Incorporation of enzyme cofactor model systems into designed polypeptides

Martin Kjellstrand

Licentiate thesis

Department of Organic Chemistry, Göteborg University
Göteborg 1997

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ABSTRACT

A NAD^+ analogue has been synthesised and incorporated into a folded polypeptide with a helix-loop-helix motif by a site-selective self-functionalisation reaction. The success of the incorporation reaction was shown by electrospray mass spectroscopy and two-dimensional NMR spectroscopy. The solution structure of the polypeptide was not affected by the incorporation of the cofactor model as shown by NMR and CD spectroscopy. The rate of reduction of the peptide-bound NAD^+ analogue by sodium dithionite and its stability in aqueous solution were compared to that of free 1-methylnicotinamide. The peptide-bound NAD^+ analogue was reduced faster and its lifetime was increased by more than a factor of three. The modified peptide is water soluble which is a requirement for the creation of a turnover system.

NP-42, a 42-residue polypeptide, has been designed to bind substrates for a NADH-mediated reduction reaction by the introduction of binding residues in a reactive site. NP-42 has been synthesised and the NAD^+ analogue has been incorporated by a site-selective self-functionalisation reaction as shown by ES-MS.

PP-42, a 42-residue polypeptide, has been designed to bind pyridoxal phosphate, or a synthetic analogue of pyridoxal, by the introduction of binding residues in a reactive site. PP-42 has been synthesised and found to bind pyridoxal phosphate covalently as an aldimine as shown by UV spectroscopy and the appearance of a peak in the mass spectrum corresponding to the molecular weight of the peptide-bound pyridoxal phosphate.

Preparations have been made for the synthesis of a cyclic hexapeptide containing a pyridoxal residue, for use as a transaminase mimic.

Keywords: NAD^+, NADH, enzyme mimic, folded polypeptides, transamination, pyridoxal, design, site-selective, self-functionalisation, incorporation.

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LIST OF PAPERS

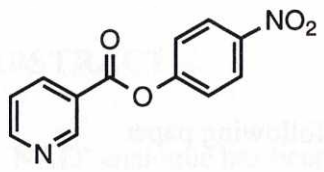
This thesis is based, in part, on the work presented in the following paper

The site-selective incorporation of a NAD^+ cofactor mimic into a folded helix-loop-helix motif.

Martin Kjellstrand, Klas Broo, Linda Andersson, Cecilia Farre, Åke Nilsson, and Lars Baltzer*.

J. Chem. Soc., Perkin Trans. 2, Submitted for publication.

Molecular structures of the compounds investigated



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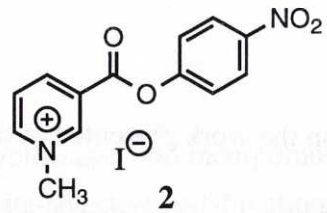

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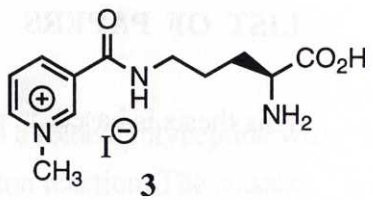

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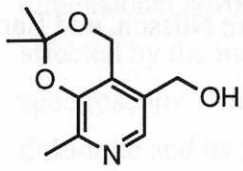

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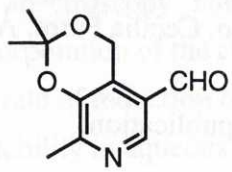

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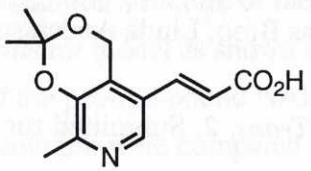

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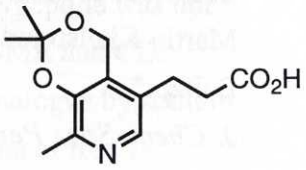

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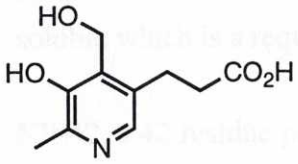

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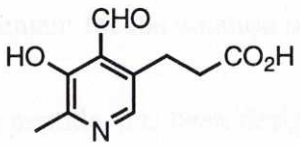

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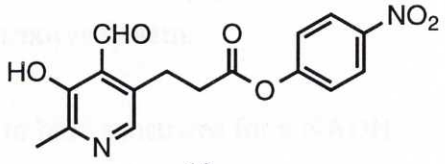

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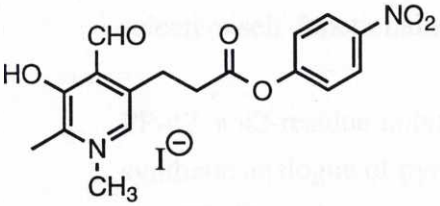

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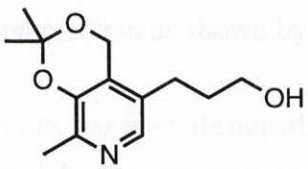

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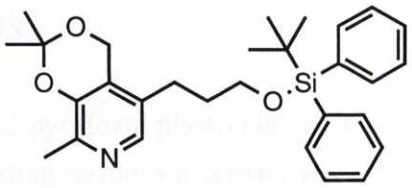

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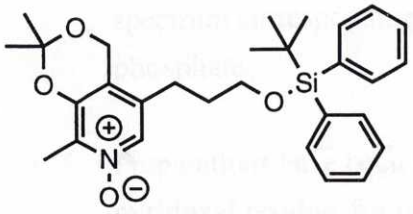

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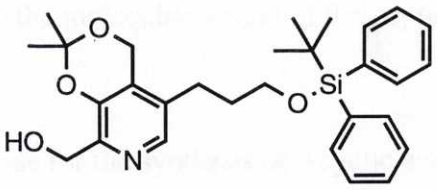

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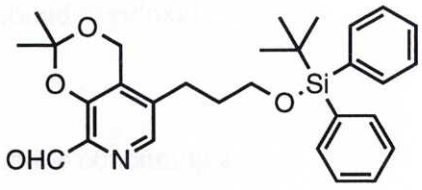

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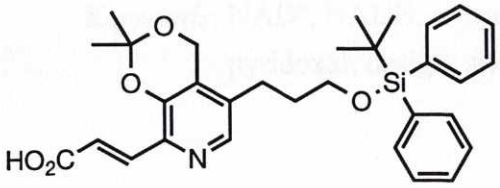

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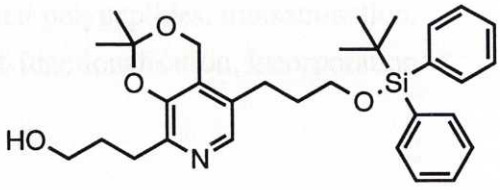

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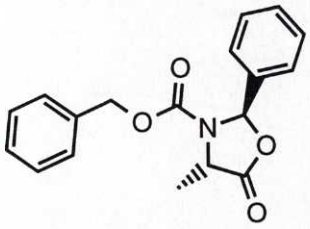

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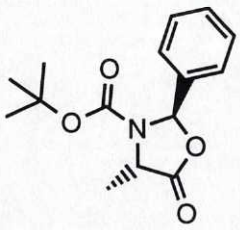

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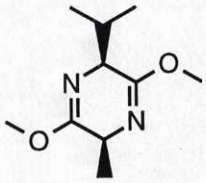

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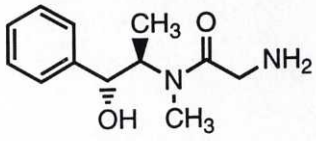

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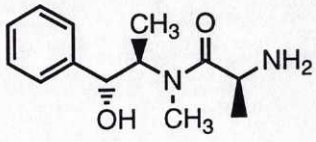

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LIST OF ABBREVIATIONS

AcO	acetoxy
Ac_2O	acetic anhydride
Aib	amino isobutyric acid
Ala	L-alanine
Arg	L-arginine
Asn	L-asparagine
Asp	L-aspartic acid
t-Boc	*tert*-butoxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
n-BuLi	*n*-butyl lithium
Cbz	benzyloxycarbonyl
CbzCl	benzyl chloroformate
CD	Circular Dichroism
DCC	*N,N'*-dicyclohexyl carbodiimide
d.e.	diastereomeric excess
DIPA	*N,N*-diisopropylamine
DMAP	*N,N*-dimethlamino pyridine
DMF	*N,N*-dimethyl formamide
DMSO	dimethyl sulfoxide
e.e.	enantiomeric excess
Et	ethyl
Et_3N	triethyl amine
EtOH	ethanol
EtOAc	ethyl acetate
Fmoc	fluorenyl methoxycarbonyl
Gln	L-glutamine
Glu	L-glutamic acid
Gly	glycine
His	L-histidine
HLADH	horse liver alcohol dehydrogenase
HOAc	acetic acid
HOBt	hydroxy benzotriazol
Ile	L-*iso*-leucine
LDA	lithium diisopropylamide
LDEA	lithium diethylamide
LDH	L-lactate dehydrogenase

Leu	L-leucine
LHMDS	lithium hexamethyldisilylamide
Lys	L-lysine
MCPBA	*meta*-chloroperoxybezoic acid
MeCN	acetonitrile
MeI	methyl iodide
MeNAH	1-methyl-1,4-dihydronicotinamide
MeOH	methanol
NAD	nicotinamide adenine dinucleotide
NAH	1,4-dihydro nicotinamide
NBS	*N*-bromosuccinimide
Nle	L-norleucine
NOESY	Nuclear Overhauser Effect SpectroscopY
Orn	L-ornithine
Orn-NAH	(1-methyl)-nicotinoyl ornithine
Ph	phenyl
Phe	L-phenylalanine
pNP	*para*-nitrophenol
PPal	pyridoxal phosphate
Pam	pyridoxamine
Pol	pyridoxine
pyr	pyridine
TBDMS	*tert*-butyl dimethyl silyl
TBDMSCl	*tert*-butyl dimethyl silyl chloride
TBDPS	*tert*-butyl diphenyl silyl
TBDPSCl	*tert*-butyl diphenyl silyl chloride
TBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium-tetrafluoroborate
TFA	trifluoro acetic acid
TFAA	trifluoro acetic anhydride
TFAB	trifluoro acetyl benzene (α,α,α-trifluoro acetophenone)
THF	tetrahydrofuran
TMS	trimethylsilyl
TOCSY	Totally Correlated SpectroscopY

TABLE OF CONTENTS

1. Introduction.

The catalytic function of many enzymes is dependant on the incorporation of cofactors[1] that have functionalities not otherwise available to the enzyme. The NAD^+/NADH cofactor couple, i.e. the oxidised and reduced forms of nicotinamide adenine dinucleotide, is the hydride-transfer reagent found in the dehydrogenase class of enzymes, that catalyse reduction reactions of carbonyl compounds, and oxidation reactions also, in metabolic systems[2]. NAD^+ functions as a hydride acceptor, whereas NADH functions as a hydride donor. The cofactor is non-covalently bound to the enzyme in the active site, and is capable of transferring the hydride stereospecifically. In the alcohol dehydrogenases the substrate is bound in the active site by coordination to a zinc ion in a hydrophobic pocket, Figure 1. The zinc ion also has an important role in

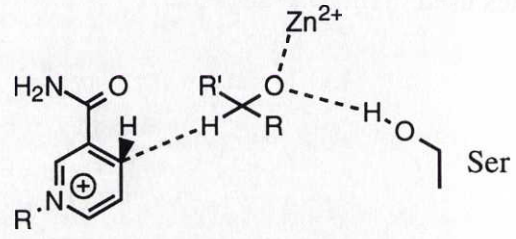

Figure 1. Outline of the NAD^+-substrate-zinc coordination.

the transition state of the hydride transfer reaction. The NAD^+/NADH-dependent enzymes have been the targets of many enzyme mimics capable of stereoselective reduction of carbonyl compounds.

The interest in enzyme mimics[3], such as the cofactor-dependent enzyme mimics, is due to our interest in learning more about the structure-function relationships and mechanisms of the enzymes. There is also a need for novel catalysts capable of catalysing reactions not catalysed by native enzymes, and catalysts that are soluble in organic solvents or attached to inert polymers, while retaining their functionality. The cofactors are ideal for enzyme-mimicking studies in that they are readily available and easily modified[4].

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[1] Dugas, H. *Bioorganic Chemistry, 3rd edition*, **1996**, Springer-Verlag, New York.
[2] Fersht, A. R. *Enzyme Structure and Mechanism*, 2nd ed.**1985**, W. H. Freeman, New York.
[3] Murakami, Y.; Kikuchi, J.-I.; Hisaeda, Y.; Hayashida, O. *Chem. Rev.*, **1996**, *96*, 721.
[4] Metzler, A. E. *Biochemistry, The Chemical reactions of Living Cells*, Chap. 8. **1977**, Academic Press, New York

Key features of enzymes targeted in enzyme mimickry include achieving reaction rate enhancements, substrate recognition, turnover, and stereospecific catalysis. An enzyme mimic with the properties of a native enzyme has not yet been achieved.

These features are difficult to implement in model systems for several reasons. Reaction rate enhancements require the ability to bind the substrate in close proximity to the reactive part of the cofactor. The substrate recognition of enzymes is dependent on the three-dimensional structure of the reactive site, in many cases a hydrophobic crevice. Turnover requires a means of regenerating the catalyst after the first cycle is complete. Stereospecific catalysis requires the substrate to approach the cofactor in one, and only one, way. To achieve these features in enzyme mimics, cofactors are often coupled to organic template structures that are designed and synthesised to mimic one or more of the features mentioned above. The attachment of these template structure lowers the solubility of the cofactor in aqueous solution. Solubility in water is in many cases a requirement for a system to be capable of turnover.

Catalysts capable of stereospecific hydride transferreactions have been reported to give enantiomeric excesses in the region of 95-98%. Metal ions have been incorporated, although the structure of the metal ion-substrate complexes remain unsolved. Substrate recognition has not been attempted. Turnover has not been reported and solubility is as problem, as most reported NAD^+/NADH-mimicking reactions are carried out in organic solvents such as acetonitrile or DMSO.

Folded polypeptides have so far not been used in spite of the fact that they will ensure solubility of the peptide-bound cofactor, they are large enough to allow the incorporation of groups capable of substrate recognition and transition state binding. The exposed residues of polypeptides may be varied by minor modifications of the amino acid sequence that leave the tertiary structure unchanged. The catalytic groups available to enzymes are also available to polypeptides.

This thesis presents the first examples of polypeptide-based cofactor model systems. These model systems have been designed with the future development towards turnover-capable systems in mind.The design of the model systems have been done from a thematic structure-function viewpoint, mimicking the structure of the active sites of relevant enzymes as closely as possible.

2. The NAD$^+$/NADH cofactor and model systems.

The dehydrogenase class of enzymes perform redox reactions in metabolic systems[1] by utilising the NAD$^+$/NADH cofactor couple for hydride-transfer reactions, Figure 2.

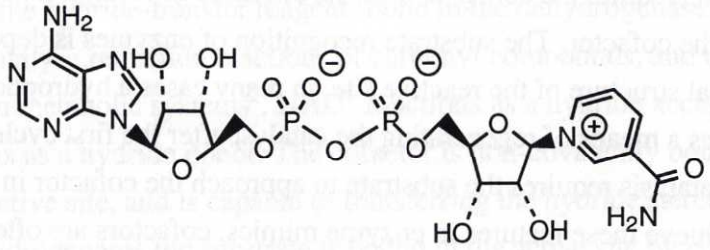

Figure 2. Nicotinamide adenine dinucleotide, NAD$^+$.

The residue responsible for the catalytic activity is the nicotinamide moiety, which exists in two redox forms, Figure 3.

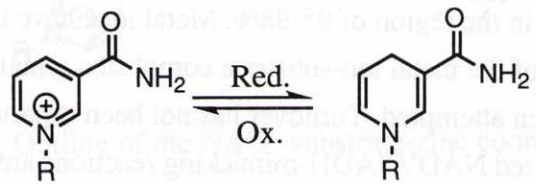

Figure 3. The active moiety of NAD$^+$/NADH. R = adenine nucleotide.

The reduced cofactor (NADH) can deliver one of the hydrons in 4-position of the nicotinamide residue as a hydride ion to a carbonyl group and reduce it to the corresponding alcohol. In the reverse reaction, the oxidised form of the cofactor (NAD$^+$) can abstract a hydride from a substrate in an oxidation reaction[ref]. The hydrogens at position 4 in the NADH dihydropyridine ring are prochiral[5], Figure 4.

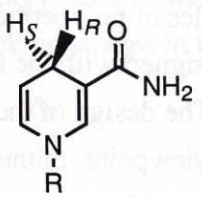

Figure 4. The prochiral 4-hydrogens of NADH.

[5] see reference 2, p 226.

