CHRISTIAN HEISS

I. Synthesis and Evaluation of Mechanism Based Inhibitors of Kynureninase.
II. Asymmetric Reductions of Ethynylketones by Secondary Alcohol Dehydrogenase from *Thermoanaerobacter ethanolicus*.

(Under the direction of ROBERT S. PHILLIPS)

In chapter I of this dissertation, the synthesis of some new inhibitors of the pyridoxal phosphate dependent enzyme kynureninase and the assessment of their kinetic parameters is described. Ten different compounds were prepared and their activity as substrates or as inhibitors was tested.

Chapter II presents the enantioselective reduction of ethynylketones and ethynylketoesters by secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (SADH). Ethynylhydroxyesters were obtained in optically pure form and represent useful chiral building blocks. The substrate dependence of enantioselectivity was elucidated and a refined active site model was proposed.

Chapter III describes the effect of the mutation cysteine 295 to alanine on the enantioselectivity of the reduction of ethynylketones by SADH. In this chapter, the temperature dependence of the enantioselectivity of SADH oxidations of secondary alcohols is also presented.

In the appendix, the formation of a benzisoxazole by cyclization of the aminonitrile derived from 2-nitrocinnamaldehyde by Strecker reaction is presented.

INDEX WORDS: Kynureninase, *Pseudomonas fluorescens*, Alcohol dehydrogenase, Thermophilic enzymes, Asymmetric reduction, Ethynylketones, *Thermoanaerobacter ethanolicus*.

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by

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CHRISTIAN HEISS

Approved:

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Date

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Dean of the Graduate School

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Date

DEDICATION

To the Lord Jesus Christ

To my wife Becky, my children, and my parents

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to my research advisor Professor Robert S. Phillips for being a wonderful boss who gave me direction and at the same time appreciated my own thoughts and ideas. Unfailingly, he took time to help me with problems that arose and made every effort to answer my questions. I would also like to thank Professor George Majetich for his excellent teaching and his valuable advice and my other committee members Professors J. Philip Bowen, Allen D. King, and Thomas E. Johnson for their cooperation.

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I would like to thank my father and my mother for their invaluable advice, their continued interest in my education and for their complete support of all of my decisions; I know that, at times, this must have been difficult. My wife Becky and my two daughters Danielle and Clara deserve special thanks for being a wonderful, understanding family to me. By providing a loving and joyful home, they caused my mind and my heart to recuperate whenever they were weighed down by difficulties in my work.

It is clear that with all these acknowledgments I ultimately thank God for bringing the above mentioned people and many more into my life and for watching over me and staying by my side regardless of whether I adhered to his principles or not. Without him I

cannot even draw a single breath but “I can do all things through Christ which strengt h-eneth me.” (Philippians 4:13)

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LIST OF ABBREVIATIONS

ADH	alcohol dehydrogenase
BTMSA	*bis*(trimethylsilyl)acetylene
CBADH	*Clostridium beijerinckii* alcohol dehydrogenase
CoA	coenzyme A
DHP	3,4-dihydro-2*H*-pyran
DIBAH	diisobutylaluminum hydride
DMSO	dimethylsulfoxide
ee	enantiomeric excess
GC	gas chromatography
HLADH	horse liver alcohol dehydrogenase
HPLC	high perfomance liquid chromatography
NAD	nicotinamide adenine dinucleotide
NADH	1,4-dihydronicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide 2'-phosphate
NADPH	1,4-dihydronicotinamide adenine dinucleotide 2'-phosphate
NMDA	*N*-methyl D-aspartate
NMR	nuclear magnetic resonance
PLP	pyridoxal 5'-phosphate
PMP	pyridoxamine
PPTS	pyridinium *p*-toluenesulfonate
SADH	*Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase
TBADH	*Thermoanaerobacter brockii* alcohol dehydrogenase
THP	tetrahydropyranyl

TMS	trimethylsilyl
TRIS	*tris*(hydroxymethyl)aminomethane
YADH	yeast alcohol dehydrogenase

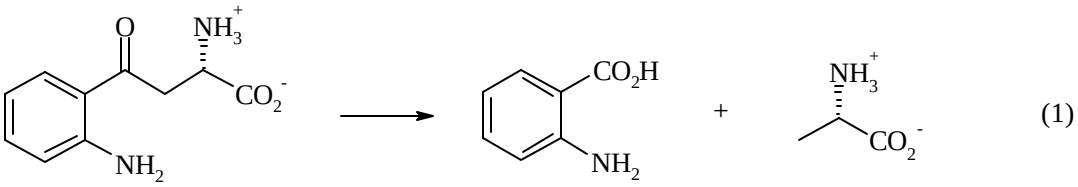
INTRODUCTION AND LITERATURE REVIEW

SECTION 1

The Enzymology of Kynureninase

Biochemical Aspects, Inhibition, and Catalytic Mechanism

Kynureninase [EC 3.7.1.3] is a pyridoxal 5'-phosphate (PLP) dependent enzyme that plays a role in the metabolism of L-tryptophan. Kynureninase occurs in nature as either of two different types, inducible or constitutive kynureninase. Inducible kynureninase is part of the metabolic degradation of L-tryptophan in bacteria and fungi. It also catalyzes the hydrolytic γ,δ-cleavage of L-kynurenine to give anthranilic acid and L-alanine (eq. 1). It is



(1)

called inducible because its expression depends on the presence of L-tryptophan in the growth medium.[1] Inducible kynureninase is inactivated by L-alanine.[2] The inactivation is caused by conversion of PLP to pyridoxamine 5'-phosphate by transamination with alanine which in turn is converted to pyruvic acid (Fig. 1). Added PLP or pyruvate protects the enzyme from this inactivation.

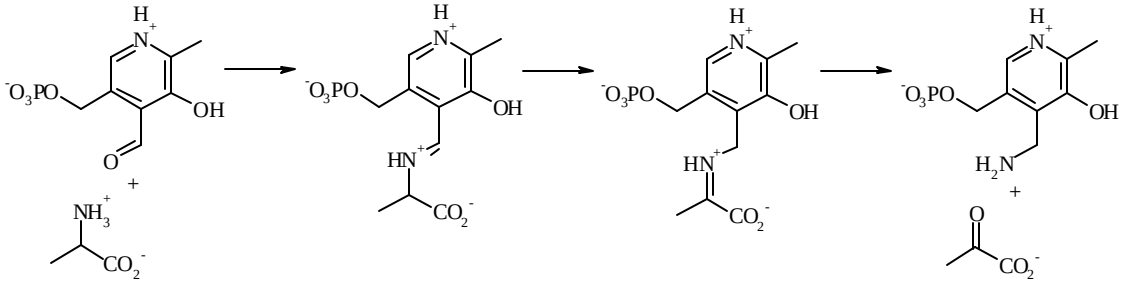


Figure 1. Transamination of PLP and L-alanine by kynureninase

Yeast and mammals possess only constitutive kynureninase whereas both types of kynureninase are present in fungi. Figure 2 summarizes the pathway of L-tryptophan metabolism in which constitutive kynureninase plays a central role. L-Tryptophan is oxidized by tryptophan oxygenase to *N*-formyl-L-kynurenine, which is deformylated by formamidase to give L-kynurenine. L-Kynurenine is then subjected to one of two possible transformations. It is either cyclized to kynurenic acid by the enzyme kynurenine aminotransferase or hydroxylated in the 3-position by kynurenine 3-hydroxylase. The resulting 3-hydroxykynurenine is then cleaved by kynureninase to produce 3-hydroxyanthranilic acid and alanine. 3-Hydroxyanthranilic acid is further converted to quinolinic acid by 3-hydroxyanthranilate oxygenase–catalyzed oxidation and ring-cleavage followed by non-enzymatic isomerization and ring-closure. Quinolinic acid is converted to NAD via the intermediates nicotinic acid and nicotinamide. Constitutive kynureninase is not inactivated by alanine but is inhibited by 3-hydroxyanthranilate.[3] This inhibition is possibly involved in the regulation of the tryptophan-NAD biosynthetic pathway. Quinolinic acid, one of the intermediates in this pathway, is an *N*-methyl-D-aspartate (NMDA) receptor-agonist[4] that acts as a neurotoxin, causing lesions and death of nerve cells in the brain.[5] It has been postulated that a high level of quinolinic acid is responsible for the degeneration of nerve cells in the pathology of a number of neurological disorders such as Huntington's disease,[6] stroke,[7] AIDS-related dementia,[8] and inflammatory brain diseases.[9] Kynurenic acid, on the other hand, is an NMDA receptor-antagonist that counteracts the deleterious effects of quinolinic acid, thereby representing a neuroprotective agent. The dichotomy between quinolinic acid on the one hand and kynurenic acid on the other hand has raised interest in the modification of the kynurenine pathway of NAD biosynthesis from L-tryptophan using suitable enzyme inhibitors.Within these efforts, kynurenine 3-hydroxylase and kynureninase have been the most extensively studied targets for such inhibitors. Blocking of one of these enzymes should have a twofold effect. First, the levels of the neurotoxin quinolinic acid should be lowered, and second, those of its

