2}$ from $^+NHEt_3$ counter ions), 43.63 (d) (1-C), 27.18 (5-C), 8.60 ($\underline{CH_3}CH_2$ from $^+NHEt_3$ counter ions); δ_P(101MHz, D_2O) 0.29 (s); *m/z* (+FAB) 228(MH^+, 52%), 211(28), 194(100), 186(73), 167(22) (Found: MH^+, 228.06369. $C_{12}H_{15}NO_6P$ requires 228.06369).

5.3.2 The Briggs Test for Phosphate[237]

A sample (0.33 ml) was removed from every alternate ion exchange fraction. These were evaporated to dryness in an oven then concentrated H_2SO_4 (2 drops) was added to each dried sample. The samples were heated to 100°C for 30 minutes, then 0.5 ml solution A, 0.25 ml solution B and 0.25 ml solution C were added to each sample. The samples were shaken and heated gently, a blue colour indicates the presence of phosphate.

Solution A

Ammonium molybdate (2.5 g) was dissolved in water (30 ml) then water (20 ml) containing concentrated sulphuric acid (7.5 ml) was added to the mixture.

Solution B

Hydroquinone (0.5 g) was dissolved in water (100 ml) and one drop of concentrated sulphuric acid was added to retard oxidation.

Solution C

Sodium sulphite (4 g) was dissolved in water (20 ml). This solution must be kept tightly stoppered or made fresh.

REFERENCES

References

1. P.A. Levene and H. Mandel, *Chem.Ber.*, 1908, **41**, 1905.
2. J. Liebig, *Annalen.*, 1847, **62**, 317.
3. F. Miescher, *Hoppe-Seyler's Med. Chem. Untersch.*, 1871, 441.
4. P.A. Levene and W.A. Jacobs, *Chem.Ber.*, 1911, **44**, 746.
5. G. Embden and M. Zummermann, *Z. Physiol. Chem.*, 1927, **167**, 137.
6. H. Schmitz, R.B. Hurlbert and V.R. Potter, *J. Biol. Chem.*, 1954, **209**, 41.
7. E.E.B. Smith and G.T. Mills, *Biochim. et Biophys. Acta.*, 1954, **13**, 386.
8. H.L. Segal, *Biochim. et Biophys. Acta.*, 1956, **21**, 194.
9. S. Osawa, V.G. Allfrey and A.E. Mirsky, *J. Gen. Physiol.*, 1957, **40**, 491.
10. R.L. Potter, S. Schlesinger, V. Buettner-Janusch and L. Thompson, *J. Biol. Chem.*, 1957, **226**, 381.
11. P.A. Levene, *J. Biol. Chem.*, 1918, **33**, 425; 1919, **40**, 415.
12. E. Chargaff, B. Magasanik, E. Vischer, C. Green, R. Doniger and D. Elson, *J. Biol. Chem.*, 1950, **186**, 51.
13. W.E. Cohn, *J. Am. Chem. Soc.*, 1949, **71**, 2275; 1950, **72**, 1471; 1950, **72**, 2811.
14. P.A. Levene and L.W. Bass, *Nucleic Acids, A.C.S. Monograph No. 56*, Reinhold, New York, 1931.
15. A.M. Michelson, *The Chemistry of Nucleosides and Nucleotides*, Academic Press, London, 1963.
16. C.E. Carter, *J. Am. Chem. Soc.*, 1951, **73**, 1537.
17. R. Klimek and J.K. Parnas, *Biochim. Z.*, 1937, **292**, 356.
18. B. Lythgoe and A.R. Todd, *J. Chem Soc.*, 1944, 592.
19. A.C. Paladini and L.F. Leloir, *Biochem. J.*, 1952, **51**, 426.
20. E. Volkin, J.X. Khym and W.E. Cohn, *J. Am. Chem. Soc.*, 1951, **73**, 1533.
21. P. Horn, V. Luzzati and K.N. Trueblood, *Nature*, 1959, **183**, 880.
22. J. Kraut and L.H. Jensen, *Nature*, 1960, **186**, 798.
23. C.E. Carter and W.E. Cohn, *Fed. Proc.*, 1949, **8**, 190.
24. W.E. Cohn, *Science*, 1949, **109**, 377.
25. W.E. Cohn, *J. Am. Chem. Soc.*, 1950, **72**, 1471; 1950, **72**, 2811.
26. W.E. Cohn, *J. Cellular Comp. Physiol.*, 1951, **38**, 21.
27. H.S. Loring, N.G. Luthy, H.W. Bortner and L.W. Levy, *J. Am. Chem. Soc.*, 1950, **72**, 2811.