The transfer of a hydride ion from NADH to a substrate, or from a substrate to NAD^+, is direct and stereospecific[6]. In the L-dehydrogenases, the hydride transfer proceeds from H_R via a *si*-face attack to produce products of *S* configuration.

The alcohol dehydrogenases are metalloenzymes that utilise zinc ions for their catalysis[7]. The Zn^{2+} -ion is situated at the bottom of a hydrophobic pocket that joins the catalytic site with the nucleotide-binding part, Figure 5, and is bound to the enzyme by two cysteine residues and a histidine. A water molecule in the free enzyme

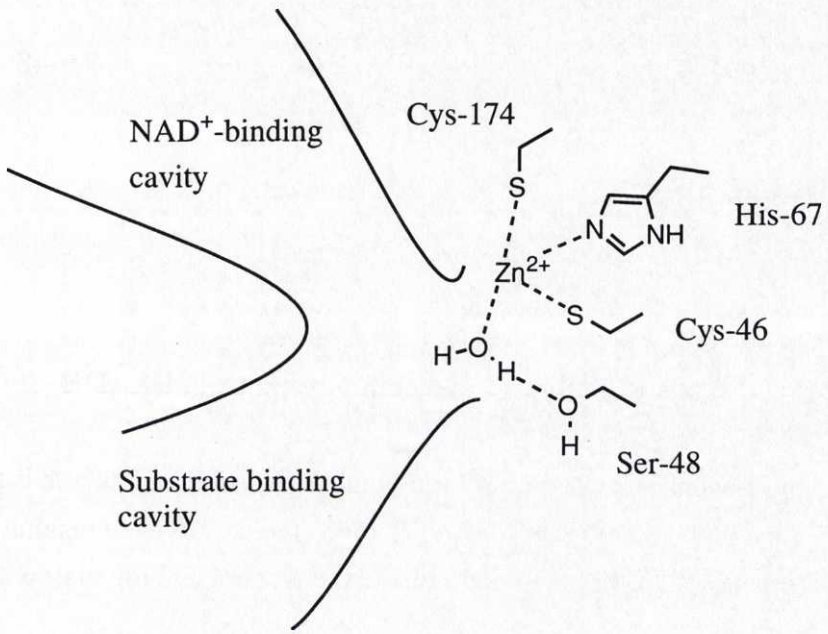

Figure 5. Sketch of the active site of HLADH apoenzyme.

is bound by the Zn^{2+} -ion and a serine residue in the free holoenzyme. The water is displaced by the substrate in the enzyme-substrate complex, Figure 6.

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[6] Fischer, H. F.; Conn, E. E.; Vennesland, B.; Westheimer, F. H.; *J. Biol. Chem.* **1953**, *202*, 687.
[7] Brändén, C.-I.; Jornvall, H.; Eklund, H.; Furugren, B. *The Enzymes* , **1975**, *11*, 104.

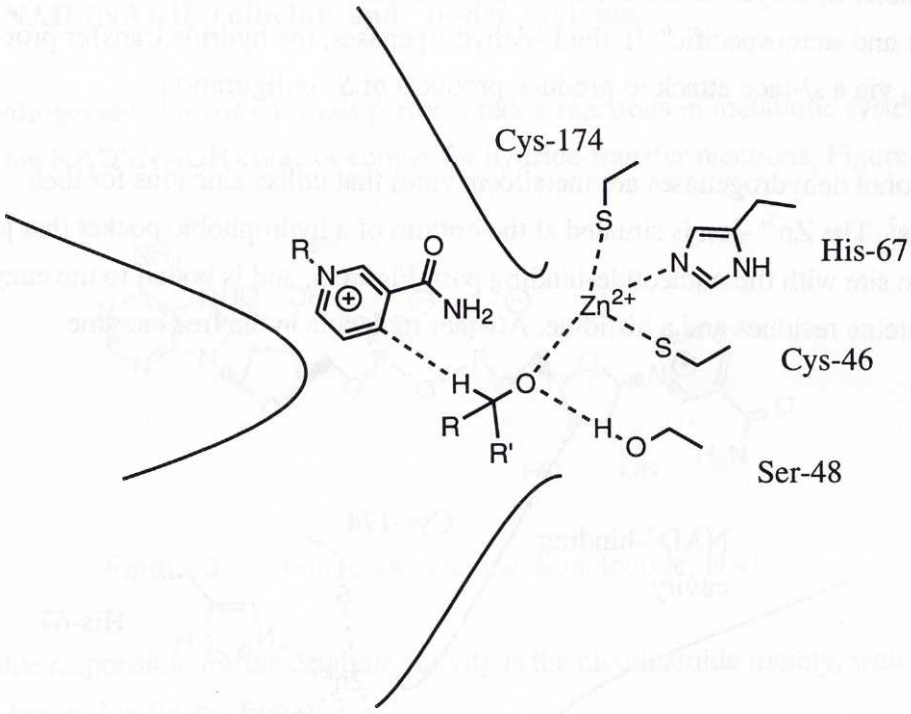

Figure 6. Outline of the ternary complex in HLADH.

L-Lactate dehydrogenase lacks the Zn^{2+} ion in the active site, and its role is taken over by His-195 and Arg-109[8], Figure 7. Arg-171 binds the carboxylate residue of the substrate. The role of His-195 is to deprotonate the alcohol and together with Arg-109

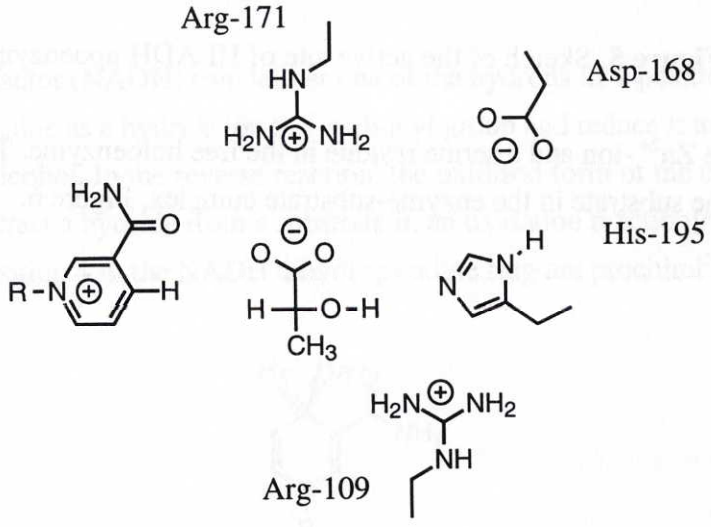

Figure 7. Sketch of the active site of LDH.

[8] Parker, D. M.; Holbrook, J. J. *Pyridine Nucleotide Dependent Dehydrogenases*, **1977**, Sund, H. (ed), Walter de Gruyter, Berlin, p 485.

stabilise and bind the alcoholate anion. The hydride ion is then delivered stereospecifically to the *R* face of the NAD^+ moiety, Figures 8 and 9.

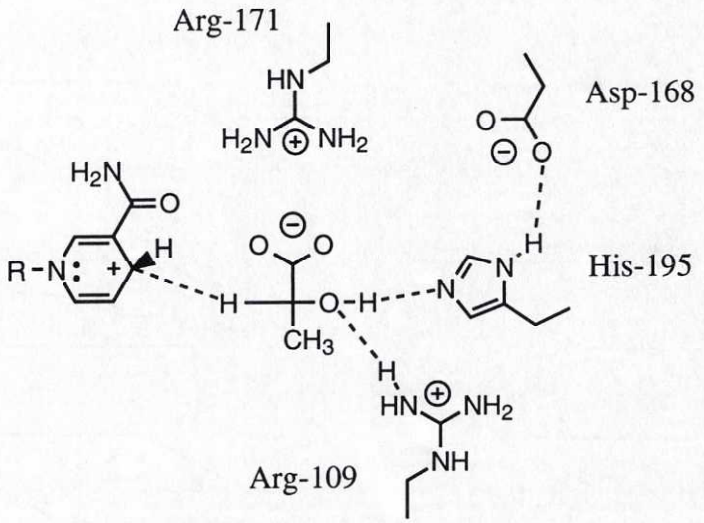

Figure 8. The stereospecific hydride transfer in LDH.

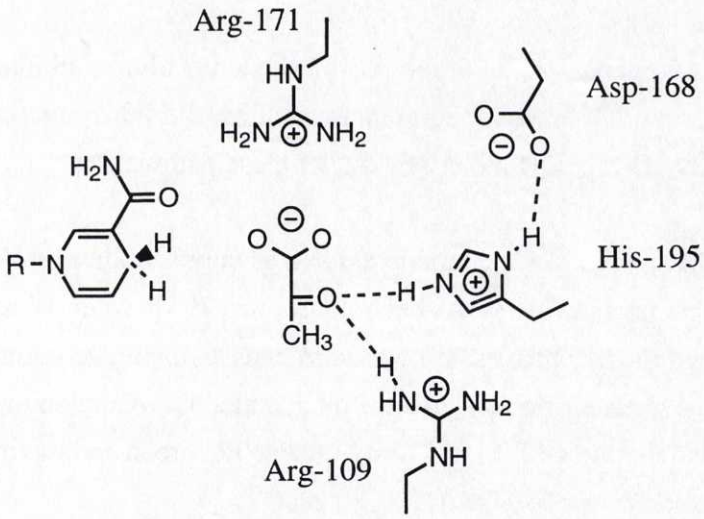

Figure 9. Structure of the ternary complex of LDH after hydride transfer.

The stereospecific transfer of the hydride ion is the basis for the stereospecificity of the dehydrogenases.

Model systems designed to mimic dehydrogenase activity must be able to incorporate some of the control mechanisms used for stereospecificity.
To achieve this in enzyme mimics, an alternative way of imposing the required three-dimensional restrictions upon the hydride transfer must be found. In the system of a

simple chiral NAH derivative interacting with ethyl benzoylformate, there are four possible interactions leading to hydride transfer, Figure 10. The *si*-face attacks both yield the (*R*)-mandelate as product, and the *re*-face

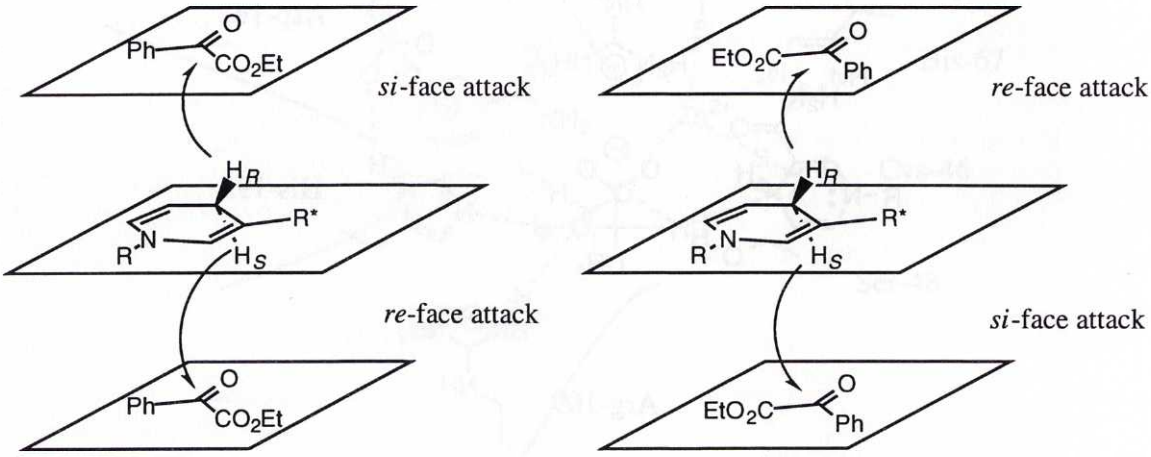

Figure 10. The four possible interactions between leading to hydride transfer. R* is the chiral functionality.

attacks yield the (*S*)-mandelate. In order to achieve stereocontrol, all interactions but one must be disfavored. The enzyme mimic must allow the substrate to approach and interact with the NAD^+/NADH moiety by one of these pathways.

The NAD^+/NADH system has been modelled extensively[9]. Studies of NAD^+ model systems for mechanistic purposes has been done since 1951, when Westheimer[10] and co-workers studied the mechanism of hydride transfer by using deuterium as a tracer to investigate the mechanism of NAD^+-mediated ethyl alcohol oxidation to acetaldehyde. Later on, Westheimer studied the reduction of malachite green and methylene blue by a simple NADH cofactor model, BNAH, Figure 11[10b].

[9] Yasui, S.; Ohno, A. *Bioorg. Chem.* **1986**, 70.

[10] a) Westheimer, F. H.; Fisher, H. F.; Conn, E. E.; Vennesland, B. *J. Am. Chem. Soc.* **1951**, *73*, 2403.

b) Westheimer, F. H.; Mauzerall, D. *J. Am. Chem. Soc.* **1955**, *77*, 2261.

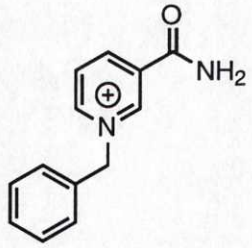

Figure 11. The NAD^+ model system BNAH

Ohno and co-workers have shown that in e.g BNAH reductions of ethyl benzoylformate in acetonitrile, the reaction does not proceed unless an equimolar amount of Mg^{2+} ion is present[11], and that the reaction rate of the reduction of trifluoroacetophenone is dependent on the concentration of Mg^{2+} ion[12].
van Eikeren and co-workers reported rate enhancements in the reduction of TFAB by 1-*n*-propylnicotinamide (PNAH) of a factor of 5000 when changing the reaction medium from DMSO to water[13].

The Hantzsch[14] 1,4-dihydropyridine esters were capable of reducing organic compounds via a hydride transfer mechanism, Figure 12.

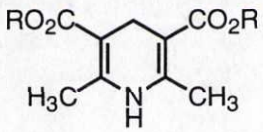

Figure 12. A Hantsch ester. R = alkyl

The Hantsch ester was reported to reduce puruvic acid nonenzymatically, albeit in low yield.

In attacking the problem of cofactor stability at low pH, Shinkai and co-workers replaced the pyridine ring by a quinoline derivative in order to enhance stability under acidic conditions[15], Figure 13. This model was

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[11] Onishi, Y.; Kagami, M.; Ohno, A. *J. Am. Chem. Soc.* **1975**, *97*(10), 4766.
[12] Ohno, A.; Yamamoto, H.; Oka, S. *J. Am. Chem. Soc.* **1981**, *103*, 2041.
[13] van Eikeren, P.; Grier, D. L. *J. Am. Chem. Soc.* **1975**, *98*, 4655.
[14] a) Braude, E. A.; Hannah, J.; Linstead, R. *J. Chem. Soc.* **1960**, 3249.
b) Braude, E. A.; Hannah, J.; Linstead, R. *J. Chem. Soc.* **1960**, 3257.
c) Norcross, B. E.; Klinedinst, Jr., P. E.; Westheimer, F. H. *J. Am. Chem. Soc.* **1962**, *84*, 979.
[15] Shinkai, S.; Hamada, H.; Manabe, O. *Tetrahedron Lett.* **1979**, *16*, 1397.

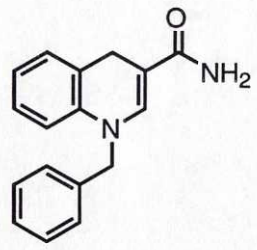

Figure 13. The acid-stable quinoline-based NADH model of Shinkai.

used in the synthesis of α-amino acids from α-keto acids.

Others have worked on the stereochemical selectivity of the reaction. Meyers[16] attached a glyoxylic acid residue to a modified cofactor analogue, Figure 14, and made the Zn^{2+}-mediated hydride transfer reaction intramolecular. The intramolecularity had the advantage of not only inducing stereoselectivity, but also enhancing the reaction rate considerably.

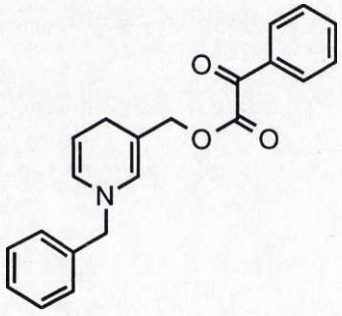

Figure 14. The intramolecular NADH model used by Meyers.

An example of stereochemical control through substrate binding rather than through the formation of a NADH-metal ion-substrate complex is the cyclodextrine-based NADH model system, Figure 15, investigated by Toda and co-workers[17]. These analogues gave significant rate enhancements in the

[16] Meyers, A. I.; Brown, J. D. *J. Am. Chem. Soc.* **1987**, *109*, 3155.
[17] Yoon, C.-J.; Ikeda, H.; Kojin, R.; Ikeda, T.; Toda, F. *J. Chem. Soc., Chem. Commun.* **1986**, 1080.