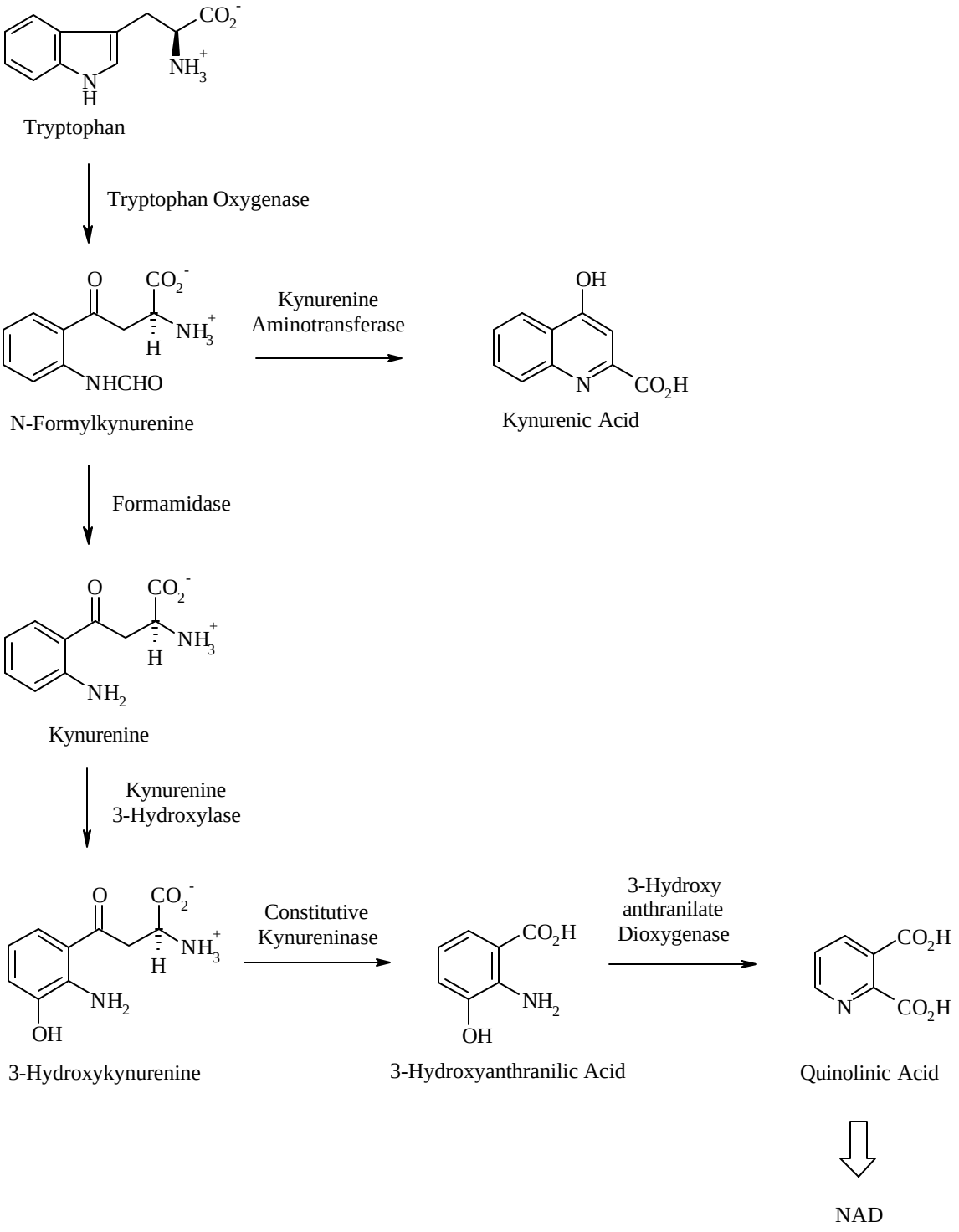


Figure 2. Metabolism of L-tryptophan in mammals, yeast, fungi

antagonist kynurenic acid should be raised. Thus, effective inhibitors promise to be useful in the treatment of the above mentioned neurological disorders.

Inducible kynureninase has been isolated from *Pseudomonas fluorescens,*[10] *Pseudomonas marginalis,*[11] and *Neurospora crassa,*[12] the constitutive enzyme has been purified from *Saccharomyces cerevisiae,*[13] *Neurospora crassa,*[14] and hog liver.[15] Recently, kynureninase from *Pseudomonas fluorescens,*[16] as well as that from rat and human[17] have been sequenced, cloned, and expressed.

The mechanism of kynureninase has been subject to a considerable amount of research and speculation. Braunstein[18] proposed a mechanism (Fig. 3) in which, after initial formation of the aldimine from PLP and kynurenine and conversion into the tautomeric ketimine, which is the Schiff base of an α,γ-diketo acid, water adds to the carbonyl and the γ,δ-bond is cleaved to give anthranilic acid and the ketimine of pyruvic acid which is converted into alanine.

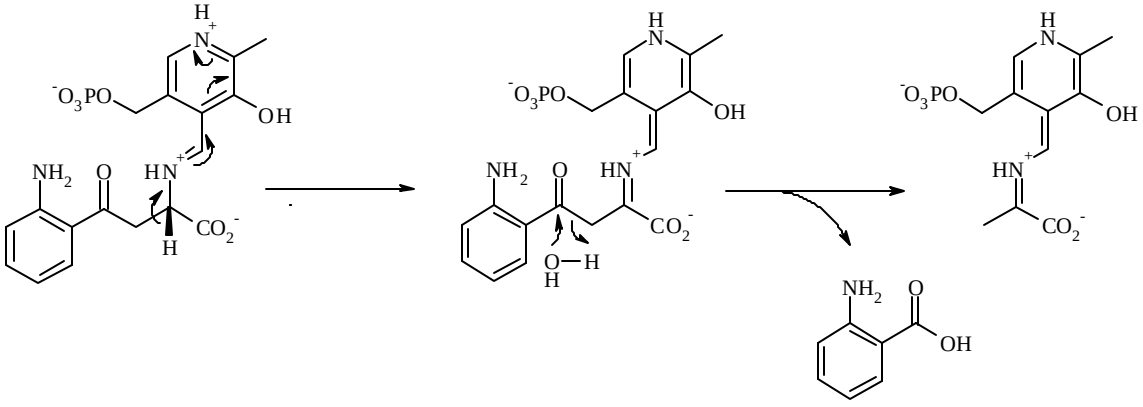


Figure 3. Mechanism for kynureninase reaction proposed by Braunstein

On the basis of the mechanism of the other PLP-dependent enzymes serine dehydrase, tryptophanase, and cysteine desulfhydrase, Longenecker and Snell[19] proposed a mechanism (Fig. 4) in which the kynurenine ketimine is cleaved first into the *o*-amino

benzoyl anion and the aldimine of α-aminoacrylic acid. Then, these two products undergo a non-enzymatic redox reaction to give anthranilic acid and alanine.