28. D.M. Brown and A.R. Todd, *J. Chem. Soc.*, 1952, 44.
29. W.E. Cohn, *J. Am. Chem. Soc.*, 1950, **72**, 1471.
30. C.E. Carter, *J. Am. Chem. Soc.*, 1950, **72**, 1466.
31. D.M. Brown, L.J. Haynes and A.R. Todd, *J. Chem. Soc.*, 1950, 3299.
32. D.M. Brown, D.I. Magrath and A.R. Todd, *J. Chem. Soc.*, 1952, 2708.
33. D.M. Brown and A.R. Todd, *J. Chem. Soc.*, 1952, 52.
34. L. Shuster and N.O. Kaplan, *J. Biol. Chem.*, 1953, **201**, 535.
35. J.X. Khym and W.E. Cohn, *J. Am. Chem. Soc.*, 1954, **76**, 1818.
36. J.X. Khym, D.G. Doherty and W.E. Cohn, *J. Am. Chem. Soc.*, 1954, **76**, 5523.
37. D.M. Brown, G.D. Fasman, D.I. Magrath and A.R. Todd, *J. Chem. Soc.*, 1954, 1448.
38. J. Kumamoto, J.R. Cox and F.H. Westheimer, *J. Am. Chem. Soc.*, 1956, **78**, 4858.
39. H.G. Khorana, G.M. Tener, R.S. Wright and J.G. Moffatt, *J. Am. Chem. Soc.*, 1957, **79**, 430.
40. J.R. Cox, R.E. Wall and F.H. Westheimer, *Chem. and Ind.*, 1959, 929.
41. C.A. Dekker and H.G. Khorana, *J. Am. Chem. Soc.*, 1954, **76**, 3522.
42. E.W. Sutherland and T.W. Rall, *J. Biol. Chem.*, 1958, **232**, 1065.
43. D.F. Ashmann, R. Lipton, M.M. Melicow and T.D. Price, *Biochem. Biophys. Res. Comm.*, 1963, **11**, 330.
44. M. Smith, G.I. Drummond and H.G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 698.
45. E. Fischer, *Chem. Ber.*, 1914, **47**, 3193.
46. P.A. Levene and R.S. Tipson, *J. Biol. Chem.*, 1937, **121**, 131.
47. P.A. Levene and R.S. Tipson, *J. Biol. Chem.*, 1935, **111**, 313.
48. P.A. Levene and R.S. Tipson, *J. Biol. Chem.*, 1934, **106**, 113.
49. F.R. Atherton, H.T. Openshaw and A.R. Todd, *J. Chem. Soc.*, 1945, 382.
50. P. Brigl and H. Muller, *Chem. Ber.*, 1939, **72**, 2121.
51. T. Jachimowicz, *Biochem. Z.*, 1937, **292**,356.
52. J.M. Gulland and G.I. Hobday, *J. Chem. Soc.*, 1942, 231.
53. G.R. Barker and J.M. Gulland, *J. Chem. Soc.*, 1940, 746.
54. M. Yoshikawa, T. Kato and T. Takenishi, *Tetrahedron Lett.*, 1967, **50**, 5065.
55. K.I. Imai, S. Shoichiro, K. Takanohashi, Y. Furukawa, T. Masuda and M. Honjo, *J. Org. Chem.*, 1969, **34**, 1547.
56. D.M. Brown, A.R. Todd and S. Varadarajan, *J. Chem. Soc.*, 1956, 2388.
57. M. Smith and H.G. Khorana, *J. Am. Chem. Soc.*, 1959, **81**, 2911.
58. A. Harden and W.J. Young, *Proc. Roy. Soc. B.*, 1906, **78**, 369.