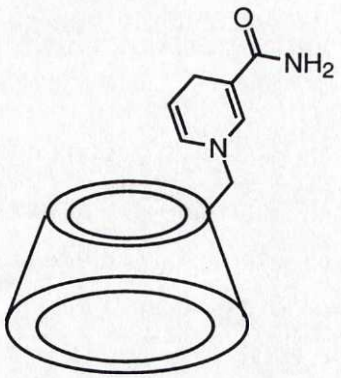

Figure 15. The cyclodextrine-bound NADH model of Toda.

reduction of ninhydrin in comparison with that in the reduction by NADH.

Another approach to the problem of inducing chiral selection in a NAD^+/NADH model system was the introduction of C_2 and D_2 symmetry by Wennerström and co-workers[18]. The C_2-symmetric NADH model, Figure 16, reduced methyl benzoylformate in DCM with an e.e. of 95 %.

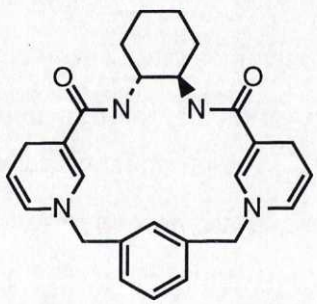

Figure 16. The C_2-symmetric NADH model used by Wennerström *et al.*

These model systems have all attempted to mimic some aspect of the dehydrogenases. Partial solutions to the problems of stereoselectivity, metal coordination, and rate enhancements were proposed. The problem of achieving a turnover-capable system has so far not been solved, probably because most NAD^+/NADH mimics are insoluble in water. The most efficient way of regenerating the reduced form of the cofactor, by sodium dithionite, requires water as solvent.

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[18] a) Skog, K.; Wennerström, O. *Tetrahedron Lett.* **1992**, *33*, 1751.
b) Skog, K.; Wennerström, O. *Tetrahedron* **1994**, *50*, 8227.
c) Skog, K.; Wennerström, O. *Tetrahedron Lett.*, submitted for publication.

3. The pyridoxal cofactor and model systems.

The cofactor pyridoxal phosphate (PPal) is an important factor in the metabolism of α-amino acids[19]. PPal condenses with an amino acid to form a Schiff base aldimine, which is transformed into a ketimine through an allylic rearrangement. The ketimine is hydrolysed by water, to form pyridoxamine and a keto acid, Figure 17.

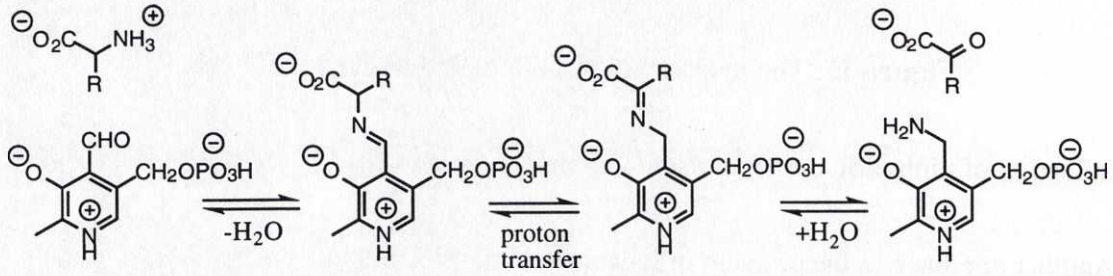

Figure 17. The reaction sequence of the pyridoxal- or pyridoxamine-mediated transamination.

The reaction is performed by a class of enzymes known as aminotransferases that are essential for the biosynthesis of α-amino acids. The exception is L-glutamic acid, for which the nitrogen is provided by ammonia, through an NADH-mediated reductive amination of 2-oxoglutarate. In the aminotransferase catalysed reactions a proton from the solvent is incorporated at the C_α of the new amino acid[20], and the β hydrogen of the amino acid is not a participant in the reaction[21].The pyridoxal is initially bound covalently as an aldimine Schiff base to the enzyme[22], and is then displaced by the substrate amino acid, under the formation of a new Schiff base as shown in figure 18.

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[19] a) Snell, E. E.; di Mari, S. J. *The Enzymes*, **1976**, *2*, 335.
b) Davis, L.; Metzler, D. E. *The Enzymes*, **1972**, *7*, 33.
[20] Hilton, M. A.; Barnes, Jr., F. W.; Enns, T. *J. Biol. Chem.*, **1956**, *219*, 83.
[21] a) Meister, A. *Nature (London)*, **1957**, *168*, 1119.
b) Sprinson, D. B.; Rittenberg, D. *J. Biol. Chem.*, **1950**, *184*, 405.
c) Gisolia, S. B.; Burris, R. H. *J. Biol. Chem.*, **1954**, *210*, 109.
[22] Hughes, R. C.; Jenkins, W. T. Fischer, E. H. *Proc. Natl. Acad. Sci. USA*, **1962**, *48*, 1615.

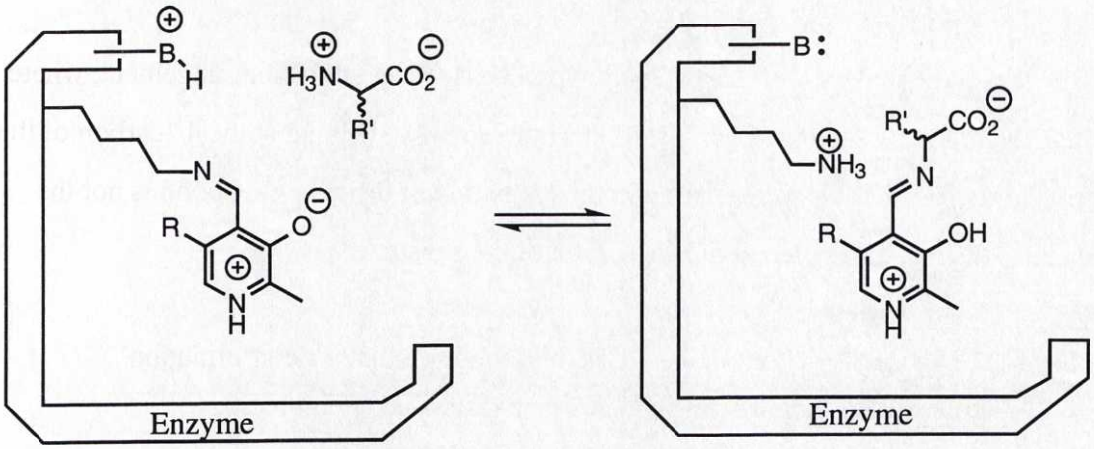

Figure 18. The binding of the substrate amino acid to the enzyme-bound pyridoxal. R = $CH_2OPO_3H^-$, R'= amino acid side chain.

The reaction has been thouroughly investigated in aspartate aminotransferase[23]. The pyridoxal-bound substrate is stabilised through the interactions with two arginine residues, Arg-292 and Arg-386. The phenolic group is hydrogen-bonded to the hydroxyl of Tyr-225 while the pyridine ring nitrogen is hydrogen-bonded to Asp-222. The 5'-phosphate group is bound by several amino acid residues, including Arg-266 and Ser-255, Figure 19.

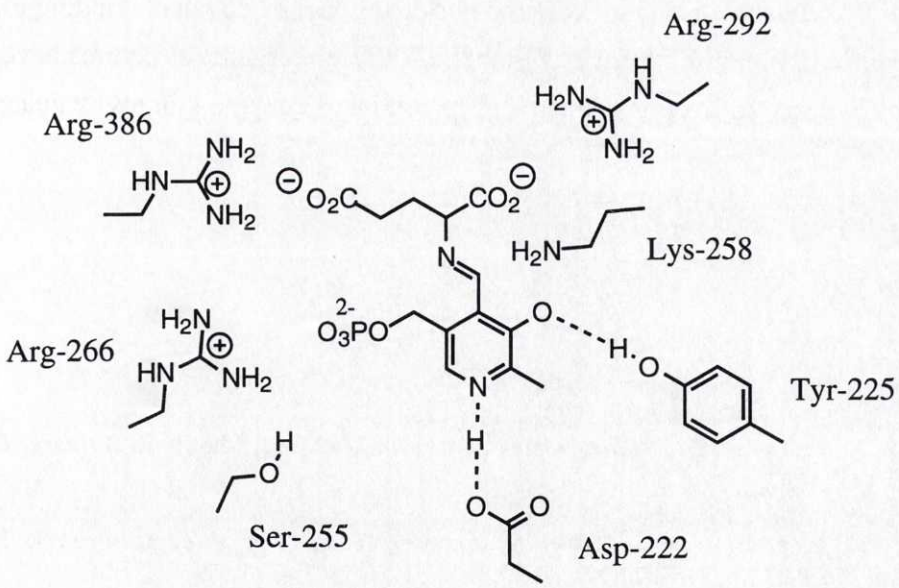

Figure 19. Outline of the active site of aspartate aminotransferase.

[23] Kirsch, J. F.; Eichele, G.; Ford, G. C; Vincent, M. G.; Jansonius, J. N.; Christen, P. *J. Mol. Biol.*, **1984**, *174*, 497.

The PPal-based enzymatic transamination proceeds via an allylic rearrangement, where the C_α-H bond of the amoni acid is broken, and a new C-H bond at the 4'-carbon of the cofactor is formed. The proton used in the formation of the new C-H bond is not the same as the one abstracted at the C_α-carbon of the substrate.

The C-H bond that is broken in the allylic rearrangement is in a conformation perpendicular to the pyridine ring-plane when abstracted[23], Figure 20.

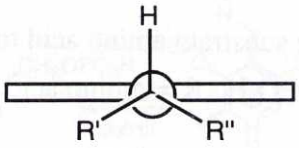

Figure 20. The C_α-H bond is perpendicular to the pyridine ring plane.

Non-enzymatic transamination[24] of α-amino acids can occur in presence of free cofactor as well as with the holoenzyme, but with much lower rates and selectivities. In many cases metal ions (e.g. Al^{3+}) accelerate the transamination[25].

Breslow[26,27] attached a cyclodextrine to pyridoxamine, by covalent bonding to the 5'-hydroxy group, Figure 21. A large number of different organic structures have also been attached to pyridoxamines, and their potential as enzyme mimics evaluated[26,27].

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[24] Martell, A. E. *Acc. Chem. Res.*, **1989**, *22*(4), 115.

[25] Martell, A. E.; Taylor, P. A. *Inorg. Chim. Acta*, **1988**, *152*, 181. Martin, R. B. *Inorg. Chem.* **1987**, *26*(14), 2197.

[26] a) Breslow, R. *Chemica Scripta* **1987**, *27*, 555.
b) Breslow, R.; Czarnik, A. W.; Lauer, M.; Leppkes, R.; Winkler, J.; Zimmerman, S. *J. Am. Chem. Soc.* **1986**, *108*, 1969.

[27] a) Breslow, R.; Czarnik, A. W.; Zimmerman, S. C. *J. Am. Chem. Soc.* **1983**, *105*, 1694.
b) Breslow, R. *Ann. NY Acad. Sci.* 471 (Int. Symp. Bioorg. Chem. **1985**), 60.
c) Weiner, W.; Winkler, J.; Zimmerman, S. C.; Czarnik, A. W.; Breslow, R. *J. Am. Chem. Soc.* **1985**, *107*, 4093.
d) Breslow, R.; Chmielewski, J.; Foley, D.; Johnson, B.; Kumabe, N.; Varney, M.; Mehra, R. *Tetrahedron* **1988**, *44*(17), 5515.
e) Breslow, R.; Canary,, J. W.; Varney, M.; Waddell, S. T.; Yang, D. *J. Am. Chem. Soc.*, **1990**, *112*, 5212.

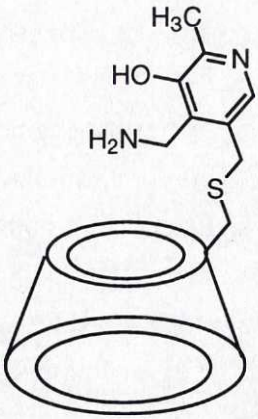

Figure 21. The cyclodextrine derivative used by Breslow.

Murakami and co-workers have attached pyridoxine moieties to vesicle bilayers[28], and attempted to control the stereochemistry of the reaction. Small, 6-residue, polypeptides functionalised with pyridoxal and pyridoxamine have been used for transamination studies by Imperiali *et al*[29].

Wu[30] studied the reactivity of a pyridoxamine derivative with an attached propellane designed to function as a bifunctional catalyst, Figure 22. One nitrogen was expected to abstract a proton, and the other nitrogen was expected to donate a proton. The work by Wu was a thorough investigation of the inter- versus intra-molecularity of the proton transfer step in the aldimine-ketimine equilibrium of the transamination reaction.

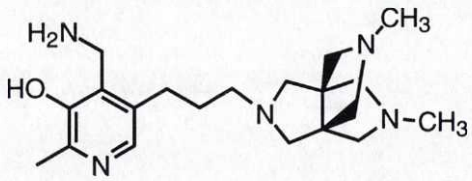

Figure 22. The pyridoxamine derivative employed by Wu.

The propellane moiety functioned as proton abstractor and donor in the intramolecular transamination reaction, but no enhancements were observed, possibly due to coordination of the Zn^{2+} ion to the amine side chain.

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[28]Murakami, Y.; Kikuchi, J.-I.; Akiyoshi, K.; Imori, T. *J. Chem. Soc. Perkin Trans., 2* **1985**, 1919.
[29]a) Imperiali, B.; Roy, R. S. *J. Org. Chem.*, **1995**, *60*, 1891
b) Imperiali, B.; Roy, R. S. *Tetrahedron Lett.*, **1996**, *37*, 2129.
[30] a) Wu, Y.-K., Thesis, Göteborg University, **1991**.
b) Wu, Y.-K., Ahlberg, P. *Acta Chem. Scand.*, **1992**, *46*, 60.

4. Outline of the model systems in the present investigation

Folded polypeptides are good templates for the engineering of enzyme mimics. The peptide surface can be designed to incorporate similar functional groups as are present in the active site of the enzyme. The functional groups in the reactive site can be varied systematically by changing amino acid residues in the sequence to optimise binding and reactivity. Folded polypeptides are soluble in water, and the solubility is not effected by the incorporation of e.g. a NAD^+/NADH analogue. Solubility is in some cases a requirement for the creation of a catalyst capable of turnover. Polypeptides are readily available due to the advanced state of solid phase peptide synthesis technology.

RA-42 is a 42-residue polypeptide that folds into a helix-loop-helix motif which dimerises in solution into an amphiphilic antiparallel dimer[31]. RA-42 was found to react rapidly and site-selectively with mono-*p*-nitrophenyl fumarate to form an amide at the side chains of lysine and ornithine residues[32]. This reaction was used for the incorporation of a nicotinoyl moiety in an attempt to create a NAD^+/NADH model as a first step towards peptide-based enzyme mimics.

An alternative approach is the use of cyclic hexapeptides as templates. They have well-defined structures and are straightforward to synthesise and purify. The incorporation of a pyridoxal derivative, functionalised as a diamino acid derivative, in a cyclohexapeptide is expected to result in a transaminase mimic, which can be designed to bind amino acids stereoselectively and to organise catalytic groups in a predetermined reactive geometry.

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[31] Lundh, A.-C., Thesis, Göteborg University, **1995**.
[32] Broo, K., Thesis, Göteborg University, **1997**.

5. Polypeptide design, structure, and reactivity.

5.1. Rational design of polypeptides

The interest in *de novo* design of polypeptides with well-defined tertiary structures stems from the importance of protein folding. By design and study of small polypeptides, with less than 100 residues, the understanding of protein folding mechanisms can be enhanced. The design of tailor-made proteins for catalysis and recognition is therefore. It is now possible to design small polypeptides that have well-defined tertiary structure and introduce catalytic functions, and a small number of polypeptides with such properties has been reported.

5.2. Design and structure of SA-42 and RA-42

SA-42[33], a polypeptide with 42 amino acid residues, was previously designed to fold into a helix-loop-helix motif that would dimerise in solution to form a four-helix amphiphilic antiparalell bundle. The design of the helical system was based on amino acids with helix propensity[34,35]. Helical dipole stabilisation[36] and capping, as well as salt bridge formation was used to further stabilise the helices. A loop between the two helical segments was designed by the introduction of amino acids with helix-breaking properties. Hydrophobic interactions between amino acid side chains was the driving force in the stabilisation of the helical structure and dimerisation [37]. The hydrophobic core contained phenylalanine, leucine, isoleucine and norleucine residues. The amino acid sequence was varied and included 16 different amino acids, in order to facilitate structural investigation and resonance assignment by NMR spectroscopy. Two phenylalanine residues were placed at the end of helix II, to yield chemical shift dispersion and to function as NOE reporter. Studies of SA-42 in solution by both NMR and CD spectroscopy as well as by ultracentrifugation revealed that it does form an amphiphilic helix-loop-helix motif that dimerises in an antiparalell fashion.