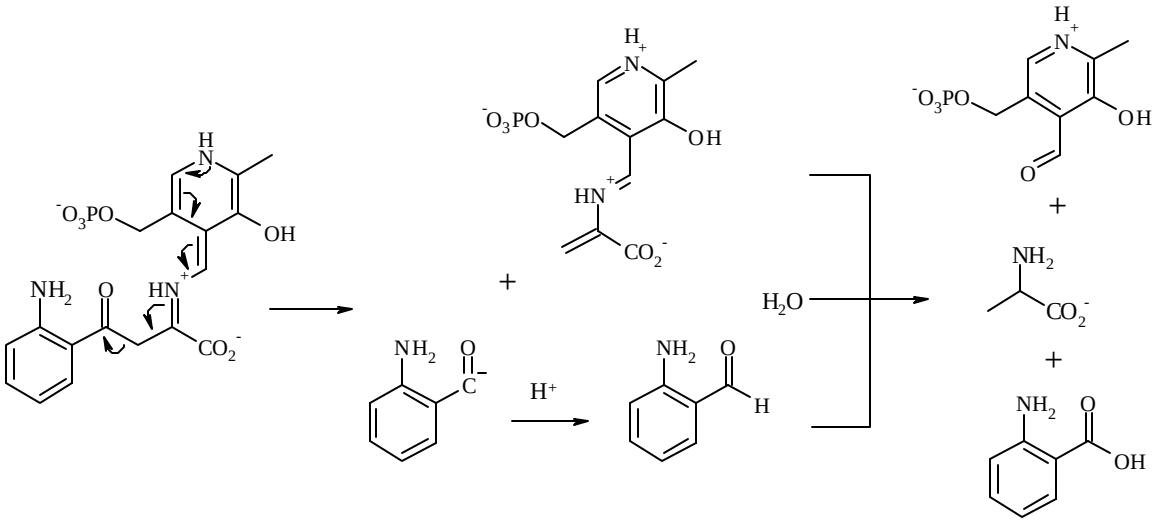
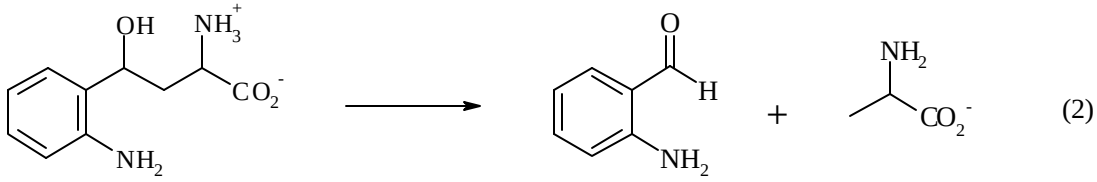


Figure 4. Mechanism for kynureninase proposed by Longenecker and Snell

Walsh[20] and Akhtar *et al*.[21] speculated that the reaction proceeds through an acyl-enzyme intermediate resulting from attack of a nucleophilic enzyme residue on the carbonyl group of the kynurenine ketimine; a molecule of water would then attack the anthranoyl group and liberate anthranilic acid.

Tanizawa and Soda[22] found that dihydrokynurenine acts as a substrate for kynureninase and is cleaved to give alanine and *o*-aminobenzaldehyde in a *retro*–aldol reaction (eq. 2). If the reaction follows Longenecker's mechanism, the carbanion of *o*-amino



(2)

benzyl alcohol should initially be produced by a β-elimination. The resulting *o*-amino-benzyl alcohol would be too stable to undergo oxidation to the aldehyde. Similarly, Walsh's acyl–enzyme mechanism cannot account for the cleavage of dihydrokynurenine due to the absence of the electrophilic carbonyl group.

Bild and Morris found that addition of an excess of benzaldehyde to the reaction mixture of kynurenine and kynureninase results in formation of 2-amino-4-hydroxy-4-phenylbutanoic acid (Fig. 5).[23] The product arises from an aldol-type condensation of benzaldehyde with incipient alanine formed in the cleavage of L-kynurenine. This reaction does not take place if benzaldehyde and added L-alanine are incubated with kynureninase.

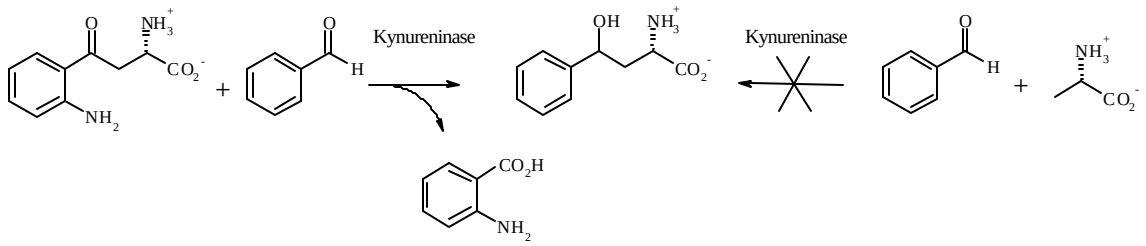


Figure 5. Aldol-reaction catalyzed by kynureninase

Phillips and Dua examined the aldol and retro-aldol reactions in more detail and found that only (4*R*)-dihydro-L-kynurenine acts as a substrate for kynureninase with a K_m of 1.5 μM and a V_{max} about 9 % of that of L-kynurenine.[24] The 4*S* diastereomer is not cleaved by kynureninase; it acts, however, as a potent competitive inhibitor of the enzyme with an apparent K_i of 0.3 μM. The 4*R* diastereomer also is a kynureninase inhibitor with a K_i of 1.4 μM. Based on these results, it was concluded that the reaction proceeds through a *gem*-diolate intermediate in accordance with the mechanism proposed by Braunstein. Kishore had found that suicide substrate inhibitors modified an active site carboxylate which he had postulated to be responsible for the abstraction of the

α-proton.[25] However, Palcic's finding that the α-proton of L-kynurenine is scrambled between the α- and β-positions in the produced L-alanine suggests that a polyprotic base like a lysine ε-amino group likely abstracts the α-proton.[26] Taking these data into account, a mechanism (Fig. 6) was put forth that represents an extension of Braunstein's mechanism. In the *retro*–aldol cleavage (path A), the hydroxy group in (4*R*)-dihydro-L-kynurenine takes the place of the water molecule in the cleavage of L-kynurenine (path B).

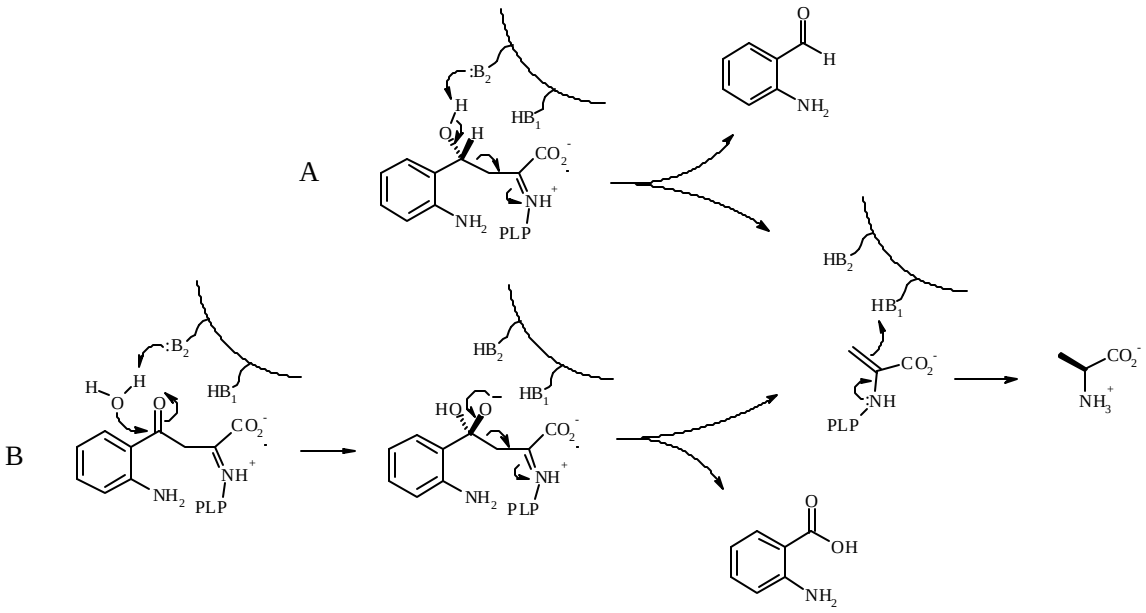


Figure 6. Mechanisms of retro-aldol (A) and L-kynurenine cleavage (B) reactions

In agreement with the proposed intermediacy of a *gem*–diolate in the mechanism, Dua and Phillips[27] found that *S*-(2-aminophenyl) L-cysteine *S,S*-dioxide is a highly potent inhibitor of kynureninase with a K_i of 0.07 μM.