59. H. Von Euler, H. Albers and F. Schlenk, *Z. Physiol. Chem.*, 1936, **240**, 113.
60. O. Warburg and W. Christian, *Biochem. Z.*, 1936, **287**, 291.
61. R. Markham and J.D. Smith, *Biochemical J.*, 1951, **49**, 401.
62. O. Warburg and W. Christian, *Biochem. Z.*, 1931, **242**, 206.
63. T.P. Singer and E.B. Kearney, *Adv. Enzymol.*, 1954, **15**, 79.
64. C.H. Fiske and Y. Subbarow, *Science*, 1929, **70**, 381.
65. K. Lohmann, *Naturwiss.*, 1929, **17**, 624; *Biochem. Z.*, 1935, **282**, 120.
66. B. Lythgoe and A.R. Todd, *Nature*, 1945, **155**, 695.
67. R. Bergkvist and A. Deutsch, *Acta. Chem. Scand.*, 1953, **7**, 1307.
68. R. Bergkvist and A. Deutsch, *Acta. Chem. Scand.*, 1954, **8**, 1889.
69. R.L. Potter, S. Schlesinger, V. Beuttner-Janusch and L. Thompson, *J. Biol. Chem.*, 1957, **226**, 381.
70. F. Schlenk, *J. Biol. Chem.*, 1942, **146**, 619.
71. R.U. Lemieux and J.W. Loun, *Can. J. Chem.*, 1963, **41**, 889.
72. A. Kornberg and W.E. Pricer, *J. Biol. Chem.*, 1954, **206**, 129.
73. J. Preiss and P. Handler, *J. Biol. Chem.*, 1958, **233**, 488; **233**, 493.
74. J. Baddiley and A.R. Todd, *J. Chem. Soc.*, 1947, 648.
75. R.W. Chambers and H.G. Khorana, *J. Am. Chem. Soc.*, 1958, **80**, 3749.
76. V.M. Clark, G.W. Kirby and A.R. Todd, *J. Chem. Soc.*, 1957, 1497.
77. T. Ueda and E. Ohtsuka, *Chem. and Pharm. Bull. (Tokyo)*, 1959, **7**, 740.
78. J. F. Koerner and S. Varadarajan, *J. Biol. Chem.*, 1960, **235**, 2688.
79. R.W. Chambers, *J. Am. Chem. Soc.*, 1959, **81**, 3032.
80. R.W. Chambers, P. Shapiro and V. Kurkov, *J. Am. Chem. Soc.*, 1960, **82**, 970.
81. P. Lengyel and R.W. Chambers, *J. Am. Chem. Soc.*, 1960, **82**, 752.
82. J.G. Moffatt and H.G. Khorana, *J. Am. Chem. Soc.*, 1961, **83**, 649.
83. N.A. Hughes, G.W. Kenner and A.R. Todd, *J. Chem. Soc.*, 1957, 3733.
84. L.J. Haynes, N.A. Hughes, G.W. Kenner and A.R. Todd, *J. Chem. Soc.*, 1957, 3727.
85. R. Jeck, P. Heik and C. Woenckhaus, *FEBS Letters*, 1974, **42**, 161.
86. C.P. Fawcett and N.O. Kaplan, *J. Biol. Chem.*, 1962, **237**, 1709.
87. J.T. Slama and A.M. Simmons, *Biochem.*, 1988, **27**, 183.
88. P. Karrer, P. Frei and H. Meerwein, *Helv. Chim. Acta.*, 1937, **20**, 79.
89. E.P. Abraham, *Biochem. J.*, 1939, **33**, 543.
90. S.M.H. Christie, G.W. Kenner and A.R. Todd, *J. Chem. Soc.*, 1954, 46.
91. F.M. Heunnekens and G.L. Kilgour, *J. Am. Chem. Soc.*, 1955, **77**, 6716.

92. C. De Luca and N.O. Kaplan, *J. Biol. Chem.*, 1956, **223**, 569.

93. H.G. Khorana and J.G. Moffatt, *J. Am. Chem. Soc.*, 1958, **80**, 3756.

94. F. Lipmann, *J. Biol. Chem.*, 1945, **160**, 173.

95. J. Baddiley, *Adv. in Enzymol.*, 1955, **16**, 1.

96. F. Lipmann, *Bacteriol. Rev.*, 1953, **17**, 1.

97. E.E. Snell and G.M. Brown, *Adv. in Enzymol.*, 1953, **14**, 49.

98. H.G. Khorana and J.G. Moffatt, *J. Am. Chem. Soc.*, 1961, **83**, 663.

99. A.M. Michelson, *Biochim. et Biophys. Acta.*, 1961, **50**, 605.

100. W. Gruber and F. Lynen, *Annalen.*, 1962, **659**, 139.

101. Y.F. Shealy and J.D. Clayton, *J. Am. Chem. Soc.*, 1966, **88**, 3885.

102. T. Kusaka, *J. Antibiot.*, 1968, **21**, 255.

103. S. Yaginuma, N. Muto, M. Tsujino, Y Sudate, M. Hayashi and O. Masiru, *J. Antibiot.*, 1981, **34**, 359.

104. *Enzyme Nomeclature*, Academic Press, New York, 1978.