A reactive site for ester hydrolysis was included on the surface of the folded SA-42, using His-15, Lys-19, Gln-26, and Gln-30. The reactive site was positioned close to the β-turn in the hairpin motif. The reactive site however did not enhance the rate of the ester hydrolysis beyond that of the 4-methylimidazole catalysed reaction

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[33] Olofsson, S., Thesis, Göteborg University, **1994**.
[34] O'Neil, K. T.; DeGrado, W. F. *Science*, **1990**, *250*, 669.
[35] Chou, P.Y.; Fasman, G. D. *Biochemistry*, **1974**, *13*, 222.
[36] Hol, W. G. J.; van Duijnen, P. T.; Berendsen, H. J. C. *Nature*, **1978**, *273*, 443.
[37] a) Kellis, Jr. J. T., et al. *Nature*, **1988**, *333*, 784.

The studies of Lundh[31] led to the design and synthesis of RA-42, a 42-residue peptide which performs acyl transfer reactions of activated esters. The structural basis of RA-42 was the hydrophobic core of SA-42, but the reactive site was moved towards the middle of the motif, in order to transfer it from the less stable helix ends. In RA-42 ornithine residues were introduced, to replace the glutamine residues of the reactive site of SA-42, in order to enhance the hydrolytic capacity of the peptide. The reactive site of RA-42 was designed with a histidine residue at position *i* (His-11) and an ornithine residue in position *i*+4 (Orn-15), Figure 23.

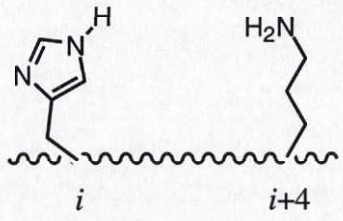

Figure 23. Outline of the reactive site of RA-42

The purpose of the design was to obtain a reactive center with a nucleophilic catalyst, the imidazolyl group of the histidine, and a flanking positively charged hydrogen bond donor that could bind the acyl residue as an amide.

5.3 The reactivity of RA-42 and LA-42.

RA-42 reacted with mono-*p*-nitrophenyl fumarate in 10 % TFE at pH 5.85 and 293 K under the release of p-nitrophenol with a second-order rate constant of $28*10^{-3}$ dm^{-3} mol^{-1} s^{-1} which is 8.3 times larger than the the second-order rate constant of the 4-methyl-imidazole catalysed reaction, $3.38*10^{-3}$ dm^{-3} mol^{-1} s^{-1}. The reaction was studied over a range of pH, in order to identify the reactive residues. The pH profile of the reaction showed that the histidine was the catalytically active amino acid. The reaction products were identified by NMR spectroscopy and mass spectroscopy and it was found that the acyl group of the substrate formed an amide of the side chain of an Orn or a Lys residue.

The acyl transfer reaction proceeds via the initial and rate determining formation of an acyl intermediate under the release of p-nitrophenol. In the next step the acyl group is transferred in a fast intramolecular transfer to the flanking ornithine residue.

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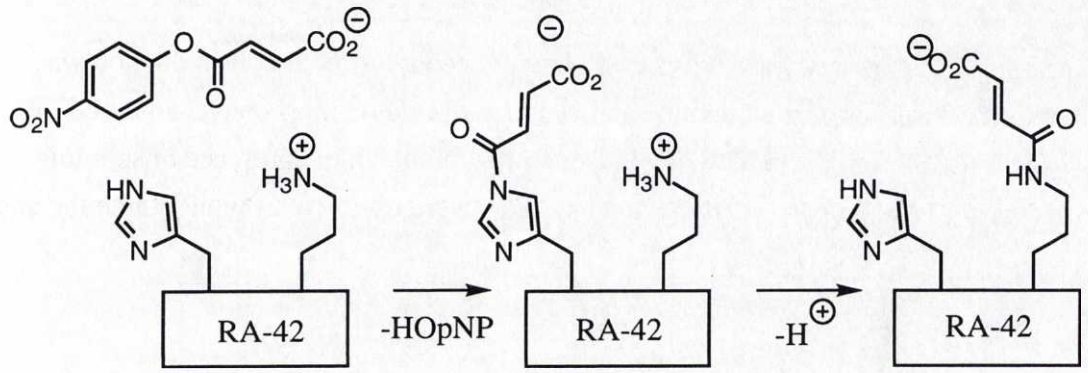
b) Chotia, C. *Nature*, **1974**, *248*, 338.

5.4. The site selectivity of RA-42.

There are three residues in RA-42 with side chains that can form amide bonds, Orn-15, Lys-19 and Orn-34. Orn-15 was the final recipient of the acyl group. Broo[32] established that in RA-42 one equivalent of fumarate acylates only Orn-15[38] by trypsin-catalysed site-selective cleavage of the acylated peptide and identification of the fragments by ES-MS. The same results were observed by NMR spectroscopy. The reaction was found to be site-selective and only residues in position *i*+4 or *i*-3 relative to the histidine in position *i* were shown to be amidated.

The proposed reaction sequence for the acyl transfer reaction is shown in Scheme 1.



Scheme 1. Proposed reaction sequence for the acyl transfer reaction of RA-42

5.5. NAD^+ analogue incorporation into RA-42 and LA-42.

In order to exploit the functionalisation reaction in cofactor incorporation, a NAD^+/NADH cofactor analogue was synthesised. The system chosen was a *p*-nitrophenyl ester of *N*-methylnicotinic acid, Figure 24. Initially,

[38] Broo, K.; Allert, M.; Andersson, L.; Erlandsson, P.; Stenhagen, G.; Wigström, J.; Ahlberg, P.; Baltzer, L. *J. Chem. Soc. Perkin Trans. 2*, **1997**, 397.

Figure 24. The *p*-nitrophenyl ester of *N*-methylnicotinic acid.

RA-42 was used, but then LA-42 was used because it was slightly more reactive. The only difference between RA-42 and LA-42 is that Orn-15 in RA-42 has been replaced by a lysine. The modified LA-42 was studied with NMR and CD spectroscopy in order to determine the effect of the modification on the secondary and supersecondary structure.

Once the NAD^+ moiety was incorporated into the peptide structure, its reduction was attempted. A survey of the literature showed that this would most conveniently be accomplished through a reduction with sodium dithionite in the presence of sodium carbonate[39]. For comparison, two model systems were used, 1-methylnicotinamide and N_ε-(*N*-methyl-nicotinoyl)-ornithine, Figure 25.

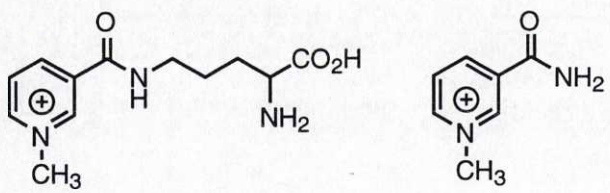


Figure 25. The model systems used in the comparison reactions

[39] Blankenhorn, G.; Moore, E. G. *J. Am. Chem. Soc.* **1980**, *102*, 1092.

6. The design and structure of the peptides NP-42 and PP-42.

6.1. Design of NP-42

With the successful incorporation and reduction of the NAD^+ analogue into LA-42, the next logical step was the design of a polypeptide to be used as a dehydrogenase mimic. The design of NP-42 was based on the structure of RA-42. The hydrophobic core of RA-42 was kept unchanged, whereas the active site was moved towards the amino- and carboxy-terminii, away from the loop, in order to increase the surface area of the peptide on which to place side chains for substrate and transition-state binding. As a structural model for this reactive site, lactate dehydrogenase (LDH) described by Adams[40] was used, Figure 26.

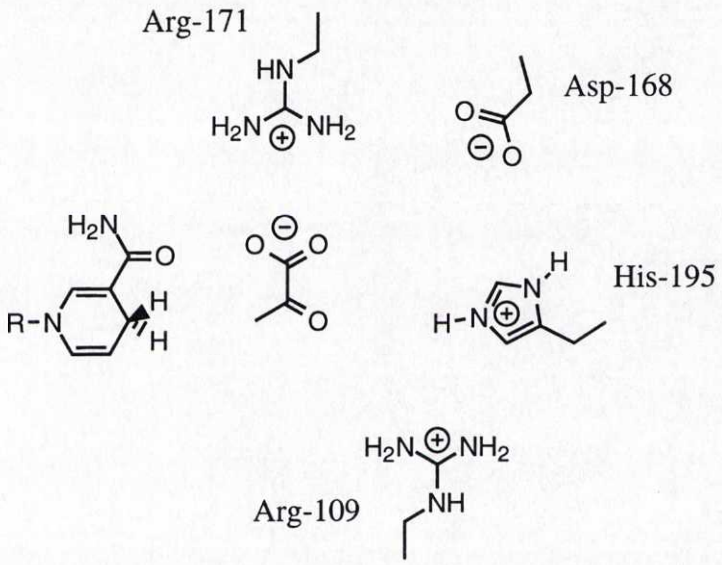


Figure 26. Sketch of the active site of lactate dehydrogenase, LDH.

There are two main differences between the reactive site of the designed peptide and the active site of LDH.The active site of LDH is situated within a hydrophobic pocket in the enzyme whereas the reactive site of NP-42 is situated on the surface of the peptide, and the number of positions available for amino acid side chains on NP-42 is much smaller than in LDH. The amino acid sequence of NP-42 is shown in Figure 27. The underscored residues are designed to be parts of the reactive site. To keep the reactive site design as simple as

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[40] Adams, M. J. *Enzyme Mechanisms*, Page, M. I.; Williams, A. (Eds.), **1987**, Royal Soc. Chem. London.

Asn-Ala-Ala-Asp-Nle-Glu-His-Ala-Ile-Arg-Lys-Leu-Ala-Glu-Arg-Nle-Ala-Ala-Gly-
1 7 11 15 19
-Gly-Pro-Val-Asp-
20 23
Gly-Ala-Arg-Ala-Phe-Ala-Glu-Phe-Arg-Arg-Ala-Leu-Arg-Glu-Ala-Nle-Gln-Ala-Ala
42 34 30 24

Figure 27. The amino acid sequence of NP-42.

possible, arginine residues were chosen both for substrate binding and transition state stabilisation, and as a proton donor in the carbonyl reduction reaction. The positioning of the residues is as shown in Figure 28.

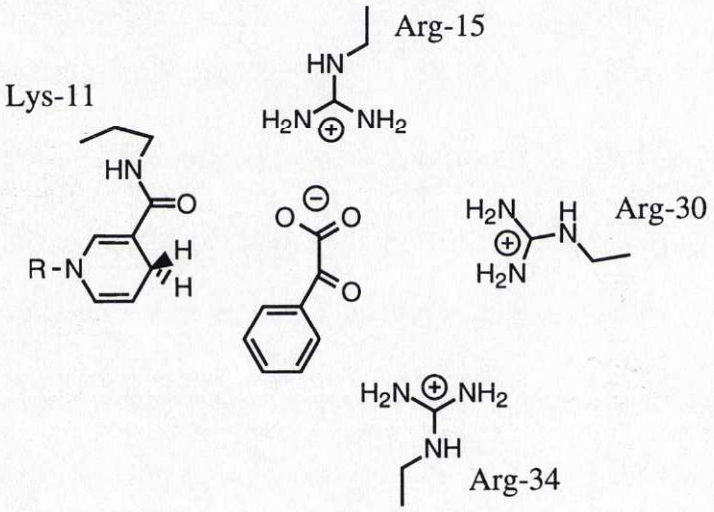


Figure 28. Suggested reactive site structure of NP-42 according to presynthetic molecular modelling studies

In the designed model Lys-11 is the residue which carries the incorporated 1,4-dihydronicotinamide moiety, and Arg-15 binds the carboxylate residue of the substrate. Arg-30 and Arg-34 can also be used for binding the substrate. The Aib residues used in RA-42 were replaced by alanines in NP-42.

6.2. Design of PP-42

In widening the scope of polypeptide-based enzyme mimics a transaminase mimicking peptide, PP-42, was designed. PP-42 is also based on the structure of RA-42.

Molecular modelling studies indicated that the positioning of the His-Lys reactive site at positions 7 and 11 would be the most advantageous in the design of a transaminase-mimicking peptide. This positioning would, like in NP-42, increase the number of available side chains that would be used to make up the reactive site.
In order to keep the pyridoxal ring parallel to the peptide plane, formation of an internal aldimine would be necessary. The reactive site was loosely modelled after the active site of aspartate transaminase[41]. Two 'extra' lysine residues, at positions 15 and 30, were introduced. They were positioned far enough from His-7 to ensure minimal possibility of diacylation and were used to maximise the possibility for intramolecular aldimine formation and to have one lysine function as base in the transamination reaction. Two arginine residues and a glutamic acid were also made part of the reactive site, to bind the cofactor and to bind in the transition state, Figure 29.

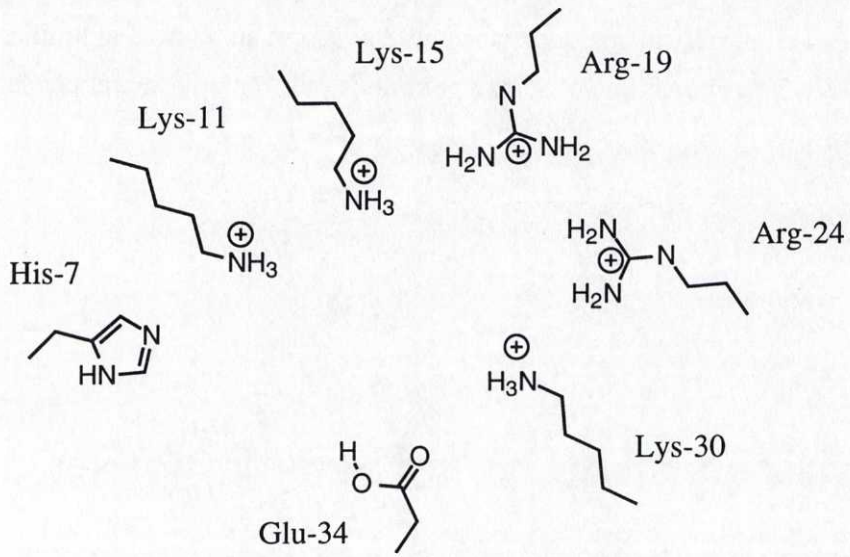


Figure 29. Placement of amino acid residues in PP-42 according to molecular modelling studies

Here, as in NP-42, the Aib residues were replaced by alanines. The amino acid sequence of PP-42 is shown in Figure 30, with the amino acid residues of the reactive site underscored.

[41] Smith, D. M.; Thomas, N. R.; Gani, D. *Experentia*, **1991**, 1104.

Asn-Ala-Ala-Asp-Nle-Glu-His-Ala-Ile-Arg-Lys-Leu-Ala-Glu-Lys-Nle-Ala-Ala-Arg-
1 7 11 15 19
-Gly-Pro-Val-Asp-
20 23
Gly-Ala-Arg-Ala-Phe-Ala-Glu-Phe-Glu-Arg-Ala-Leu-Lys-Glu-Ala-Nle-Gln-Ala-Ala
42 34 30 24

Figure 30. The amino acid sequence of PP-42.

Modelling studies of the distances between side chains suggested that a chain-elongated pyridoxal analogue would be a good choice for incorporation, as a longer side chain would increase the possibility for interaction with other groups in the reactive site.

The incorporated pyridoxal analogue is supposed to form an internal aldimine, to either Lys-15 or Lys-30. An example of such an aldimine, between pyridoxal phosphate and Lys-15, is shown in Figure 31.

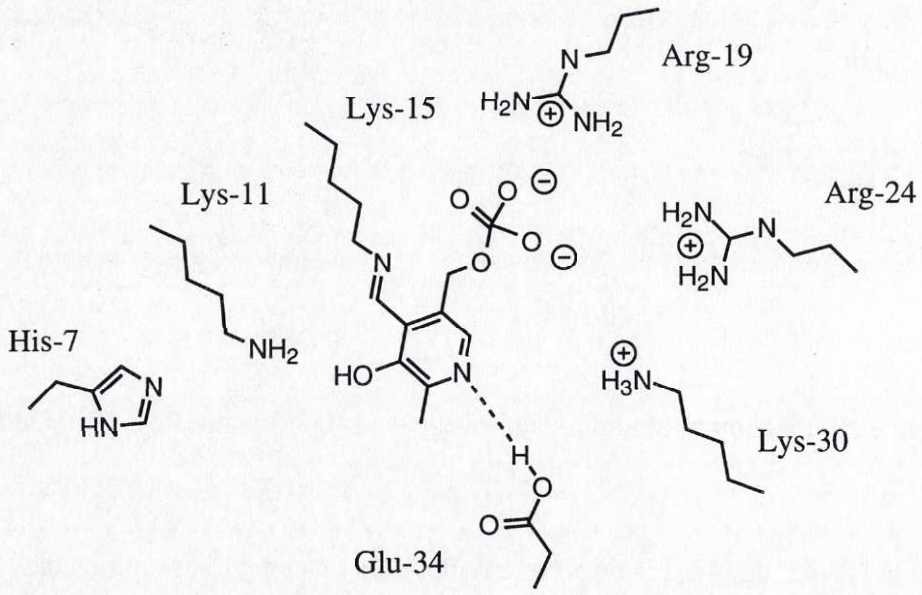


Figure 31. Outline of a pyridoxal phosphate-Lys-15 internal aldimine of PP-42.