Recently, in additional studies from our laboratory, the effects of pH and of isotopic substitution on the kinetics of kynureninase from *Pseudomonas fluorescens* were studied.[28] The pH–dependence of k_{cat} and k_{cat}/K_m indicated the involvement of only a single base in the reaction mechanism. Neither a substrate kinetic isotope effect nor a

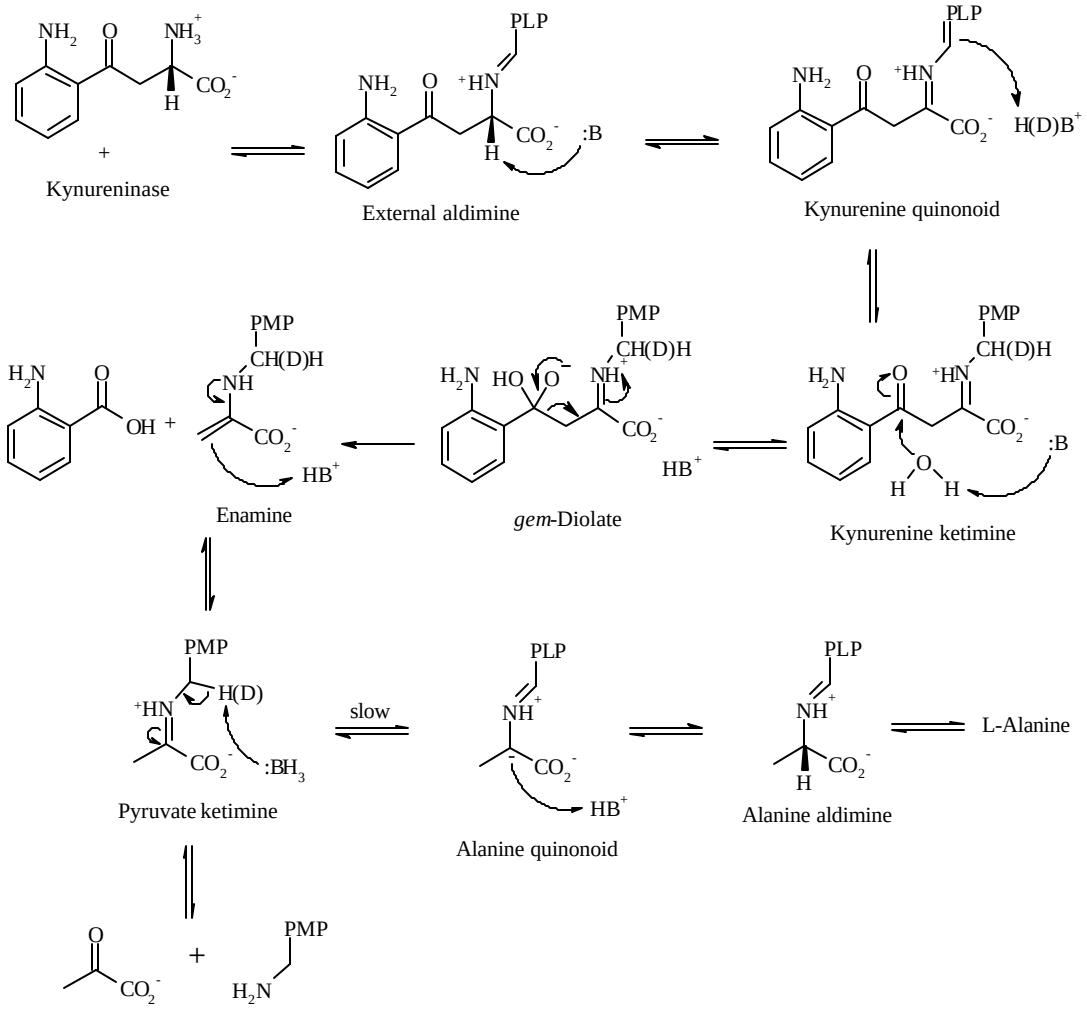


Figure 7. Mechanism of Kynureninase proposed by Phillips *et al.*

significant solvent isotope effect on k_{cat}/K_m was seen, but a large solvent isotope effect on k_{cat} (^{D}k = 6.56) was found. These data, together with the finding from rapid quench experiments that there is a burst of anthranilic acid in the pre–steady state phase of the reaction suggest that the rate–determining step occurs after the first irreversible step which is the release of anthranilic acid. It was proposed that the rate-limiting step is the proton abstraction from C4' of a pyridoxamine–pyruvate ketimine intermediate. In a study by Phillips *et al.*[29] rapid-scanning stopped-flow spectrophotometry was used to detect the quinonoid and ketimine intermediates through which the mechanism had been postulated to proceed. The L-kynurenine quinonoid intermediate forms very rapidly and decays by protonation at C4' by a solvent exchangeable acid, as evidenced by the observed solvent isotope effect (^{D}k = 2.14). These results led to a new, modified mechanistic model which is shown in Fig. 7.

In chapter II of this dissertation, the synthesis of new kynureninase inhibitors and their kinetic evaluation are described.

SECTION 2

Thermostable Alcohol Dehydrogenases as Catalysts in Organic Synthesis

Alcohol dehydrogenases have been of considerable interest in the quest for efficient methodologies for the asymmetric synthesis of chiral building blocks (chirons) in high enantiomeric excess. Among these, horse liver alcohol dehydrogenase (HLADH) [EC 1.1.1.1] has probably received the most attention. HLADH is a dimer with a molecular weight of 80 kDa composed of two identical subunits consisting of 374 amino acids each.[30] The enzyme requires NAD^+ or NADH as cofactor. It reduces both aldehydes[31] and ketones to the corresponding primary or secondary alcohols, but is most

useful for reductions of carbo- and heterocyclic (containing oxygen or sulfur atoms, but not nitrogen) ketones[32] which have been carried out with high enantioselectivity.

Selective asymmetric reductions of one of the carbonyl groups of decalindiones have yielded potentially useful chirons.[33] Oxidations have also been carried out with HLADH. Primary α-amino and α-hydroxyalcohols have been oxidized enantioselectively to give chiral α-substituted aldehydes and *meso*–diols have been oxidized to chiral lactones.[34] The observed enantioselectivity in the reactions of HLADH follows Prelog's rule[35] which states that the *pro-R* hydride of NADH is transferred to the *re*–face of the carbonyl substrate giving rise to (*S*)-alcohols. Based on results from reductions of cyclohexanone and decalin derivatives, Prelog proposed a diamond-lattice model[35] which allows one to rationalize and predict the stereochemical outcome of HLADH–catalyzed reactions (Fig. 8). The active site of the enzyme is represented by a section of a diamond-lattice. A potential substrate should be placed in the space of the lattice with the oxygen-bearing carbon in a fixed position. The orientation of the substrate is limited by the "walls" and the "forbidden" position. If in an orientation of substrate some of these regions are occupied by parts of the molecule, that orientation is considered unfavorable. The diamond lattice model has been superceded in its usefulness by Jones' cubic space section model[36] which was mainly derived from the X-ray crystal structure of HLADH[37] (Fig. 9). Here, the active site is represented by layers of cubes of arbitrary size (although cubes with the length of an edge of 1.3 Å have been found practical). Some of the cubes are considered forbidden, some unfavorable, and some freely accessible by substrate. Like in the diamond lattice model, the carbinol is fixed in place and stereochemistry and determines the location of the carbonyl of the substrate, which is then oriented to avoid any penetration into forbidden areas. Yeast alcohol dehydrogenase (YADH) [EC 1.1.1.1] has also been used extensively for the asymmetric reduction of ketones. Although in most cases, it is employed in whole cells,[38] there are some reports of the use of purified YADH.[39] The whole cell reactions suffer from the disadvantage of low enantiomeric