105. H.B. White, *Evolution of Coenzymes and the Origin of Pyridine Nucleotides, in the Pyridine Nucleotide Coenzymes*, J. Everse, B.M. Anderson and K. You (eds), Academic Press, New York, 1982.

106. F. Schlenk, *Naturwissenschaften*, 1937, **25**, 270.

107. H.C. Lee and R. Aarhus, *Biochim. et Biophys. Acta.*, 1993, **1164**, 68.

108. R.A. Mackieth, R. McCague, H.F. Olivo, C.F. Palmer and S.M. Roberts, *J. Chem. Soc., Perkin Trans. 1*, 1993, 313.

109. E. Fischer, *Chem. Ber.*, 1914, **47**, 3196.

110. K.H. Scheit, *Nucleotide Analogs*, John Wiley and Sons, New York, 1980.

111. K.A. Shoberu and S.M. Roberts, *J. Chem Soc., Perkin Trans. 1*, 1992, 2419.

112. E.A. Saville Stones, S.D. Lindell, N.S. Jennings, J.C. Head and M.J. Ford, *J. Chem Soc., Perkin Trans. 1*, 1991, 2603.

113. M.G.J. Beets and E.A. Drukker, *Recueil.*, 1953, **72**, 247.

114. H. Paulsen and U. Maab, *Chem. Ber.*, 1981, **114**, 346.

115. J. C. Jagt and A.M. Van Leusen, *J. Org. Chem.*, 1974, **39**, 564.

116. S. Daluge and R. Vince, *J. Org. Chem.*, 1978, **43**, 2311.

117. P.E. Morgan, R. McCague and A. Whiting, *J. Chem. Soc., Chem. Comm.*, 1996, 1811.

118. S.J.C. Taylor, A.G. Sutherland, C. Lee, R. Wisdom, S. Thomas, S.M. Roberts, and C. Evans, *J. Chem. Soc., Chem. Comm.*, 1990, 1120.

119. D.A.H. Van Maarschalkerwaart, N.P. Willard and G.J. Koomen, *Nucleosides and Nucleotides*, 1990, **9(6)**, 787.

120. S. Daluge and R. Vince, *Tetrahedron Lett.*, 1976, **35**, 3005.

121. O. Caamano, A. Eirin, F. Fernandez, G. Gomez and E. Uriarte, *Heterocycles*, 1988, **27(12)**, 2839.

122. R.C. Cermak and R. Vince, in *Nucleic Acid Chemistry, Improved and New Synthetic Procedures, Methods and Techniques, Part Three*, John Wiley and Sons, New York, 1986.