7. Design of the cyclic hexapeptide

7.1. The cyclic hexapeptide structure

The choice of a cyclic hexapeptide as the basis for design and synthesis of a transaminase mimic was based upon the need to control the stereochemistry and the distances between functional groups. Many cyclic hexapeptides have stable, well-defined structures. The synthesis of hexapeptides is relatively short and the purification is straightforward.

7.2 Design of the cyclic hexapeptide

Many cyclic hexapeptides have a β-turn-β-turn conformation, Figure 32.

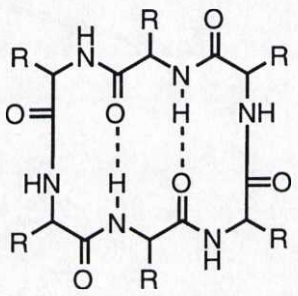


Figure 32. Schematic conformation of a cyclic hexapeptide

A pyridoxine derivative can be incorporated as a bridge that restricts conformational degrees of freedom in the peptide. The outline of this peptide is shown in figure 33.

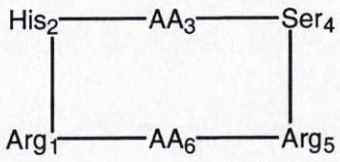


Figure 33. Outline of the designed cyclohexapeptide.

The amino acids AA-3 and AA-6 are the residues that are coupled to the pyridoxine derivative. Arg-1 and His-2 can be used for either catalytic functions and Ser-4 and Arg-5 were incorporated to enhance solubility,. His-2 was designed to function as the proton abstractor and donator in the transamination reaction, whereas Arg-1 was

designed to bind to the carboxylate function of the substrate amino acid. The schematic structure of the designed cyclohexapeptide is shown in Figure 34.

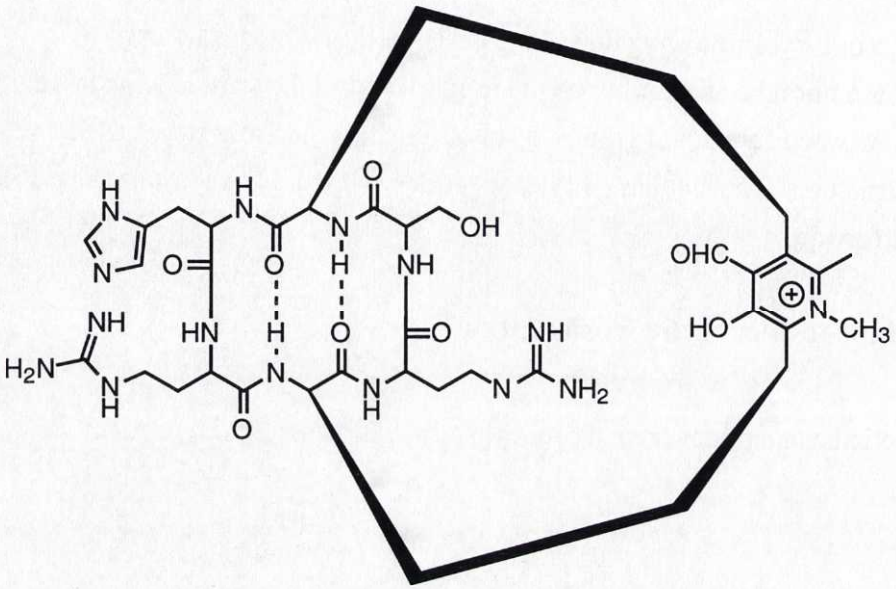


Figure 34. Designed cyclohexapeptide based transaminase mimic.

The protecting groups on the two amino acid residues of the pyridoxine derivative must be different if the the pyridoxine-amino acid moieties are to be coupled at different positions in the sequence of the peptide. When the first amino acid residue is deprotected and coupled the other must remain protected.

8. Design and synthesis of the cofactor model systems

8.1. Design of the two-armed pyridoxine derivative for incorporation into a cyclohexapeptide

In order to incorporate a structurally well-defined pyridoxine function into a cyclic hexapeptide linkages to the cyclic peptide had to be constructed. The length of the linkages was determined by molecular modelling and a three-carbon chain was found to be . The 2- and 5-positions of the pyridine ring were designated as attachment points for the linkages as they are not essential for reactivity. The coupling of the linkage chains to the amino acid moieties must be done sequentially in order to be able to use different protection groups for the two amino acids, and use of different protection groups on the two amino acid functions is required for sequential insertion into the amino acid sequence during the peptide synthesis. The structure of the designed pyridoxine derivative is shown in Figure 35.

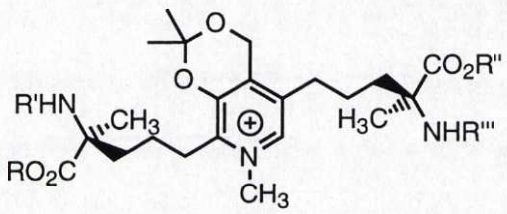
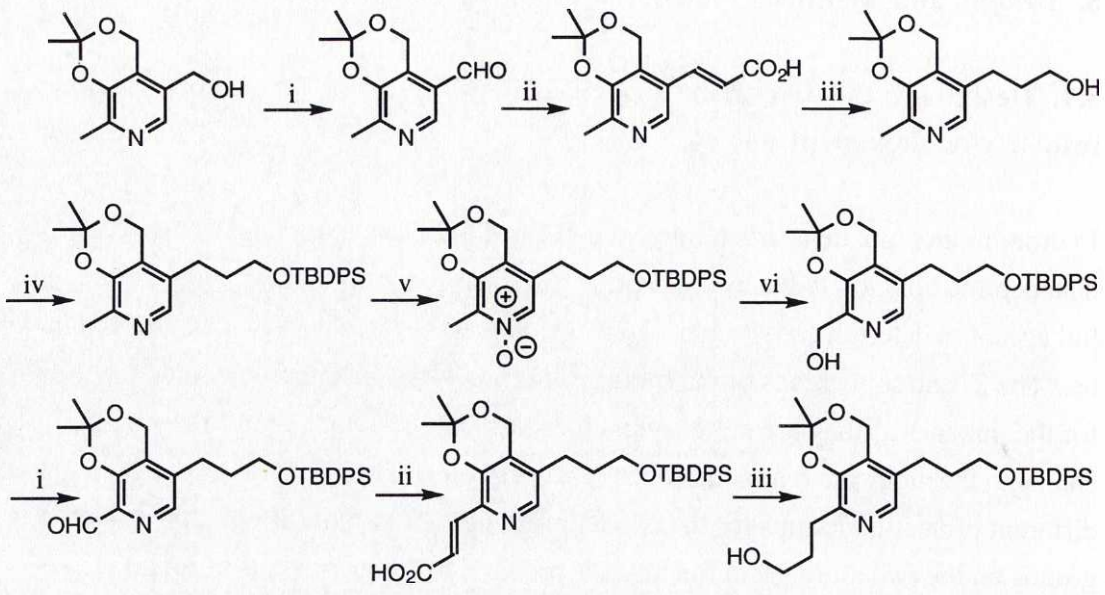


Figure 35. The designed two-armed pyridoxine derivative. R-R´´´ are different protecting groups

8.2. Synthesis of a two-armed pyridoxine derivative for incorporation into a cyclic hexapeptide

The synthetic sequence for the designed pyridoxine derivative was mainly based on methods published by Korytnyk[42], Scheme 2.

[42] a) Korytnyk, W. *J. Med. Chem.*, **1965**, *8*, 112.
b) Korytnyk, W.; Srivastava, S. C.; Angelino, N.; Potti, P. G. G.; Paul, B. *J. Med. Chem.*, **1973**, 16, 1096.



Scheme 2. The synthetic path of the two-armed pyridoxine derivative.

The synthesis started from the 3-*O*-α^4-isopropylidenepyridoxine[43] by oxidation to the corresponding aldehyde using MnO_2, (i). The aldehyde was then transformed to the α,β-unsaturated acid through a Knoevenagel condensation with malonic acid, (ii). The unsaturated acid was reduced directly to the saturated alcohol by $LiAlH_4$ in THF, (iii). The alcohol was protected as a tert-butyldiphenyl silyl ether through a DMAP-catalysed reaction[44], (iv). Oxidation of the silyl ether at the pyridine nitrogen using MCPBA, (v), yielded the *N*-oxide, which was transformed to the 2'-alcohol in a TFAA-mediated rearrangement, (vi). The 2'-alcohol was then oxidised with MnO_2, (i), the formed aldehyde was condensed with malonic acid, (ii), and the formed unsaturated acid was reduced with $LiAlH_4$, (iii), similarly to what was described for the 5-position.

The resulting alcohol was then to be transformed into a halide and asymmetrically alkylated onto an amino acid moiety. The TBDPS protecting group would then be removed, and a second asymmetric alkylation performed to yield the designed derivative.

8.3. Design of the pyridoxal derivatives for incorporation into PP-42

In order to expand the cofactor incorporation concept to include transamination, two pyridoxal and one pyridoxamine derivatives were developed, Figure 36.

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[43] Wu, Y.-K.; Ahlberg, P. *Acta Chem. Scand.*, **1989**, *43*, 1009.
[44] Chaudhary, S. K.; Hernandez, O. *Tetrahedron Lett.*, **1979**, 99.

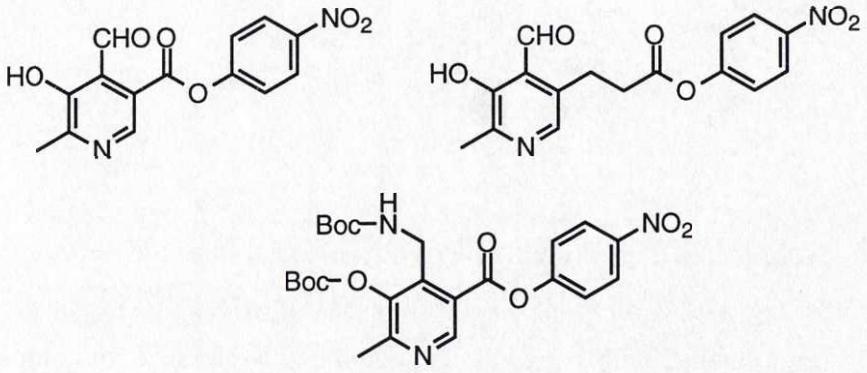

Figure 36. The three derivatives of pyridoxal and pyridoxamine designed for incorporation in PP-42

The compounds were to be incorporated using the site-selective functionalisation reaction.

8.4. Synthesis of the NAD$^+$ model systems and NAD$^+$ incorporation derivative

The *p*-nitrophenyl ester of *N*-methylnicotinic acid, **2**, Figure 37, was prepared in two synthetic steps. The first reaction was the esterification of nicotinic acid with *p*-nitrophenol using DCC and pyrrolidinopyridine as coupling reagents[45]. The ester was methylated with methyl iodide in DMF. The product was used both for incorporation

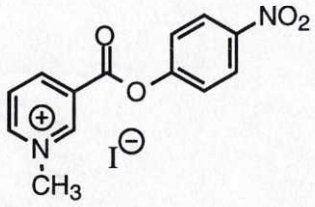

Figure 37. The *p*-nitrophenyl ester of *N*-methylnicotinic acid, **2**.

into the peptides and as reagent in the synthesis of **3**, Figure 38. **3** was prepared by first making a copper chelate of L-ornithine and then adding the *p*-nitrophenyl ester **2** to the chelate.

[45] Hassner, A.; Alexanian, V. *Tetrahedron Lett.*, **1978**, 4475.

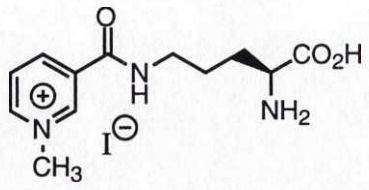

Figure 38. N_{ε}-(*N*-methylnicotinoyl)-L-ornithine iodide, **3**.

3 was to be used together with 1-methylnicotinamide as a reference for comparisons of the rate of reduction and of stability.

9. Asymmetric amino acid synthesis

9.1. The Mutter approach to α-methylated α-amino acid synthesis

The pyridoxine derivative designed for incorporation into the cyclohexapeptide was to be alkylated asymmetrically with two α-amino acid moieties. There are a large number of methods of amino acid synthesis in the literature that give both good chemical yields and products of high optical purity[46]. Those most suited to our purposes were the methods of Mutter[47], Schöllkopf[48], and Myers[49].

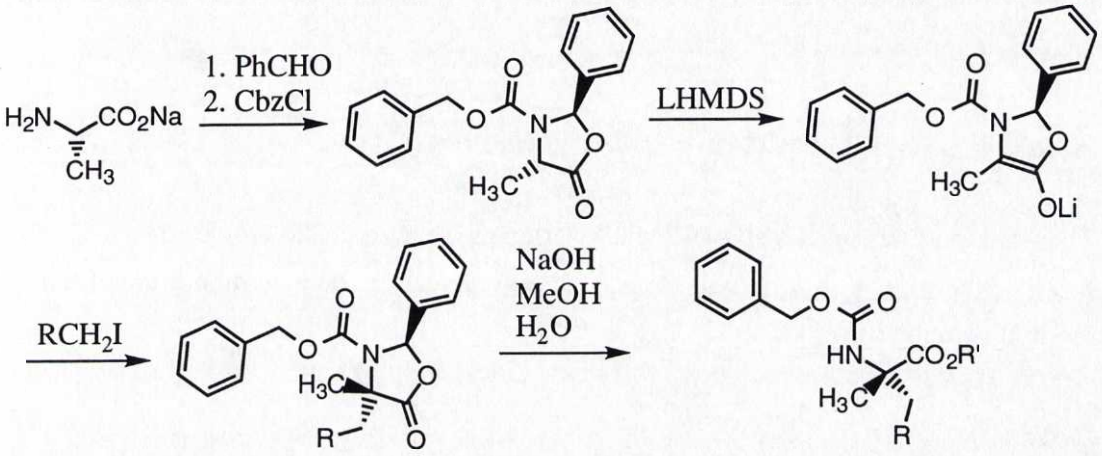

Figure 39. Mutter's Oxazolidinone method of asymmetric α-amino acid synthesis. R'=H or CH_3.

This synthesis according to Mutter, Figure 39, is rather straightforward, with the only apparent sensitive step being the enolisation of the oxazolidinione. The ring opening method shown in Figure 39 is the simplest of the alternatives, the other being catalytic hydrogenation.

9.2. The Schoellkopf *bis*-lactim ether method

The Schoellkopf *bis*-lactim ether method begins with the synthesis of an asymmetric *bis*-lactim ether of either alanine and valine, or of glycine and valine.

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[46] For two comprehensive reviews of asymmetric α-amino acid synthesis, see
a) Williams, R. M., *Synthesis of optically active α-amino acids, Vol. 7 of Organic Chemistry Series*; Baldwin, J. E.; Magnus, P. D. (Eds); Pergamon Press, Oxford, 1989.
b) Duthaler, R. O. *Tetrahedron*, **1994**, *50*, 1539.

[47] Altmann, E.; Nebel, K.; Mutter, M. *Helvetica Chim. Acta*, **1991**, *74*, 800.

[48] a) Schöllkopf, U.; Hinrichs, R.; Lonsky, R. *Angew. Chem. Int Ed. Engl.* **1987**, *26*, 143.
b) Schöllkopf, U.; Lonsky, R. *Synthesis*, **1983**, 675.

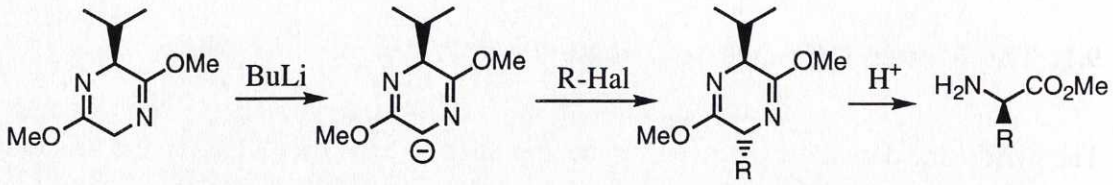

Figure 40. Schoellkopf's *bis*-lactim ether method.