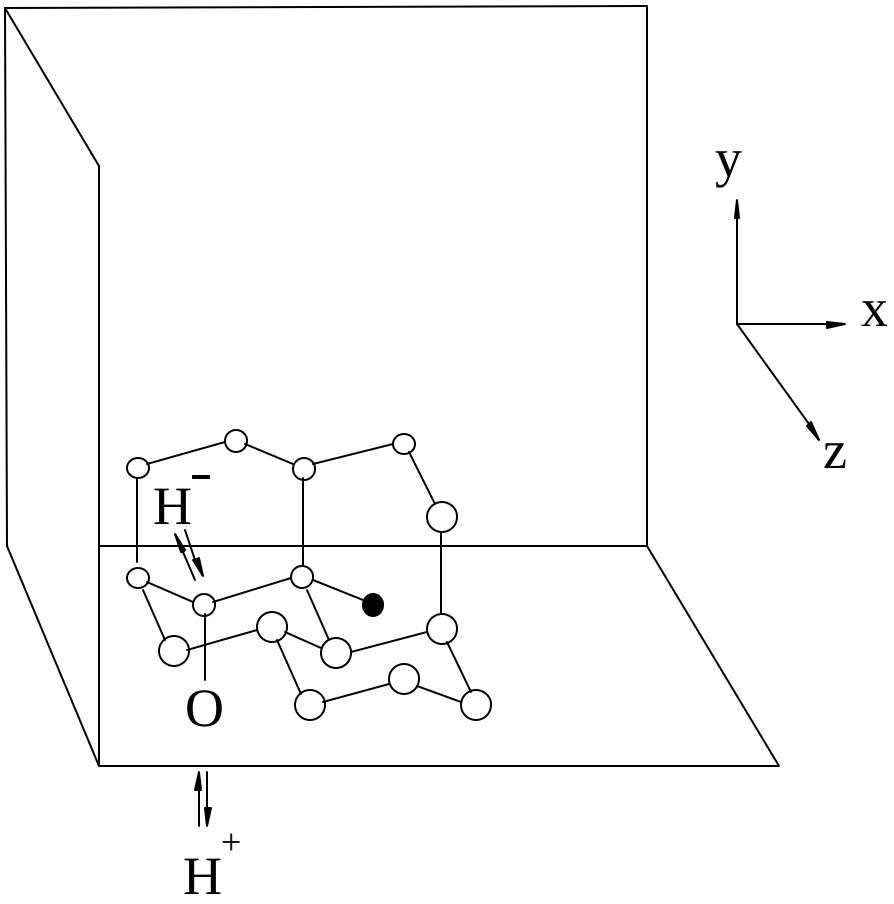


Figure 8. Diamond lattice model for HLADH, • = “forbidden” position

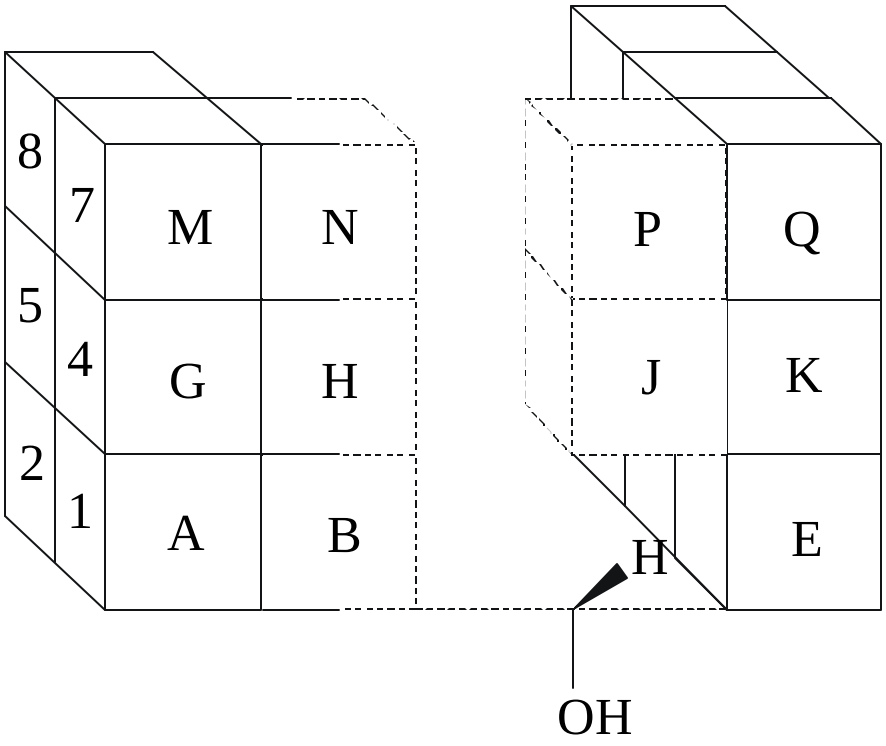


Figure 9. Cubic space section model for HLADH

excess, probably due to the presence of several different dehydrogenases with different specificity. Apart from reductions of simple aldehydes and ketones, YADH has been used to reduce ketoesters,[40] haloketones,[41] and cyclic sulfur-functionalized ketones.[42] Both HLADH and YADH are quite sensitive to heat and to the presence of organic solvents.

The alcohol dehydrogenase from *Thermoanaerobacter brockii* (TBADH) [EC 1.1.1.2] does not suffer from these drawbacks.[43] TBADH has been used for the asymmetric reduction of acyclic ketones containing a variety of functional groups including esters, acetals, ethers,[44] halides,[45] non-conjugated alkenes and alkynes,[46] furans,[47] and isoxazoles.[48] Several total syntheses have employed chirons obtained by TBADH–catalyzed reduction.[49]

TBADH was the first alcohol dehydrogenase isolated from a thermophilic bacterium. Since its discovery several other dehydrogenases from thermophilic organisms have been found including those from *Bacillus stearothermophilus,*[50] *Zymomonas mobilis,*[51] *Sulfolobus solfataricus,*[52] and *Thermoanaerobacterium sp*.[53] These enzymes are most active at temperatures around 90 °C and are stable for extended periods at temperatures between 25 and 70 °C. The present study focusses on a secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus* which was first described by Bryant and Ljungdahl.[54] The sequence of this enzyme is 99 % identical with that of TBADH.[55] Both of these enzymes are homotetramers consisting of four identical subunits each of which is made up of 352 amino acids and has a molecular weight of 37.7 kDa. The crystal structure of TBADH (1YKF.PDB) was solved in 1995 and shows that the subunits are characterized by the presence of a deep cleft that separates the catalytic domain from the cofactor binding domain.[56] The monomers are folded similarly to HLADH, although the enzymes are only 27 % identical. While HLADH contains two zinc atoms, one catalytic and one structural, TBADH and SADH only have the catalytic zinc atom. The subunits associate to tetramers through interactions on the part of the protein that is opposite to the domain–separating cleft, which remains accessible from the

surface of the tetramer. Both TBADH and SADH require $NADP^+$ or NADPH as cofactor. The cofactor is bound to the enzyme with its adenosine moiety in the cofactor binding domain, while the rest of the molecule is positioned in the domain-separating cleft with the nicotinamide ring in close proximity to the catalytic zinc atom. Comparison of the structures of TBADH and HLADH revealed that the difference in cofactor specificity lies partly in substitution of an aspartate residue (Asp223) that, in HLADH, makes hydrogen bonds with two hydroxyl groups of the adenosine ribose of NADH, while in TBADH a glycine (Gly198) in the same place leaves room for the phosphate group of NADPH. Hydrogen bonds are established between this phosphate and the residues Gly198, Ser199, Arg200, and Tyr 218. A sequence comparison between TBADH and an alcohol dehydrogenase from the mesophilic *Clostridium beijerinckii* (CBADH) showed 75 % sequence identity between the two enzymes. Interestingly, in eight of the positions that are not conserved, TBADH has prolines in the place of other amino acids. It has been hypothesized that proline substitutions are partially responsible for the increased thermal stability of enzymes from thermophilic bacteria. Studies using site–directed mutagenesis on CBADH indicated that at least two of the eight additional prolines in TBADH contribute to its thermal stability.[57]