123. L.F. Fieser and M. Fieser, *Reagents for Organic Synthesis, Volumes 1-6*, John Wiley and Sons, New York.

124. V. Van Rheenan, R.C. Kelly and D.Y. Cha, *Tetrahedron Lett.*, 1976, 1973.

125. N.A. Milas and S. Sussman, *J. Am. Chem. Soc.*, 1936, **58**, 1302.

126. N.A. Milas and S. Sussman, *J. Am. Chem. Soc.*, 1937, **59**, 2345.

127. N.A. Milas, S. Sussman and H.S. Mason, *J. Am. Chem. Soc.*, 1939, **61**, 1844.

128. K.A. Hofmann, *Chem.Ber.*, 1912, **45**, 3329.

129. K.A. Hofmann, O. Ehrhart and O. Schneider, *Chem.Ber.*, 1913, **46**, 1657.

130. F.M.C. Corporation, *Netherlands Appl.*, 74, 11, 150, 1976; *Chem. Abstr.*, 1976, **86**, P4925.

131. F.A. Cotton and G. Wilkinson, *Advanced Inorganic Chemistry, 3rd ed.*, Interscience, New York.

132. H.O. House, *Modern Synthetic Reactions, 2nd ed.*, Benjamin, San Francisco, CA, 1972.

133. M. Schroder, *Chem. Rev.*, 1980, **80**, 187.

134. R. Criegee, *Justis Liebigs Ann. Chem.*, 1936, **522**, 75.

135. R. Criegee, B. Marchand and H. Wannowius, *Justis Liebigs Ann. Chem.*, 1942, **550**, 99.

136. B.L. Kam and N. Oppenheimer, *J. Org. Chem.*, 1981, **46**, 3268.

137. J.E. Taylor, D. Williams and K. Edwards, *Can. J. Chem.*, 1984, **62**, 11.

138. A.J. Fatiadi, *Synthesis*, 1987, 85.

139. T.G. Traylor, *Acc. Chem. Res.*, 1969, **2**, 152.

140. S.J. Cristol, T.C. Morrill and R.A. Sanchez, *J. Org. Chem.*, 1966, **31**, 2719.

141. H.C. Brown, J.H. Kawakami and K.T. Liu, *J. Am. Chem. Soc.*, 1970, **92**, 5536.

142. G. Alvernhe, D. Anker, A. Laurent, G. Haufe and C. Beguin, *Tetrahedron*, 1988, **44**, 3551.

143. N. Koga, T. Ozawa and K. Morokuma, *J. Phys. Org. Chem.*, 1990, **3**, 519.

144. H.C. Brown and J.H. Kawakami, *J. Am. Chem. Soc.*, 1970, **92**, 201.

145. H.C. Brown, J.H. Kawakami and K.T. Liu, *J. Am. Chem. Soc.*, 1973, **95**, 2209.

146. J. Kollonitsch, O. Fuchs and V. Gabor, *Nature*, 1955, **175**, 346.

147. Farbenfabriken Bayer, *Patent Specification 684, 801*, Date of Application Dec. 6, 1950.

148. M. Ikbal, C. Cerceau, F. Le Goffic and S. Sicsic, *Eur. J. Med. Chem.*, 1989, **24**, 415.

149. A. Hampton, *J. Am. Chem. Soc.*, 1965, **87**, 4654.

150. J. Hes and M.P. Mertes, *J. Org. Chem.*, 1974, **39**, 3767.

151. T. Sowa and S. Ouchi, *Bull. Chem. Soc. Jap.*, 1975, **48**, 2084.

152. W.H. Dawson, R. L. Cargill and R. Dunlop, *J. Carbohydr. Nucleosides Nucleotides*, 1977, **4**, 363.

153. A. Kennedy, *J. Biol. Chem.*, 1956, **222**, 185.

154. P. Karrer, G. Schwartzenbach, F. Benz and U. Solmssen, *Helv. Chim. Acta.*, 1936, **19**, 811.

155. H. Fischer, E.E. Conn, B. Vennesland and F.H. Westheimer, *J. Biol. Chem.*, 1953, **202**, 687.

156. H. Fischer, E.E. Conn, B. Vennesland and F.H. Westheimer, *Fed. Proc.*, 1952, **11**, 211.

157. M.E. Pullman and S.P. Colowick, *J. Biol. Chem.*, 1954, **206**, 121.

158. M.E. Pullman, A. San Pietro and S.P. Colowick, *J. Biol. Chem.*, 1954, **206**, 129.

159. F.H. Westheimer and D. Mauzerall, *J. Am. Chem. Soc.*, 1955, **77**, 2261.

160. H.E. Dubb, M. Saunders and J.H. Wang, *J. Am. Chem. Soc.*, 1958, **80**, 1767.

161. F.H. Westheimer and R.F. Hutton, *Tetrahedron*, 1958, **3**, 73.

162. F.H. Westheimer, H. Fischer, E.E. Conn and B. Vennesland, *J. Am. Chem. Soc.*, 1951, **73**, 2043.