The *bis*-lactim ether is alkylated, through the sequence shown in Figure 40, with a suitably active alkyl halide, yielding a *trans* product. After the alkylation, the *bis*-lactim ether is hydrolysed by acid to give an amino ester hydrochloride, or, if a stronger acid is used, an amino acid hydrochloride.

9.3. The pseudoephedrine method of Myers

The final method considered was the alkylation of the amino acid amides of pseudoephedrine, developed by Myers and co-workers[49]. The general outline of the method is given in figure 41.

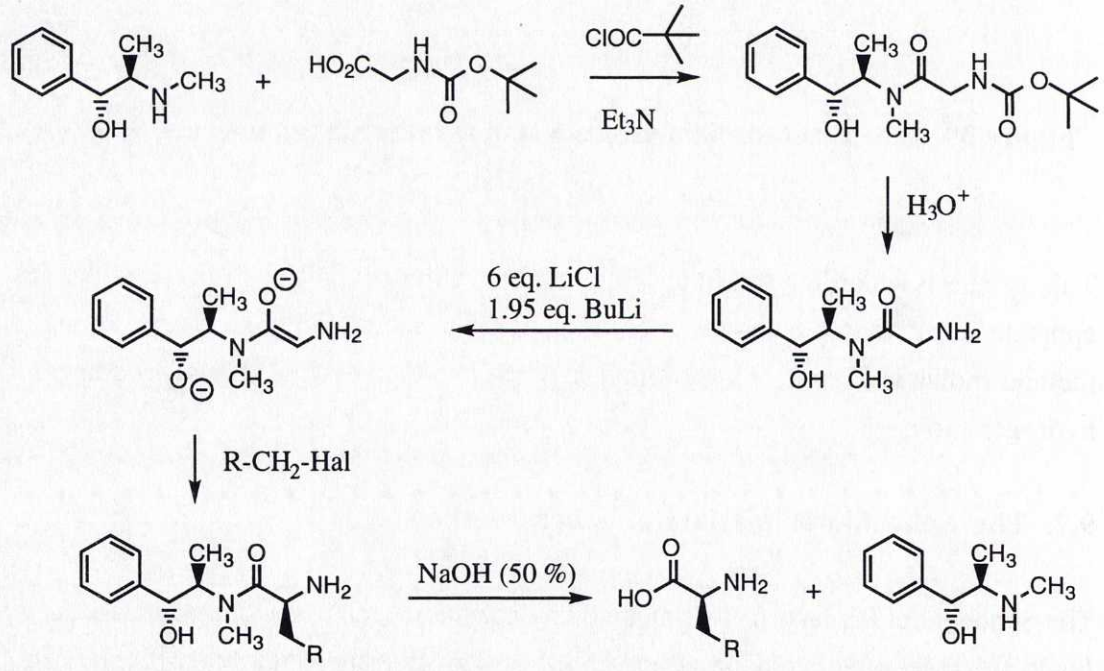

Figure 41. The Myers' pseudoephedrine synthesis of optically pure amino acids.

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[49] a) Myers, A. G.; Bryant, H.; Yang, H. C.; Gleason,J. L. *J. Am. Chem. Soc.* **1994**, *116*, 9361.
b) Myers, A. G.; Gleason, J. L.; Yoon, T. *J. Am. Chem. Soc.* **1995**, *117*, 8488.
c) Myers, A. G.; Yoon, T.; Gleason, J. L. *Tetrahedron Lett.* **1995**, *36*, 4555.
d) Myers, A. G.; Gleason, J. L. *J. Org. Chem.* **1996**, *61*, 813.

A variation of this method, using alanine instead of glycine, was also considered.

In these methods the authors report obtaining optically pure amino acids in good yield by coupling of a glycine or an alanine with an auxiliary, to form a chiral derivative. deprotronation of this derivative with a strong base, and alkylation of the anionic intermediate. This preferentially occurs on one face of the glycine or alanine derivatives. Cleavage of the auxiliary from the amino acid residue produces the new optically pure amino acid. The optical purity of the products reported in the range of 95-99% e.e. and the chemical yields are between 70 to 90 %.

10. Results and discussion.

10.1. Incorporation of a NAD^+ analogue into RA-42 and LA-42

RA-42 is a 42-residue polypeptide designed to fold into a helix-loop-helix motif and dimerise to form a four-helix bundle. Its solution structure has been determined by ^1H NMR and CD spectroscopy and the formation of the helix-loop-helix motif has been established. Its helical content was determined by CD spectroscopy and its mean residue ellipticity at 222 nm was -18 800 deg cm^2 $dmol^{-1}$.
RA-42 reacts with mono *p*-nitrophenyl fumarate in a second-order reaction under the release of *p*-nitrophenol to form an amide at the side chain of the flanking ornithine.
LA-42 is a 42-residue peptide with a structure identical to that of RA-42 except that Orn-15 is replaced by Lys-15.

RA-42 was reacted with a thirteenfold excess of the *p*-nitrophenyl ester of *N*-methyl nicotinic acid under in aqueous 50 mM sodium acetate buffer at pH 5.9 and 293 K. The reaction was monitored spectrophotometrically at 320 nm but the second-order rate constant for the release of *p*-nitrophenol was not determined due to precipitation in the cuvette.

LA-42 was reacted with a thirteenfold excess of the *p*-nitrophenyl ester of *N*-methyl nicotinic acid in aqueous 50 mM sodium acetate buffer at pH 5.9. The amount of ester was calculated by comparing the pseudo-first order rate constants for the peptide catalysed reaction and the background reaction. In cases where the peptide is available only in small amounts, the pesudo-first order rate constant for the peptide is approximated from the concentration of peptide and the second order rate constant of 4-methylimidazol multiplied by five. The products were analysed by ES-MS, and the transformed mass spectrum showed one peak with a molecular weight of 4509.35 D, corresponding to the mass of LA-42 (4390.10) plus the mass of *N*-methylnicotinic acid (138.20) minus the mass of water (18.02). The incorporation of the nicotinoyl residue was successful and no diacylated product was found. A second peak, corresponding to the unreacted LA-42 was also present in the spectrum. The relative intensities indicated approximately 60-75 % conversion of the peptide and it was therefore reacted with a sixtyfold excess of the ester. The excess of substrate was estimated from the concentration of the unreacted LA-42 (approx. 0.24 mM) and the ratio of the calculated pseudo-first order rate constants (approx. 50). The modified LA-42, LA-42NAD, was purified by size-exclusion chromatography on a Sephadex G-25 fine column using 0.1% TFA as the eluent. LA-42NAD was obtained in a yield of 70 % based on the amount of peptide used as starting material. The NMR spectroscopic investigations of

LA-42NAD showed no significant change in structure upon functionalisation, and the difference between the CD spectra of LA-42NAD and LA-42 was within the experimental error limits.

10.2. Reduction of RA-42NAD and LA-42NAD into RA-42NADH and LA-42NADH

RA-42NAD was treated with a tenfold excess of both sodium dithionite and sodium carbonate in 50 mM sodium phosphate buffer under a nitrogen atmosphere at 293 K and pH 7. The reduction was monitored spectrophotometrically at 340 nm. 1-Methylnicotinamide and N_{ε}-(*N*-methylnicotinoyl)-L-ornithine were also reduced under similar conditions for comparisons of reduction rates and stabilities of the reduced species with those of RA-42NAD. The reaction rate of the reaction was difficult to determine, as precipitation of the *red* form occurred. Comparison of the stabilities indicated that the reduced form of LA-42NAD is approximately three times as stable as free 1-methyl-1,4-dihydronicotinamide. The ornithine derivative was also reduced, but the results were not reproducible.

LA-42NAD was treated with a sixteenfold excess of sodium carbonate and sodium dithionite (1:1) in 5 vol% TFE in 50 mM sodium phosphate buffer in 90 % H_2O:10 % D_2O (v/v) under nitrogen atmosphere at pH 7. The reduction was followed by ^1H NMR spectroscopy, where the disappearance of the pyridine resonances at 8.10 and 9.19 ppm was observed, in agreement with the UV spectroscopic results. A small amount of *N*-methyl nicotinic acid was also present as an impurity, but was not reduced by the sodium dithionite.

10.3. Reduction of TFAB by LA-42NADH

A tenfold excess of α,α,α-trifluoroacetyl benzene (TFAB) was added to the reaction mixture described above, and the reduction was monitored by ^{19}F NMR. The substrate appears as a singlet at 1920 ppm, and the reduction product as a doublet at -440 ppm. relative to TFE. The reduction of TFAB was insignificant compared to that accomplished by reported model systems[11] and comparable to the background reduction induced by sodium dithionite[50].

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[50] a) de Vries, J. G.; van Bergen, T. J.; Kellogg, R. M. *Synthesis*, **1977**, 246.
b) de Vries, J. G.; Kellogg, R. M. *J. Org. Chem.*, **1980**, *45*, 4126.

10.4. NAD^+ incorporation into NP-42

NP-42 was reacted with a thirteen fold excess of the *p*-nitrophenyl ester of *N*-methyl nicotinic acid in aqueous 50 mM sodium acetate buffer at 293 K and pH 5.9. The products were analysed by ES-MS, and the transformed mass spectrum showed one peak with a molecular weight of 4635.3 D, corresponding to the mass of NP-42 (4515.73) plus the mass of *N*-methylnicotinic acid (138.20) minus water (18.02). No diacylated product was found. A second peak, corresponding to the unreacted NP-42 was also observed in the spectrum.

10.5. Incorporation of pyridoxal phosphate into PP-42.

PP-42 was reacted with PPal in 50 mM Bis-Tris buffer at pH 5.9, and the formation of the aldimine was monitored by UV spectroscopy. When the aldimine absorbance was much larger than that of the free pyridoxal, a sample was analysed by ES-MS. The transformed mass spectrume showed a peak at 4762,98 D, which corresponds to the mass of PP-42 (4533.71) plus the mass of pyridoxal phosphate (247.1) less the mass of water (18.02). A peak representing free PP-42 was also present in the mass spectrum, and the relative intensities indicated that approximately 25% of the peptide had been modified.

10.6. Synthesis of the two-armed pyridoxine derivative

To incorporate the pyridoxine derivative in a cyclic hexapeptide, a method of coupling the pyridoxine derivative to amino acid moieties was needed. Several methods of asymmetric synthesis of α-amino acids were tried.

The oxazolidinone compound was prepared in modest yields, approximately 30 %. Attempts were made to utilise N-protecting groups other than Cbz, usinc Alloc-Cl and *t*-Boc-Cl. The *t*-Boc derivative was formed in small amounts, but could not be isolated. The Alloc derivative was not formed at all. The alkylation sequence failed to produce any significant amount of product. After several attempts, a 20 % yield of the *n*-pentyl alkylated oxazolidinone was isolated.

Alkylation of the *bis*-lactim ether was performed according to the literature[ref] and the yield was within the range reported in the literature. The work-up methods were not suitable to the functional groups required of the project.

The alkylations of the pseudoephedrine glycinamide proceeded according to the literature, but the alaninamide alkylations failed to produce any significant amount of product.

10.7. Discussion

The amino acid syntheses showed that preparing a pyridoxine derivative, functionalised with an amino acid moiety at the end of each of two elongated side chains, would be time-consuming as well as difficult, although the synthesis of the pyridoxine derivative itself was performed. The project was abandoned in favour of the post-synthetic modification of polypeptides.

NAD^+/NADH model systems reported in the literature are mainly nicotinamides coupled to some form of organic structure that are introduced to induce stereoselective hydride transfer, rate enhancements, and substrate recognition. High degrees of stereoselectivity have been achieved in many cases by attaching the nicotinamide moiety to chiral auxiliaries[51]. High reaction rates have also been achieved[15] in some cases, but not substrate recognition which has rarely been attempted. The introduction of organic template structures requires complex asymmetric syntheses in most cases, and the model systems are therefore often difficult to prepare.

The NAD^+ form of the cofactor is soluble in water, whereas the NADH form is less so. When an organic structure is attached to the cofactor its solubility in water is further decreased, and the reactions of these model systems are mainly studied in solvents like CH_3CN, DCM, and DMSO. NADH-mediated hydride transfer reactions are slower in organic solvents than in water[11].

The use of folded polypeptides as template structures have many advantages. They are soluble in aqueous solutions, a likely requirement for the creation of NAD^+/NADH model systems capable of turnover. The solution structures of folded polypeptides can be solved by NMR and CD spectroscopy and polypeptides with well-defined supersecondary structures can be used to engineer reactive sites for binding and transition-state stabilisation by minor modifications of the amino acid sequence. The reactive sites of these peptides can the be systematically changed in order to optimise binding or introduce new functionalities.

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[51] Burgess, V. A.; Davies, S. G.; Skerlj, R. T. *Tetrahedron: Asymmetry*, **1991**, *2*, 299.

We have now shown the potential of polypeptide-based enzyme mimics through the first reported successful site-selective self-functionalisation of a folded polypeptide with a NAD^+ analogue and its subsequent reduction. Both the *red* and *ox* forms of the peptide-bound cofactor are soluble in water. The reduced form of the peptide-bound cofactor was also approximately three times as lomg-lived as the free 1-methyl-1,4-dihydro-nicotinamide. Furthermore we have designed a peptide-based dehydrogenase mimic, NP-42, with a more complex reactive site where the NAD^+ cofactor was also successfully incorporated. Further studies on the structure and reactivity are underway. Also, a transaminase mimic, PP-42, was designed and synthesised. The reactive site of PP-42 was designed to bind pyridoxal phosphate (PPal) both covalently and non-covalently. Preliminary results show the covalent incorporation of PPal into PP-42, and further studies of this system are also being carried out.

The design and study of enzyme mimics is a discipline that is both challenging and rewarding. The use of post-synthetic modifications of polypeptides that form stable structural motifs is a novel approach with great potential. The classic approach has been to modify peptides unselectively, by e.g. the alkylation or acylation of lysine residues, which results in di/poly-acylation of all available primary amines. The relative ease with which the compounds are prepared for incorporation, i.e. the esterification with *p*-nitrophenol, makes this method extremely convenient and it also allows the incorporation of molecules that are not able to withstand the conditions of peptide synthesis.

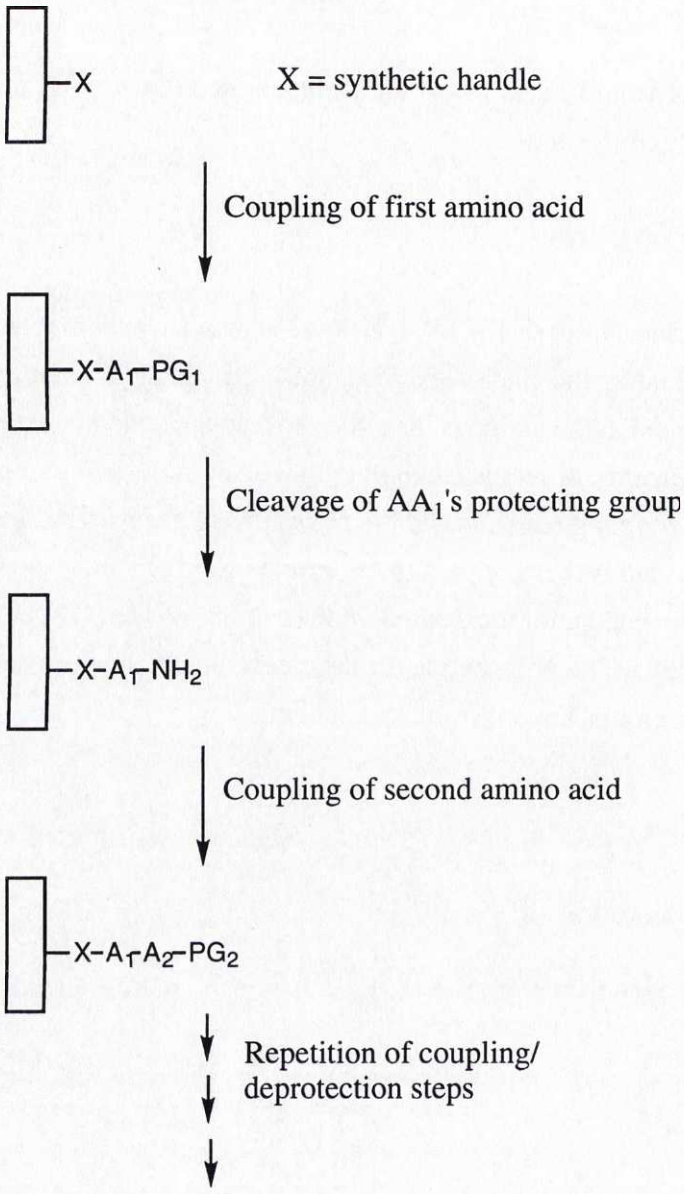
The advantage is not only the site-selectivity, but also the fact that the amide bond is likely to withstand most conditions that the peptide itself can. Furthermore, the process can be applied to almost any substrate molecule that has or can have a carboxylic acid functionality which can be esterified. The protocol also allows for design of a reactive site in close proximity to the self-functionalisation site, for use of the incorporated moiety in catalysis. This gives the method a wide utility and applicability.