Both TBADH[58] and SADH[55] have been cloned and overexpressed in *Escherichia coli*. Thus, isolation and purification are greatly simplified compared to isolation from the thermophiles themselves, due to the difference in thermal stability between the cloned enzyme and E. coli's own proteins. Thus, SADH is purified by a simple heat treatment of the cell-free extract followed by a single affinity chromatographic separation.[59]

TBADH and SADH are approximately 200 fold more specific for the oxidation of secondary alcohols compared to primary alcohols. In the reverse reaction, ketones are reduced to secondary alcohols enantioselectively, following Prelog's rule in most cases, *i. e*. the *pro-R* hydride of NADPH is transferred to the *re*–face of the substrate. However, when both ketone substituents are small, the "*anti*–Prelog" product is formed preferen-

tially. This reversal of stereochemistry has been explained by Keinan on the basis of a pocket model (Fig. 10) in which the active site comprises two alkyl binding pockets which differ in size and affinity toward alkyl groups. The smaller pocket has the higher affinity and will preferentially bind alkyl groups with larger surface area as long as they will fit into the pocket. Thus, isopropyl is bound strongly in the small pocket, but *n*-propyl is too long and prefers the large pocket.[60]

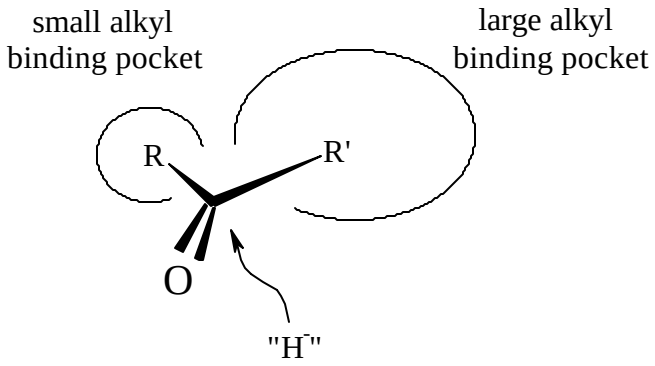


Figure 10. Pocket model for TBADH

A temperature–dependent reversal of stereochemistry was observed with SADH and 2-butanol.[61] Based on observed k_{cat}/K_m values, (*S*)-2-butanol is a better substrate than (*R*)-2-butanol at temperatures below 26 °C, whereas above 26 °C, (*R*)-2-butanol is a better substrate than (*S*)-2-butanol. At 26 °C, the specificity is lost, this temperature is therefore called the racemic temperature. 2-Pentanol and 2-hexanol exhibit a similar temperature-dependence, but their racemic temperatures are 70 °C and 240 °C, respectively. Thus, at temperatures that are feasible for the enzyme reactions, the enzyme is more specific for the (*S*)-alcohols. These findings were rationalized on the basis of thermodynamic considerations. Since the specificity of an enzyme is quantified by k_{cat}/K_m , an enantiospecificity ratio *E* can be defined as the ratio of the k_{cat}/K_m values for *R* and *S*

alcohols. According to transition-state theory, $-RT\ln E = \Delta\Delta G^{\ddagger}$, which is the difference in free energy of activation between the *R*- and *S*-alcohols. Since $\Delta\Delta G^{\ddagger} = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$ the difference in entropy of activation $\Delta\Delta S^{\ddagger}$ is responsible for the temperature dependence of enantiospecificity. This kind of temperature dependence has also been found in the reduction of ketones by TBADH.[62]

The addition of organic solvents has been shown to influence the enantiospecificity of a secondary alcohol dehydrogenase from *Thermoanaerobacterium sp*. Ket4B1.[63] An influence of the employed cofactor on the enantioselectivity of SADH has also been found.[64] Cofactor analogs are able to increase the amount of *R*-enantiomer produced. Very recently, the mutation of Ser39 → Thr shifted the observed enantioselectivity toward *R*-alcohols.[59]

Beside their activity as alcohol dehydrogenases, SADH and TBADH also exhibit reductive thioesterase activity, cleaving acetylcoenzyme A into coenzyme A and acetaldehyde, the latter being further reduced to give ethanol. In fact, acetylcoenzyme A has been suggested to be the physiological substrate of SADH.[65] The substrate activity of the large molecule acetylcoenzyme A demands that Keinan's active site pocket model be modified such that the large pocket indeed is rather an open–ended channel with the part of AcCoA that is not involved in the reaction "sticking out" from the enzyme or, alternatively, interacting with the surface of the enzyme.

In chapter II of this dissertation, the asymmetric reduction of ethynylketones and ethynylketoesters using SADH is explored. In chapter III, the effects of the mutation Cys295 → Ala on SADH–catalyzed reductions is investigated.

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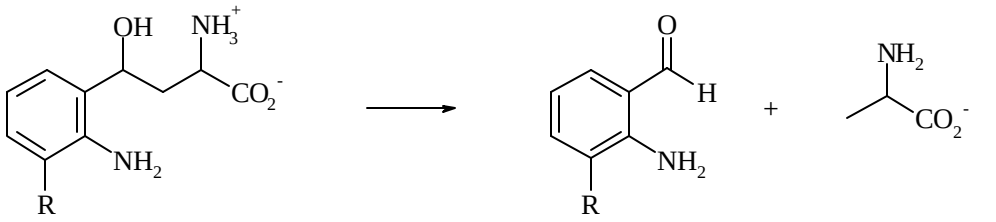
CHAPTER I

SYNTHESIS AND EVALUATION OF MECHANISM BASED INHIBITORS OF KYNURENINASE[1]

[1] Heiss, C. and Phillips, R. S. To be submitted to *Biochemistry*.

Introduction

Kynureninase [EC 3.7.1.1] is a pyridoxal 5'-phosphate (PLP) dependent enzyme that catalyzes a key step in the catabolism of tryptophan in *Pseudomonas fluorescens* and other bacteria,[1] namely the hydrolytic cleavage of L-kynurenine to anthranilic acid and L-alanine (eq. 1, R = H). As part of the biosynthetic pathway from L-tryptophan to NAD, a closely related human enzyme, 3-hydroxykynureninase, catalyzes the analogous cleavage of 3-hydroxykynurenine to produce 3-hydroxyanthranilate (eq. 1, R = OH), which

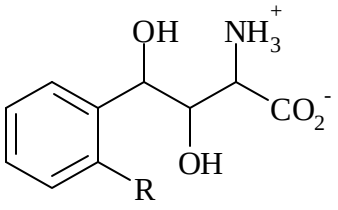


(1)

reacts with 3-hydroxyanthranilate dioxygenase to form quinolinic acid. Quinolinic acid is an *N*-methyl-D-aspartate (NMDA) receptor agonist and hence a neurotoxin. Elevated levels of quinolinic acid have been implicated in the etiology of a number of neurological disorders such as Huntington's disease,[2] AIDS-related dementia,[3] and other inflammatory brain diseases.[4] Effective and selective inhibition of 3-hydroxykynureninase represents a possible treatment of these disorders, not only due to decrease in quinolinic acid but also because of activation of an alternate pathway from L-kynurenine to kynurenic acid which is an NMDA receptor antagonist and counteracts the deleterious effects of quinolinic acid.[5]

In a previous study,[6] we synthesized and separated the two diastereomers of dihydro-L-kynurenine and found that the (4*S*)-diastereomer is a powerful competitive inhibitor of kynureninase with a K_i value of 0.3 μM. While the (4*R*)-diastereomer also displayed inhibitory activity (K_i = 1.5μM), it also acts as a substrate of kynureninase-catalyzed *retro*-aldol reaction to give *o*-aminobenzaldehyde and L-alanine. These results

suggested that the kynureninase reaction proceeds through a *gem*-diolate intermediate, as had originally been proposed by Braunstein.[7] In agreement with this assumption, we found that S-(2-aminophenyl)-L-cysteine-S,S-dioxide, which structure resembles that of the *gem*-diolate intermediate, is a very potent inhibitor with a K_i value of 0.070 μM.[8] On the basis of these results, we wondered whether a compound containing a β-hydroxy as well as an α-hydroxy group would bind more strongly to the enzyme than dihydro-L-kynurenine. The strategy of mimicking a *gem*-diolate intermediate with a vicinal diol has been successfully employed before in the design of HIV–protease inhibitors.[9] A recent study from our laboratories has shown that the *erythro*-isomer of β-methyl-L-kynurenine is a slow substrate for kynureninase.[10] This was the first time a β-substituted amino acid was examined with this enzyme. In the present paper, we wish to expand on these results and to describe the synthesis and enzymologic evaluation of various diastereomers of 2-amino-3,4-dihydroxy-4-phenylbutanoic acid (**1**) and 2-amino-3,4-dihydroxy-4-(2-aminophenyl)butanoic acid (**2**) as inhibitors of kynureninase from *Pseudomonas fluorescens*.