163. K. You, *CRC Rev. Biochem.*, 1985, **17**.

164. S.A. Benner, *Experientia*, 1982, **38**, 633.

165. K. You, L.J. Arnold Jr., W.S. Allison and N.O. Kaplan, *Trends Biochem. Sci.*, 1978, **3**, 265.

166. C. Walsh, in *Enzymatic Reaction Mechanisms*, W.H. Freeman, San Francisco, 1979.

167. K.P. Nambiar, D.M. Stauffer, P.A. Kolodziej and S.A. Benner, *J. Am. Chem. Soc.*, 1983, **105**, 5886.

168. B. Birdsall, N.J.M. Birdsall, J. Feeney and J. Thornton, *J. Am. Chem. Soc.*, 1975, **97**, 2845.

169. R. Sarma and R.J. Mynott, *J. Am. Chem. Soc.*, 1973, **95**, 1641.

170. B.D. Nageswara Rao, F.J. Kayne and M. Cohn, *J. Biol. Chem.*, 1979, **254**, 2689.

171. J.R. Knowles, *A. Rev. Biochem.*, 1980, **49**, 877.

172. W.J. Albery and J.R. Knowles, *Biochemistry*, 1976, **15**, 5631.

173. N. Oppenheimer, *J. Am. Chem. Soc.*, 1984, **106**, 3032.

174. S.A. Benner, K.P. Nambiar and G.K. Chambers, *J. Am. Chem. Soc.*, 1985, **107**, 5513.

175. B. Commoner, B.B. Lippincott and J.V. Passoneau, *Proceedings of the National Academy of Sciences (Washington)*, 1958, **44**, 1099.

176. N.O. Kaplan, M.M. Ciotti and F.E. Stoltzenbach, *J. Biol, Chem.*, 1956, **221**, 833.

177. F.L. Radkey and J.A. Donovan, *J. Biol. Chem.*, 1959, **234**, 677.

178. C.I. Branden, H. Jorvall, H. Eklund and B. Furugren, *The Enzymes*, 1975, **11**, 104.

179. A. Fersht, *Enzyme Structure and Mechanism*, W.H. Freeman and Co., 1977.

180. C.I. Branden, H. Eklund, B. Nordstrom, T. Boiwe, G. Soderlund, E. Zeppezauer, I. Ohlsson and A. Akeson, *Proc. Natl. Acad. Sci. USA*, 1973, **70**, 2439.

181. C.I. Branden and H. Eklund, in *Dehydrogenase Requiring Nicotinamide Coenzymes*, J.J. Jeffery (ed), Birkhauser Verlag, Basel, 1980, 40.

182. M.J. Adams, in *Enzyme Mechanisms*, M.I. Page and A. Williams (eds), Royal Society Of Chemistry, 1987.

183. K. Dalziel, *The Enzymes*, 1975, **11**, 2.

184. H. Theorell and B. Chance, *Acta. Chem. Scand.*, 1951, **5**, 1127.

185 C.C. Wratten and W.W. Cleland, *Biochemistry*, 1963, **2**, 935 and 1965, **4**, 2442.

186. J.P. Klinman, *J. Biol. Chem.*, 1972, **247**, 7977.

187. J.P. Klinman, *Biochemistry*, 1976, **15**, 2018.

188. J.D. Shore and H. Gutfreund, *Biochemistry*, 1970, **9**, 4655.

189. R.L. Brooks and J.D. Shore, *Biochemistry*, 1971, **10**, 3855.

190. T. Keleti, in *Pyridine Nucleotide Dependent Dehydrogenases*, H. Sund (ed), Springer-Verlag, Berlin, 1970.

191. I. MacInnes, D.C. Nonhebel, S.T. Orszulik and C.J. Suckling, *J. Chem. Soc., Perkin Trans. 1*, 1983, 2777.

192. A. Ichihara and D.M. Greenburg, *J. Biol. Chem.*, 1957, **224**, 331.

193. W.J. Black, *Canadian J. Biochem.*, 1966, **44**, 1301.

194. M. Telegdi and T. Keleti, *Acta Biochim. Biophys. Hung.*, 1968, **3**, 131.

195. M. Telegdi and T. Keleti, *Acta Physiol. Hung.*, 1964, **25**, 181.

196. B.L. Vallee, F.L. Hoch S.J. Adelstein and W.E.C. Wacker, *J. Am. Chem. Soc.*, 1956, **78**, 5879.

197. B.M. Anderson, C.P. Anderson, J.K. Lee and A.M. Stein, *Biochemistry*, 1963, **2**, 1017.

198. E. Racker, *J. Biol. Chem.*, 1950, **184**, 313.

199. H.L. Young and N. Pace, *Archives of Biochem. and Biophys.*, 1958, **75**, 125.

200. H.B. White and N.O. Kaplan, *J. Biol. Chem.*, 1969, **21**, 6031.

201. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, 1934, **56**, 658.