11. Methods

Presented here is a short overview of the methods used but a full account is, however, beyond the scope of this thesis.

11.1. Peptide synthesis

Solid phase peptide synthesis (SPPS) has now become a standard technique in peptide chemistry. The first amino acid of the sequence is coupled to a polymer resin via the carboxyl function, and the amino acids to be coupled are added one by one together with coupling reagents. After each coupling the rersin is washed to remove excess amino acid and coupling reagents and the *N*-protecting group is removed. The next *N*-protected amino acid is then coupled to the amino group of the previous amino acid. The process is repeated until the desired number of amino acids have been coupled to the growing peptide. The polypeptide is then cleaved from the polymer and the side chain protection groups are removed, see Scheme 2.



Scheme 3. Outline of the general SPPS protocol.

In Scheme 3 has been presented an outline of the peptide synthesis, but the improvements and variations are many. Commonly used *N*-protection groups are *t*-Boc (*tert*-butyloxycarbonyl)and Fmoc (9-fluorenylmethyloxycarbonyl). The main difference between *t*-Boc and Fmoc chemistry is that *t*-Boc is removed by strong acid, usually TFA, and Fmoc is removed by base. The peptide-polymer linkage must therefore be stable towards strong acids in the case of *t*-Boc chemistry and the peptide has to be cleaved from the resin with the use of liquid HF, which leads to a number of impurities and side reactions. The Fmoc protection groups are removed with piperidine, which allows the use of a peptide-to-polymer bond that is more easily cleaved than in

the *t*-Boc strategy. In the Fmoc protocol the peptide is cleaved from the polymer by TFA, which is less damaging to the peptide.

In this investigation we have used a fully automated Fmoc SPPS protocol and a continuous-flow method.

11.2. HPLC

The mixture left after cleavage of the peptide from the polymer resin contains a lot of impurities like scavengers, remnants of protecting groups, cleavage reaction by-products, and deletion peptides, i.e. peptides that are shorter than the desired peptide due to failure of one or more of the coupling steps of the synthesis, and it is necessary to purify the peptide.

Synthesised peptides are readily purified by reverse-phase HPLC using isopropanol-water mixtures as eluent.

11.3. NMR spectroscopy

In structure determinations of polypeptides there are many powerful tools, but the most versatile is NMR spectroscopy. This technique can be used to study dynamics, dimer-monomer equilibria and three-dimensional structure.

Routine one-dimensional ^{1}H and ^{19}F NMR experiments, ^{1}H NMR NOESY (Nuclear Overhauser SpectroscopY), and TOCSY (Totally Correlated SpectroscopY) experiments have been used. In combination with ES-MS and CD spectroscopy they yield sufficient data for a three-dimensional structure to be determined. The NOESY experiment gives vital information about e.g. the structure of the hydrophobic core, whereas the TOCSY spectrum gives information of the different amino acid residues and their chemical shifts.

11.4. CD spectroscopy

CD spectroscopy has been used only for comparisons ofm the mean molar ellipticities of the synthesisied or modified peptides and of RA-42 or LA-42.

11.5. Electrospray mass spectroscopy

Electrospray mass spectroscopy (ES-MS) is an excellent method for verifying the structure of a peptide. A solution containing the substance of interest is injected into the spectrometer through a capillary frit, where it is subjected to a high electric field, in the range of 10-20 kV. On the frit surface, charged micro droplets are formed and the solvent is removed by a counter current of inert gas. The ions, which now carry multiple charges, are accelerated in a magnetic field towards the mass analyser. The ions are then analysed, and their mass-to-charge ratio determined. The ES-MS spectrum shows series of peaks with the same m/z ratios, but carrying a different number of charges. These peaks are analysed after calibration and then transformed to give the molecular mass of the molecule.

12. Experimental

12.1. General

All glassware was washed thoroughly and oven-dried overnight at 150 °C or for 72 h at 55 °C. All air- and moisture-sensitive reactions were carried out in dry glassware under a positive pressure atmosphere of dry nitrogen gas. All air- and moisture-sensitive substances were transferred using gas-tight hypodermic syringes and septa. All solvents and reagents were dried and purified according to standard methods[52] when necessary, and otherwise used at commercial purity. Peptide synthesis was performed on a PerSeptive Biosystems Pioneer continuous-flow peptide synthesiser. All peptide synthesis chemicals and solvents were of the appropriate purity and were used at commercial purity. All buffer solutions were prepared from double-distilled water which was then filtered through a Millipore microfilter. All ^{1}H and ^{19}F NMR spectra were recorded on a Varian Unity 500 FT-NMR spectrometer (9.395 T), except the two-dimensional experiments, which were performed on a Varian Unity 500 FT-NMR (11.744 T) spectrometer. Melting points were determined on a Reichert hot-stage microscope and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using the sodium D-line. HRMS was performed on a VG ZabSpec FD High resolution mass spectrometer. Flash chromatography (FC) was performed on silica gel S (230-400 mesh, 0.032-0.063 mm). Kinetic UV spectroscopy were performed on a Varian Cary 1 Bio UV-visible or a Varian Cary 4 spectrophotometer. CD spectra were recorded on a JASCO J-270 spectropolarimeter in the range from 300 to 190 nm. The instrument was calibrated with d-(+)-camphor sulphonic acid.

12.2. Peptide synthesis and purification

Synthesis of NP-42

NP-42 was synthesised on Fmoc-Gly-PEG-PS resin (500 mg, 0.200 mmol/g substitution) with Fmoc-Gly attached. The entire synthesis was performed on a 0.100 millimolar scale. Fmoc chemistry was used throughout the entire synthesis. The peptide-containing resin was washed with DCM and dried *in vacuo*.The dry peptide was cleaved from the polymer with TFA (4.5 mL) using thioanisol (0.10 mL) and ethanedithiol (0.15 mL) as scavengers. The gel was then filtered, separating the peptide from the polymer, and washed with DCM. The free peptide was then precipitated with

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[52] Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*, 3rd ed. **1988**, Pergamon Press.

diethyl ether, centrifuged, and the supernatant ether removed. The crude peptide was then dissolved in water and lyophilised.

Synthesis of PP-42

PP-42 was synthesised in the same manner as NP-42.

Purification of NP-42

NP-42 was purified on a Varian Star HPLC system, using a preparative-scale C8 column. The solvent system used was 40 % isopropyl alcohol in a 0.1 % aqueous solution of TFA for the first run, and 38 % isoporopyl alcohol in in a 0.1 % aqueous solution of TFA for the second and final run. The collected fractions containing the peptide was lyophilised to yield the pure peptide.

ES-MS: 4515.729 (calc: 4516.09 $gmol^{-1}$)

Purification of PP-42

PP-42 was purified on a Varian Star HPLC system, using a preparative-scale C8 column. The solvent system used was 40 % isopropyl alcohol in a 0.1 % aqueous solution of TFA. The collected fractions containing the peptide was lyophilised to yield the pure peptide.

ES-MS: 4533.71 (calc: 4532.13 $gmol^{-1}$)

12.3. Syntheses

NAD^+ analogues and model systems

Nicotinic acid *p*-nitrophenyl ester (1)

This substance was prepared according to methods reported in the literature[41]. Nicotinic acid (0.522 g, 4.06 mmol), DCC (0.965 g, 4.68 mmol), and *p*-nitrophenol (0.650 g, 4.67 mmol) were dissolved in DCM (35 mL) and stirred at ambient temperature. 4-Pyrrolidinopyridine (63 mg, 0.4 mmol) in DCM (4 mL) was then added dropwise, causing the solution to turn yellow. The reaction mixture was stirred for six days at ambient temperature, after which it was washed with dilute HCl (aq, 1.1 M, 4 mL) and filtered to remove precipitated DCU. The filtrate was washed with HCl (30 mL, 0.4 M)

and H_2O (50 mL), dried (Na_2SO_4), and the solvent removed in vacuo to yield the crude product. After recrystallisation from ethanol colourless needles were recovered in 92% yield (0.912 g).

Mp: 171 °C

^{1}H NMR (400 Mhz, $CDCl_3$) δ 9.41 (s, 1H), 8.91 (d, 1H), 8.47 (d, 2H), 8.36 (d, 2H), 7.51 (m, 1H), 7.45 (dd, 2H)

N-Methylnicotinic acid *p*-nitrophenyl ester (2)

This synthesis was prepared according to methods found in the literature[53]. *p*-Nitrophenyl nicotinate (0.912 g, 3.74 mmol) was dissolved in DMF (5 mL), and methyliodide (0.798 g, 5.62 mmol) was added. The reaction mixture was stirred in darkness for six days at ambient temperature. The solution turned red, probably due to oxidation of the iodide ion. The product was precipitated by the addition of diethyl ether (200 mL). The yield was almost quantitative.

^{1}H NMR (400 Mhz, DMSO) δ 9.81 (m, 1H), 9.26 (d, 1H) 9.18 (d, 1H), 8.44 (d, 2H), 8.35 (t, 1H), 7.71 (d, 2H)

FAB-MS: 259 $gmol^{-1}$ (calculated: 259.072)

N_ε-(*N*-methylnicotinoyl)-L-ornithine iodide (3)

This substance was prepared using methods reported in the literature[54]. L-Ornithine hydrochloride (0.40 g, 2.3 mmol) was dissolved in boiling water (50 mL. Copper carbonate (5.1 g) was then added, rendering the pH at approximately 6. Another portion of copper carbonate (1.2 g) was then added, raising the pH to ~7.5. The undissolved residues were filtered off from the now azure blue solution. A solution of $NaHCO_3$ (10 mL, 1.3 mmol) was then added, followed by *p*-nitrophenyl-*N*-methyl nicotinate (1.43 g, 3.7 mmol). After three min. a yellowish precipitation occurred, and the solution turned green. After 26 hours, the solution was acidified to pH 5 with HCl (1.2 M), causing CO_2(g) to be released. Diethyl ether extraction (4 x 60 mL) of *p*-nitrophenol gave yellow ethereal phases, and the aqueous phase regained its azure blue color. The aqueous solution was then subjected to H_2S(g) for 30 min., causing the solution to lose its colour while a brown-black precipitate of copper sulphide was formed. The The precipitate was filtered off and water was removed from the filtrate under reduced pressure. Recrystallisation of the crude product from ethanol failed.

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[53] Murakami, Y.; Aoyama, Y.; Kikuchi, J.; Nishida, K. *J. Chem. Soc.*, **1982**, *104*, 5189.

Water was then added to the crude product, and after acidification the solution was stirred with ion exchange resin (2.04 g, Dowex 50W-X8 (H)). The mixture was left for 3-4 hours, after which the ion exchanger was packed in a column, and treated with ammonium hydroxide to release the product. Evaporation in vacuo gave the product as a crystalline substance.

Mp: (lit.)

^{1}H NMR (400 MHz, D_2O) δ 9.1 (s, 1H), 8.8 (dd, 2H), 8.0 (t, 1H), 4.4 (s, 3H), 3.75 (m, 1H), 1.8 (m, 6H)

Pyridoxal analogues

3-*O*-α^4-isopropylidenepyridoxine (4)

This substance was prepared according to the literature[43].

3-*O*-α^4-isopropylideneisopyridoxal (5)

This substance was prepared according to the literature[55].

3-*O*-α^4-isopropylidene-α^5-pyridoxylideneacetic acid (6)

This substance was prepared according to the literature[42].

3-*O*-α^4-isopropylidene-α^5-pyridoxylacetic acid (7)

This substance was prepared according to the literature[42].

α^5-pyridoxylacetic acid (8)

This substance was prepared according to the literature[42].

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[54] Sharp, J.; Goseny, I.; Rowley, A. *Practical Organic Chemistry*, 1st ed., Chapman and Hall, Cornwall, **1989**.

[55] Brooks, Jr., H. G.; Laakso, J. W.; Metzler, D. E. *J. Heterocycl. Chem.* **1966**, *3*, 126.

α^5-pyridoxalylacetic acid (9)

This substance has been prepared but not analysed.

α^5-pyridoxalylacetic acid *p*-nitrophenol ester (10)

This substance has not yet been prepared.

Syntheses of the cyclohexapeptide project

The syntheses described below start from substance **7**, 3-*O*-α^4-isopropylidene-α^5-pyridoxylacetic acid, described above.

2-(3-*O*-α^4-isopropylidenepyridox-5-yl)-ethanol (12)

This substance was prepared according to the literature[42].

3-*O*-α^4-Isopropylidenepyridox-5-yl-ethyl-*O*-*tert*-butyldiphenylsilyl ether (13)

This synthesis was based upon a general method reported in the literature[44]. (The reason for using TBDPS instead of TBDMS was that TBDMS was not able to withstand the TFAA-mediated rearrangment below.) To a stirred solution of **12** (3.66 g, 15.4 mmol) in dry CH_2Cl_2 (150 mL) was added *tert*-butyl diphenyl silyl chloride (4.00 mL, 1 eq.), triethylamine (1 eq), and a catalytic amount of DMAP. The reaction mixture was stirred under nitrogen at ambient temperature for 12-14 h. The solvent was then removed *in vacuo* and the residue taken up in ether. The ethereal solution was filtered twice to remove triethyl amine hydrochloride, washed once with water, and dried (Na_2SO_4). The solvent was evaporated to yield 7.25 g of the pure product as an oil. Yield: 99%.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (s, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 4.79 (s, 2H), 3.69 (t, 2H), 2.51 (t, 2H), 2.39 (s, 3H), 1.75 (t, 2H), 1.54 (s, 6H), 1.05 (s, 9H)

3-*O*-α^4-Isopropylidenepyridox-5-yl-ethyl-*O*-*tert*-butyldiphenylsilyl ether-1-oxide (14)

This substance was prepared using a method from the literature[42]. To a stirred solution of **13** (5.07 g, 10.6 mmol) in dry $CHCl_3$ (50 mL) was added dry MCPBA (1.1 eq) dissolved in $CHCl_3$ (25 mL) at 0 °C. The reaction mixture was then stirred at r.t. for 2 hours (until TLC indicated total consumption of reagents). The reaction mixture was then washed with $NaHSO_3$ (5%), $NaHCO_3$ (5%), and water. The solvent was removed *in vacuo*, and the crude oily product used without further purification in the next step.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (s, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 4.74 (s, 2H), 3.69 (t, 2H), 2.51 (t, 2H), 2.39 (s, 3H), 1.75 (t, 2H), 1.54 (s, 6H), 1.05 (s, 9H)

3-*O*-α^4-Isopropylidene-2'-hydroxy-norpyridox-5-yl-ethyl-*O*-*tert*-butyldiphenyl silyl ether (15)

This substance was prepared using a method from the literature[42]. The crude *N*-oxide **14** from the former step was dissolved in CH_2Cl_2 (35 mL) and the solution cooled to 0 °C. To the stirred solution TFAA (1 mL) was then added by syringe. Another portion of TFAA (2.5 mL) was added 5 minutes later. The reaction mixture was allowed to warm to room temperature and then stirred at r.t. for 8-14 h. MeOH (15 mL) was added and the reaction mixture stirred for 30 min, and then washed with Na_2CO_3 (20%) and water. The solution was dried (Na_2SO_4) and the solvent evaporated to yield 3.71 g of crystalline **15**. Yield 90 % over both steps.

When TBDMS was used as a protecting group, partial deprotection of the 5'-ethoxy group was evidenced in ^{1}H NMR, approximately 50-60 % deprotection.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (s, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 4.86 (s, 2H), 4.74 (s, 2H), 3.68 (t, 2H), 2.51 (t, 2H), 1.75 (t, 2H), 1.54 (s, 6H), 1.05 (s, 9H).

3-*O*-α^4-Isopropylidene-2-formyl-norpyridox-5-yl-ethyl-*O*-*tert*-butyldiphenyl silyl ether (16)

This substance was prepared using a method from the literature[42]. The alcohol **15** (3.70 g, 9.0 mmol) was dissolved in CH_2Cl_2 (200 mL) and MnO_2 (act.~90 %, ~7 eq, 15 g) was added. The heterogenous mixture was heated to reflux under nitrogen for 6 h (tlc monitoring). The reaction mixture was the diluted with CH_2Cl_2 (50 mL) and treated

with Celite. Celite and MnO_2 were removed by suction filtration. After evaporation of the solvent *in vacuo*, the crude aldehyde was used without further purification in the next step.