1: R = H
2: R = NH_2

Results

Chemistry. The dihydroxyamino acids **1** and **2** were prepared by an aldol-type condensation of the glycine anion equivalent, ethyl (diphenylmethylene)glycinate, and the appropriate protected α-hydroxyaldehyde. Due to the ready availability of both enantiomers of ethyl mandelate, we decided to carry out the synthesis of **1** in two separate

batches, one starting from (*S*)-ethyl mandelate (Scheme 1) and one starting from (*R*)-ethyl mandelate. The synthesis of **2**, on the other hand, was carried out with racemic mandelate. (*S*)-Ethyl mandelate (**3a**) was first protected as the THP ether (**4a**) and then subjected to reduction with diisobutylaluminum hydride to give aldehyde **5a**. The aldehyde then underwent an aldol–type condensation with the glycine anion equivalent **6**, which had been obtained from benzophenone imine according to the procedure by O'Donnell.[11] Mild acid hydrolysis[12] then removed the diphenylmethylene group and the THP group from the condensation product (**7aa**-**ac**) to give the hydrochloride salt of the amino ester (**8aa**-**ac**) which was saponified to yield **1a**. Preparative HPLC allowed separation of three diastereomers which were identified by ^{1}H-NMR as (2*S*,3*S*,4*S*)-**1** (**1aa**), (2*S*,3*R*,4*S*)-**1** (**1ab**), and (2*R*,3*S*,4*S*)-**1** (**1ac**) in a ratio of 1 : 3.5 : 1.9. The corresponding enantiomers **1ba**, **1bb**, and **1bc** were synthesized in the same fashion starting from (*R*)-ethyl mandelate.

Using the same basic synthetic route, 2-amino-3,4-dihydroxy-4-(2-aminophenyl)-butanoic acid (**2**) was synthesized as the racemate according to Scheme 2 by starting from methyl 2-nitromandelate (**11**), which was obtained by methanolysis of 2-nitrobenzaldehyde cyanohydrin (**10**). After protection as the THP ether, reduction with diisobutylaluminum hydride gave the aldehyde (**14**) and a significant amount of the primary alcohol (**13**) resulting from overreduction. The alcohol was conveniently reoxidized to the aldehyde under Swern conditions. Condensation with ethyl (diphenylmethylene)glycinate (**6**) followed by mild acid hydrolysis gave the nitrophenyl amino ester hydrochloride (**15**). Separation of the diastereomers had to be carried at this stage because trial runs showed that it could not be achieved after saponification due to increased water solubility. A separation of two fractions **A** and **B** in a ratio of 1 : 2 could be achieved by HPLC. **A** and **B** were subjected to saponification separately and then catalytic hydrogenation effected the conversion of the nitro group into the amino group in the final product **2**. ^{1}H-NMR spectroscopy showed that the **A** was a 3 : 1 mixture of 2,3-*anti*-3,4-*syn*-

diastereomer (**2c**) and 2,3-*syn*-3,4-*syn*-diastereomer (**2a**) which we were unable to separate. **B** was mostly the *anti*, *anti*-product **2b** contaminated with small amounts of **2a**,**c**, and **d**.

In efforts directed toward solving the crystal structure of kynureninase, a heavy–atom derivative of a potent inhibitor was needed. For this reason, we synthesized various bromokynurenine derivatives. 5-Bromo-L-kynurenine (**18**) was prepared as summarized in Scheme 3 by acid hydrolysis of N^{α},*N*-diacetyl-5-bromo-L-kynurenine methyl ester obtained by a procedure first described by Casnati *et al.*[13] 5-Bromo-L-kynurenine was reduced to 5-bromodihydro-L-kynurenine (**19**) with $NaBH_4$.[6] A 2 : 1 mixture of (2*S*,4*R*)-**19** (**19a**) and (2*S*,4*S*)-**19** (**19b**) was obtained. **19a** and **19b** were separated using HPLC. N-Acetyl-5-iodo-L-kynurenine methyl ester (**17b**) was prepared from *N*-acetyl-L-kynurenine methyl ester (**16b**) according to Scheme 3. Attempts to obtain 5-iodo-L-kynurenine by hydrolysis of **17b** were unsuccessful, because the iodine was lost under the acidic conditions. This observation, however, indicated that iodide could be used as an easily removable protecting group in the synthesis of 3-bromo-L-kynurenine (Scheme 4). Thus, N^{α}-acetyl-5-iodo-L-kynurenine methyl ester (**17b**) was subjected to bromination conditions and the product was found to be a 2 : 1.1 mixture of N^{α}-acetyl-3-bromo-5-iodo-L-kynurenine methyl ester (**20a**) and *N*-acetyl-3,5-dibromo-L-kynurenine methyl ester (**20b**). These were hydrolyzed together and the resulting mixture of free amino acids **21a** and **21b** was readily separated by low pressure reverse phase liquid chromatography. This synthesis of 3-bromo-L-kynurenine is shorter and more efficient than the only other one reported in the literature.[14]

Enzyme kinetics. The kinetic parameters of the compounds that were prepared are summarized in Table 1. The compounds without substitution on the phenyl ring lacked significant inhibitory activity; only **1aa** displayed a measurable effect, having a K_i of 840 μM. The other diastereomers showed no measurable inhibitory activity at concentrations up to 5 mM, the limit of solubility of these compounds. The observed K_i's of the

2-amino-3,4-dihydroxy-4-(2-amino-phenyl)butanoic acids are 3.1 μM for the 3 : 1 mixture of **2c** and **a** and 7.5 μM for **2b** (The K_i 's were corrected by dividing by 2 under the assumption that the L-amino acid is exclusively responsible for inhibition, a valid assumption in light of the fact that D-amino acids do not form an imine with enzyme-bound PLP). The 3 : 1 mixture of **2c** and **a** exhibits small substrate activity with kynureninase with a k_{cat} value of 8.9×10^{-4} s^{-1}. The compound **2b** is 4-fold less reactive with a k_{cat} value of 2.3×10^{-4} s^{-1}.

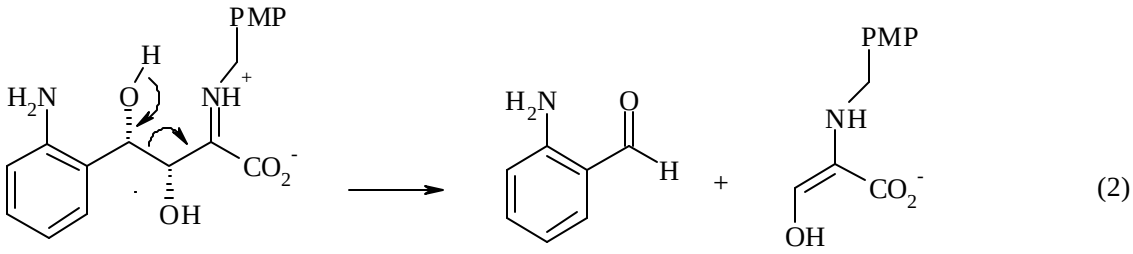
5-Bromo-L-kynurenine was found to be an excellent substrate for kynureninase with a K_m value of about half of that of L-kynurenine, whereas 3-bromo-L-kynurenine reacts fast (k_{cat} = 11 s^{-1}), but exhibits very weak binding (K_m = 2 mM).