202. N.J. Oppenheimer, L.J. Arnold and N.O. Kaplan, *Proc. Nat. Acad. Sci. USA*, 1971, **68**, 3200.

203. A.D. Winer and G.W. Schwert, *J. Biol. Chem.*, 1958, **231**, 1065.

204. Anderson, Everse and You, *The Pyridine Nucleotide Coenzymes*, Academic Press, 1982.

205. A. San Pietro, *J. Biol. Chem.*, 1955, **217**, 579.

206. N.J. Oppenheimer, L.J. Arnold and N.O. Kaplan, *Biochemistry*, 1978, **17**, 2613.

207. F.E. Hruska, A.A. Grey and I.C.P. Smith, *J. Am. Chem. Soc.*, 1970, **92**, 4088.

208. M. Legraverend and J.M. Lhoste, *Tetrahedron.*, 1984, **40**, 709.

209. D.L. Clapper, T.F. Walseth, P.J. Dargie and H.C. Lee, *J. Biol. Chem.*, 1987, **262**, 9561.

210. P.J. Dargie, M.C. Agre and H.C. Lee, *Cell Regul.*, 1990, **1**, 279.

211. H. Koshiyama, H.C. Lee and A.H. Tashijian, *J. Biol. Chem.*, 1991, **266**, 16985.

212. F.T. Walseth, R. Aarhus, R.J. Zelenznikar and H.C. Lee, *Biochim. et Biophys. Acta.*, 1991, **1094**, 113.

213. J.W. Putney, *Science*, 1993, **262**, 676.

214. A.M. White, S.P. Watson and A. Galione, *FEBS*, 1993, **318**, 259.

215. H.C. Lee, *J. Biol. Chem.*, 1993, **268**, 293.

216. S. Takasawa, K. Nata, H. Yonekura and H. Okamoto, *Science*, 1993, **259**, 370.

217. H.C. Lee, T.F. Walseth, G.T. Bratt, R.N. Hayes and D.L. Clapper, *J. Biol. Chem.*, 1989, **264**, 1608.

218. H.Kim, E.L. Jacobson and M.K. Jacobson, *Biochem. Biophys. Res. Comm.*, 1993, **194**, 1143.

219. N. Rusinko and H.C. Lee, *J. Biol. Chem.*, 1989, **264**, 11725.

220. H.C. Lee, *J. Biol. Chem.*, 1991, **266**, 2276.

221. M.R. Hellmich and F. Strumwasser, *Cell Regul.*, 1991, **2**, 193.

222. H.C. Lee and R. Aarhus, *Cell Regul.*, 1991, **2**, 203.

223. H.C. Lee and R. Aarhus, *Biochim. et Biophys. Acta.*, 1993, **1164**, 68.

224. D.L. Glick, M.R. Hellmich, S. Beushansen, P. Tempst, H. Bayley and F. Strumwasser, *Cell Regul.*, 1991, **2**, 211.

225. Q.M. Gu and C.J. Sih, *J. Am. Chem. Soc.*, 1994, **116**, 7481.

226. H. Kim, E.L. Jacobson and M.K. Jacobson, *Science*, 1993, **261**, 1330.

227. Q.M. Gu, C.J. Sih and S. Yamada, *J. Am. Chem. Soc.*, 1994, **116**, 10787.

228. G.A. Ashamu, A. Galione and B.V.L. Potter, *J. Chem. Soc., Chem. Commun.*, 1995, 1359.

229. P. Muller, M.P.N. Thi, *Helv. Chim. Acta.*, 1980, **63**, 2168.

230. M.P. Doyle, R.J. Bosch, P.G. Seites, *J. Org. Chem.*, 1978, **43**, 4120.

231. A.R. Katritzky, K. Horvath, B. Plau, *Synthesis*, 1979, 437.

232. B. Dominguez and P.M. Cullis, *Unpublished Work.*

233. J.F. Hartwig, S. Richards, D. Baranano and F. Paul, *J. Am. Chem. Soc.*, 1996, **118**, 3626.

234. W. Egan and S. Forsen, *J. Chem. Soc., Chem. Comm.*, 1973, 42.

235. D.D. Perrin and W.L.F. Armarego, *Purification of Laboratory Chemicals*, Pergamon Press, Oxford, 1988.

236. W.C. Still, M. Kahn and A. Mitra, *J. Org. Chem.*, 1978, **43**, 2923.

237. A.P. Briggs, *J. Biol. Chem.*, 1922, **53**, 13.