^{1}H NMR (400 MHz, $CDCl_3$) δ 10.24 (s, 1H), 8.18 (s, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 4.74 (s, 2H), 3.68 (t, 2H), 2.51 (t, 2H), 1.75 (t, 2H), 1.54 (s, 6H), 1.05 (s, 9H).

3-*O*-α^4-Isopropylidene-2'-carboxyvinyl-norpyridox-5-yl-ethyl-*O*-*tert*-butyldiphenyl silyl ether (17)

This substance was prepared using a method from the literature[42]. The crude aldehyde **16** was dissolved in pyridine (25 mL), and piperidine (1 mL) and malonic acid (0.94 g, 9.0 mmol) was added. The reaction mixture was heated to 80 °C for 2 h and then stirred at r.t. overnight. Work-up was performed as with **5**. Yield 3.11 g, 64 %.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.95 (d, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 6.95 (d, 1H), 4.74 (s, 2H), 3.68 (t, 2H), 2.51 (t, 2H), 1.75 (t, 2H), 1.54 (s, 6H), 1.05 (s, 9H).

3-*O*-α^4-Isopropylidene-2-(3'-hydroxypropyl)-norpyridox-5-yl-ethyl-*O*-*tert*-butyldiphenyl silyl ether (18)

This substance was prepared using a method from the literature[42]. The acid **17** (1.0 g, 1.9 mmol) was dissolved in dry THF (120 mL) and the solution added dropwise to a suspension of $LiAlH_4$ (0.5 g) in dry THF (80 mL). The reaction mixture was stirred for 1h at r.t., following which the excess hydride was destroyed with ethyl acetate followed by water. After extracting the aqueous phase with ethyl acetate, the combined organic phases were dried (Na_2SO_4) and the sovent removed in vacuo, yielding the product as a yellow oil. Yield: 0.17 g (16 %)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.46 (d, 4H), 7.38 (m, 6H), 4.74 (s, 2H), 3.68 (t, 2H), 3.05 (t, 3H), 2.71 (t, 2H), 2.51 (t, 2H), 1.65-1.75 (m, 4H), 1.54 (s, 6H), 1.05 (s, 9H).

(2*R*, 4*S*)-3-[benzyloxycarbonyl]-4-methyl-2-phenyl-1,3-oxazolidin-5-one (19)

This substance was prepared according to the literature[47].

Alkylation of (2*R*, 4*S*)-3-[benzyloxycarbonyl]-4-methyl-2-phenyl-1,3-oxazolidin-5-one (19)

Thes reactions were performed according to the literature[47].

Alkylation of the *bis*-lactim ether of cyclo-L-Val-L-Ala (21).

This reaction was performed according to the literature[48].

(*R*,*R*)-(-)-Pseudoephedrine glycineamide (22)

This substance was prepared according to the literature[49].

(*R*,*R*)-(-)-Pseudoephedrine alanineamide (23)

This substance was prepared using methods reported in the literature[49]. *N-t*-Boc-Alanine (500 mg, 2.64 mmol) and Et_3N (442 µL, 1.2 eq.) was dissolved in dry DCM (10 mL) at 0 °C and trimethylacetyl chloride (0.39 mL, 2.64 mmol) was added dropwise under vigorous stirring. After 30 min. a second portion of Et_3N (442 µL, 1.2 eq.) and (*R*,*R*)-(-)-pseudoephedrine (437 mg, 2.64 mmol) were added sequentially. The reaction mixture was stirred for 45 minutes and the volatiles were removed *in vacuo*. The reside was taken up in a methanol-water mixture (10 mL, 1:1) and cooled in an ice bath. Conc. Hcl (4 mL) was added, causing vigorous evolution of white fumes. After stirring for 3 h the methanol was removed *in vacuo* at 23 °C and the aqueous concentrate cooled in an ice bath while the pH was adjusted to to 14, using 50 % NaOH. The aqueous solution was extracted with DCM (4 x 10 mL) and the combined extracts were dried (Na_2CO_3). The solution was filtered through Celite and the solvent removed to give white crystals that were recrystallised twice from toluene. The yield was 82.2 g

Mp: 93.2-96.4 °C

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.3–7.4 (m, 5H), 4.65 (d, 1H), 4.60 (d, 1H), 4.0-4.1 (m, 0.5H), 3.8 (d, 0.5H), 3.7 (d, 1H), 2.95 (s, 1.5H), 2.80 (s, 1.5H), 1.7 (s(broad), 3H), 1.4 (d, 1.5H), 1.09 (d, 1.5H), 1.05 (d, 1.5H), 0.98 (d, 1.5H).

Alkylation of (*R*,*R*)-(-)-Pseudoephedrine glycineamide (22)

This procedure was performed according to the literature[49].

Alkylation of (*R*,*R*)-(-)-Pseudoephedrine alanineamide (23)

This procedure was performed according to methods reported in the literature[49]. The attempted alkylations of **23** was perfomed exactly as with **22**, but no product could be isolated.

12.4. Cofactor model incorporation

General procedure for cofactor model incorporation

The peptide (~4 mg)was dissolved in 400 mL of Bis-Tris buffer at pH 5.9. The substrate was then added, and the mixture stirred on a shaking plate and left overnight. The salts in the mixture was removed by passing through a column of Sephadex GD-50 size-exclusion gel. The peptide was then lyophilised and purified by HPLC.

12.5. Kinetics and reaction studies

General procedures

The incorporation kinetics were all run in 50 mM sodium acetate buffer, at pH 4.96. The formation of p-nitrophenolate ion was monitored over time by UV spectrometer at 320 nm. The kinetics were then analysed using Igor Pro.

The kinetics studies on the reduction of the NAD^+ analogues were all run in NaH_2PO_4 buffer (50 mM) at pH 7.0. The NAD^+ analogue was dissolved in buffer, which was then deoxygenated by bubbling through nitrogen gas. The sodium dithionite and sodium carbonate mixture was then dissolved in buffer and the solution deoxygenated, following which the solutions were mixed, and the absorbance at 340 nm was studied over time with the UV spectrometer. The kinetics were then analysed using Igor Pro.

Background hydrolysis reaction of *N*-methylnicotininc acid *p*-nitrophenyl ester

Sodium acetate buffer (1.2 mL, 50 mM, pH 4.96) was transferred to an UV kuvette and tempered for 30 min. The NAD^+ analogue (1.1 mg, ~2.8 µmol) was dissolved in water (1.0 mL) and from this solution, 80 µL (0.19 mM) was added to the kuvette. The formation of *p*-nitrophenolate was monitored for several half-lives.

Kinetics measurement of NAD^+ analogue incorporation into RA-42

RA-42 (1.2 mg, 27.4 µmol judging by an appr. 75 % peptide content) was dissolved in sodium acetate (1.2 mL, 50 mM, pH 4.96) and the solution was placed in an UV kuvette and tempered for 30 min. Approximately 1/5 of a molar equivalent of the NAD^+ analogue was added to the kuvette, and the formation of *p*-nitrophenolate was monitored for several half-lives. The product was purified and analysed by ES-MS, in order to determine the extent of incorporation.

Kinetics measurement of the reduction of 1-methylnicotinamide by sodium dithionite and sodium carbonate

1-Methylnicotinamide (0.21 mg, 1.2 µmol) was dissolved in sodium phosphate buffer (400 µL, 50 mM, pH 7.02) to give a 3 mM solution, and the solution deoxygenated by passing nitrogen gas through it for several minutes. Sodium dithionite (1.04 mg, 5 eq.) and sodium carbonate (0.64 mg, 5 eq) was then added under nitrogen flow, following which the formation of the 1.4-dihydronicotinamide was monitored at 340 nm for several half-lives.

12.6. List of chemicals used in this investigation

Chemical name	Abbr.	Grade	Supplier
Acetone		99 %	Riedel-de-Haen
Acetone dimethyl acetal		98 %	Aldrich
L-Alanine	Ala	99 %	Aldrich
Ammonium fluoride	NH_4F	99 %	Riedel-de-Haen
Benzaldehyde	PhCHO	99 %	Aldrich
Benzyl chloroformate	CbzCl	95 %	Aldrich

Butyllithium	BuLi	1.6 M	Aldrich
Benzyl chloride	BnCl	98 %	Fluka
tert-Butyl dimethyl silylchloride	TBDMSCl	98 %	Aldrich
tert-Butyl diphenyl silylchloride	TBDPSCl	98 %	Aldrich
Chloroform	$CHCl_3$	99.6 %	Prolabo
3-Chloroperbenzoic acid	MCPBA	50-60 %	Aldrich
Copper carbonate		99 %	MCIB
Cyclohexene		99.5 &	Riedel-de-Haen
Dichloromethane	DCM	99 %	J.T. Baker
Dicyclohexyl carbodiimide	DCC	99 %	Lancaster
Diethyl ether	Et_2O	99.5 %	Riedel-de-Haen
Diethylamine	Et_2NH	99 %	Riedel-de-Haen
Diisopropyl ether	$(i\text{-}Pr)_2O$	99 %	Aldrich
4-Dimethylamino pyridine	DMAP	99 %	Aldrich
Dimethylformamide	DMF	99 %	Aldrich
Ethanedithiol		99.5 %	Lancaster
Ethyl acetate	EtOAc	99.5 %	Riedel-de-Haen
Fmoc-Ala-OH		99.9%	Alexis
Fmoc-Arg(Pmc)-OH		99.9%	Alexis
Fmoc-Asn(Trt)-OH		99.9%	Alexis
Fmoc-Asp(OtBu)-OH		99.9%	Alexis
Fmoc-Gln(Trt)-OH		99.9%	Alexis
Fmoc-Glu(OtBu)-OH		99.9%	Alexis
Fmoc-Gly-OH		99.9%	Alexis
Fmoc-His(Boc)-OH		99.9%	Alexis
Fmoc-Ile-OH		99.9%	Alexis
Fmoc-Leu-OH		99.9%	Alexis
Fmoc-Lys(Boc)-OH		99.9%	Alexis
Fmoc-Nle-OH		99.9%	Alexis
Fmoc-Phe-OH		99.9%	Alexis
Fmoc-Pro-OH		99.9%	Alexis
Fmoc-Val-OH		99.9%	Alexis
Hexane		95 %	May & Baker
Hydrogen peroxide	H_2O_2	30 %	Riedel-de-Haen
Isopropyl alcohol	IPA	99.5 %	Prolabo
Lithium aluminum hydride	LAH	99 %	Fluka
Lithium diisopropylamide	LDA	2 M (hexane)	Aldrich
Lithium bis(trimethylsilyl)amide	LHMDS	1 M (THF)	Aldrich

Malonic acid		98 %	Lancaster
Manganese (IV) oxide	MnO_2	90 % act.	Fluka
Methanol	MeOH	99.8 %	Merck
Methyl iodide	MeI	99 %	Fluka
Methyllithium	MeLi	1.4 M	Aldrich
1-Methyl nicotinamide	Me-NAD	99 %	Lancaster
Nicotinic acid		99.5 %	Fluka
Nicotinoyl chloride hydrochloride		97 %	Aldrich
p-Nitrophenol	pNP	98 %	Lancaster
L-Ornithine hydrochloride		95 %	EGA-Chemie
n-Pentane		99 %	Riedel-de-Haen
Piperidine		98 %	XXX???
Pyridine	Pyr	99 %	Riedel-de-Haen
Pyridoxal phosphate	PPal	98 %	Aldrich
Pyridoxine hydrochloride	Pol·HCl	98 %	Aldrich
4-Pyrrolidinopyridine	Prp	98 %	Lancaster
Pyruvic acid, sodium salt	Prv-Na	99 %	Sigma
Sodium		50 % in oil	Aldrich
Sodium carbonate		99 %	Fluka
Sodium dithionite		97 %	Riedel-de-Haen
Sodium hydride	NaH	55-65 %	Fluka
Sodium phospate, monobasic	NaH_2PO_4	98 %	Aldrich
Sodium sulphate	Na_2SO_4	99 %	Riedel-de-Haen
Sulphuric acid	H_2SO_4	96 %	Labassco
TBTU		99 %	Alexis
Tetrahydrofuran	THF	99.8 %	Riedel-de-Haen
Thioanisol		99.5 %	Lancaster
Thionyl chloride	$SOCl_2$	98 %	Fluka
Triethylamine	Et_3N	98 %	Aldrich
Triethyl phosphono acetate		97 %	Fluka
Trifluoroacetic anhydride	TFAA	98 %	Fluka
α,α,α-trifluoroacetophenone	TFAB	99 %	Lancaster
2,2,2-Trifluoroethanol	TFE	99 %	Lancaster

På grund av upphovsrättsliga skäl kan vissa ingående delarbeten ej publiceras här.
För en fullständig lista av ingående delarbeten, se avhandlingens början.

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For a complete list of papers, see the beginning of the dissertation.

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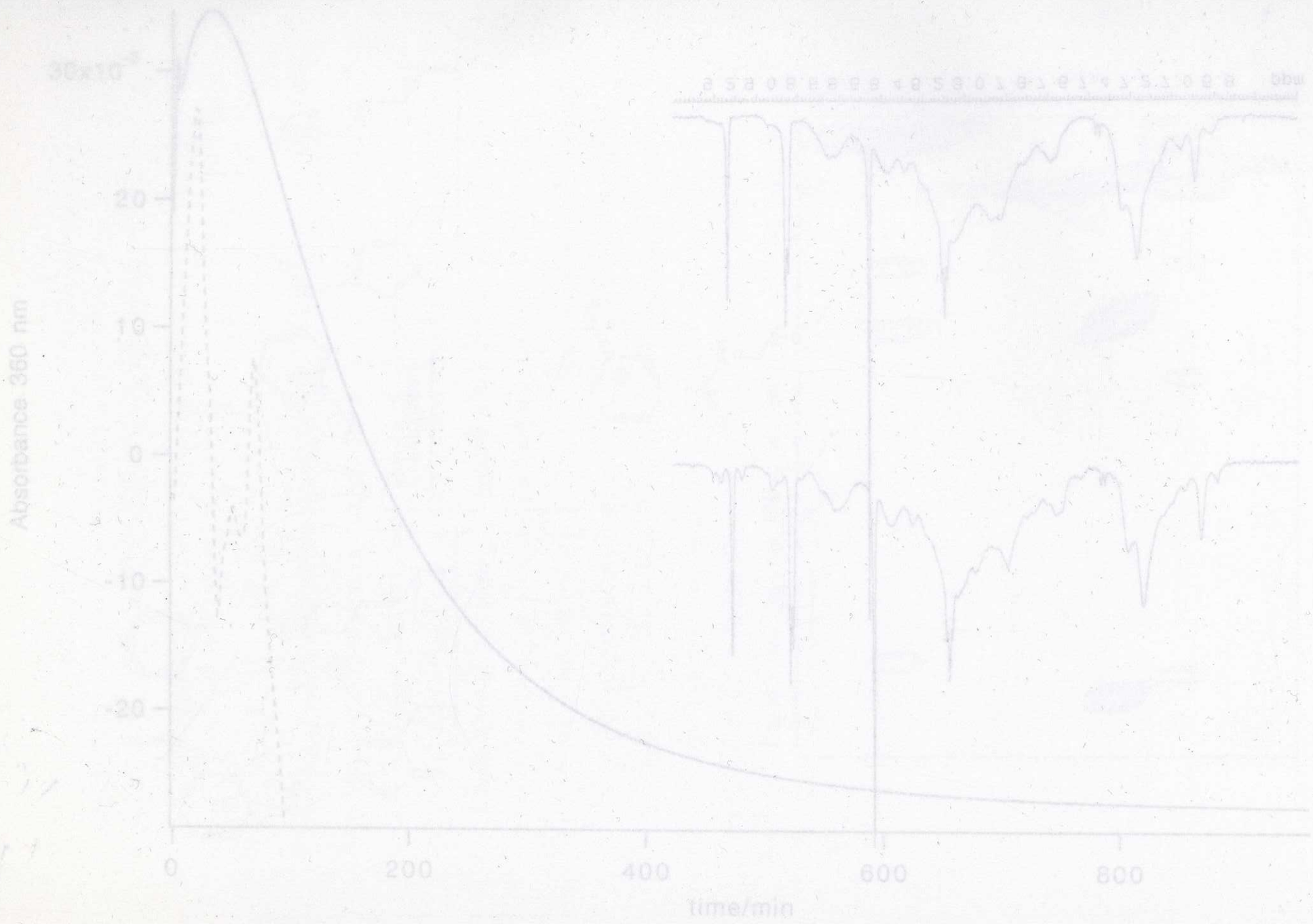
30x10⁻³
20
10
0
-10
-20
Absorbance 360 nm
0
200
400
600
800
time/min