Among the 5-bromodihydro-L-kynurenines, only **19b** acts as a slow substrate with a k_{cat} value of 4.6×10^{-3} s^{-1}. Both **19a** and **19b** are potent competitive inhibitors of kynureninase with K_i's of 0.170 and 0.055 μM, respectively.

Discussion

The results presented here show that the β-hydroxy group does not improve binding to kynureninase relative to the dihydro-L-kynurenines as evidenced by the about one order of magnitude larger observed K_i values. Unfortunately, separation of **2a** and **2c** could not be accomplished, leaving us short of experimental evidence of the different activity of the two compounds. However, the different behavior of (4*R*)- and (4*S*)-dihydro-L-kynurenine suggests that **2c**, having the same configuration as (4*S*)-dihydro-L-kynurenine, is mainly responsible for the inhibitory activity. Thus, **2a** is likely to be a substrate for kynureninase. The difference in reactivity between **2a** and **2b** is only 4-fold, in contrast to the *threo*- and *erythro*-isomers of β-methyl-L-kynurenine, where the difference was 390-fold. The difference in reactivity of the two β-methyl-L-kynurenines was rationalized by analysis of the conformation that the compounds have to assume to undergo reaction. It is required that the β-γ carbon–carbon bond be perpendicular to the

plane of the PLP-π system. In the case of the *threo*-isomer, this leads to eclipsing of the methyl group with the carboxylate group and to an increase in activation energy. In keeping with this theory, in **2b**, the carboxylate would be eclipsed with the β-hydroxy group, which would be less unfavorable than with a β-methyl group due to possible hydrogen bonding. The low reactivity of **2a** and **2b** might be explained by the formation of an unstable β-enolamine (eq. 2), in the cleavage of the β-γ carbon–carbon bond.



(2)

The behavior of the 2 isomers of 5-bromodihydro-L-kynurenine parallels that of the unsubstituted dihydro-L-kynurenines in that the (4*R*)-isomer (**19b**) acts as a substrate and the (4*S*)-isomer (**19a**) does not. Surprisingly, **19b** is also a very strong inhibitor (K_i = 55 nM), in contrast to what was seen with the two isomers of dihydro-L-kynurenine, where the (4*R*)-isomer is the weaker inhibitor. Apparently, the bromine induces a small conformational change that leads to the observed reversal of binding affinity. The impressive effect of the bromine in these compounds raises the question whether the improved binding is due to hydrophobic interactions or electronic effects. We are currently working on the synthesis of other halogenated kynurenines to elucidate this question.

Experimental

General

^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker AC 250 or AC 300, respectively. Optical rotations were measured with an Autopol IV from Rudolph Research.

Enzyme assays and kinetic experiments were performed with a Varian Cary 1E UV/visible spectrophotometer. High pressure liquid chromatography (HPLC) was carried out on an instrument with two Rainin Rabbit HP pumps and a LDC Milton Roy Spectromonitor 3000 variable wavelength detector using Gilson Unipoint software. Kynureninase was purified from *E. coli* cells containing plasmid pTZ18U with the *kyn* gene of *P. fluorescens* inserted into the linker as previously described.[15]

Kinetic measurements

Kynureninase activity was measured by following the decrease in absorbance at 360 nm due to consumption of L-kynurenine (ε = 4500 M^{-1} cm^{-1}). The competitive inhibition was measured by variation of L-kynurenine concentration at several fixed concentrations of inhibitor. K_m, V_{max}, and K_i values were determined by fitting of initial rate data to the equations 3 and 4 using the compiled FORTRAN programs HYPER and COMP of Cleland.[16] K_m and V_{max} values of **19a** were determined by running the reaction at a fixed

$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad (3)$$

$$v = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]} \qquad (4)$$

concentration (35 μM) of substrate and fitting the time course of the increase in absorbance at 360 nm due to formation of 2-aminobenzaldehyde (ε = 3900 M^{-1} cm^{-1}) to the integrated Michaelis–Menten equation. V_{max} of **2** was determined by following the increase in absorbance at 360 nm at a high substrate concentration (50 μM) relative to K_i (= K_m for slow substrates).

Chemistry

(2*S*)-2-Phenyl-2-(tetrahydropyran-2-yloxy)ethanal (5a). (2*S*)-Ethyl 2-(tetrahydropyran-2-yloxy)acetate[17] (10.6 g, 40 mmol) was dissolved in dry CH_2Cl_2 (80 ml) and cooled to –78 °C under N_2. Diisobutylaluminum hydride (48 mL of a 1.0 *M* so-

lution in hexanes) was added via dropping funnel over a period of 15 min and the mixture was stirred at –78 °C for 4 h. A saturated solution of NH_4Cl (20 mL) was added at –78 °C, followed by 1 *M* HCl (60 mL). The cooling bath was removed and stirring continued for 30 min. The mixture was extracted with CH_2Cl_2, the combined organic extracts were dried (Na_2SO_4), and evaporated. The resulting crude oil was dissolved in Et_2O and passed through a short column of silica gel. Evaporation yielded 7.93 g **5a** (90 %). ^{1}H-NMR ($CDCl_3$) δ 9.65 (d, *J* = 2.2 Hz, 1H), 9.62 (d, *J* = 1.9 Hz, 1H), 7.38 (m, 10H), 5.21 (d, *J* = 1.3 Hz, 1H), 5.05 (d, *J* = 2.1 Hz, 1H), 4.93 (t, *J* = 2.6 Hz, 1H), 4.67 (t, *J* = 3.8 Hz, 1H), 3.95 (m, 1H), 3.71 (m, 1H), 3.49 (m, 2H), 2.1 – 1.4 (m, 12H).

(4*S*)-2-Amino-3,4-dihydroxy-4-phenylbutanoic acid (1aa – 1ac). To a solution of ethyl *N*-(diphenylmethylene)glycinate (**6**) (4.06 g, 15.2 mmol) in dry EtOH (150 mL) was added at 0 °C NaOEt (15.0 mL of a 1 *M* solution in EtOH), followed by a solution of **5a** (3.30 g, 15.0 mmol) in EtOH (20 mL). After stirring for 18 h at rt, the solution was poured into ice-cold half-saturated NH_4Cl (400 mL) and extracted with Et_2O (3 × 100 mL). After drying (Na_2SO_4) and evaporation, the condensation product **7aa-ac** (6.08 g, 83 %) was obtained as a yellow oil.

Crude **7aa-ac** (2.44 g, 5.0 mmol) was dissolved in Et_2O (15 mL) and stirred vigorously for 5 h with 1 *M* HCl (6.5 mL). The layers were separated, the aqueous layer extracted with Et_2O, evaporated to dryness H_2O was added and evaporated again. Low pressure reverse phase chromatography (eluent: H_2O) gave **8aa-ac** (0.74 g, 54 %) as a colorless oil.

Substrate **8aa-ac** (0.64 g, 2.3 mmol) was dissolved in H_2O (18 mL) and the solution adjusted to and kept at pH 10 by periodic addition of 0.2 *M* LiOH. After completion of the reaction, the solution was concentrated and chromatography (reverse phase silica gel, eluent: H_2O) yielded **1aa-ac** (0.40 g, 82 %) as a white solid. Anal. Calcd for $C_{10}H_{13}NO_4 \cdot 0.25\ H_2O$: C, 55.68; H, 6.31; N, 6.49. Found: C, 55.84; H, 6.17; N, 6.54. 6

Portions of **1aa-ac** (20 mg each) were separated into **1aa**, **1ab**, and **1ac** by HPLC (isocratic at 5 % MeOH/TFA (0.1 %)).

(2*S*,3*S*,4*S*)-1 (1aa): 16 mg; mp 226-227 °C (dec.); $[\alpha]^{20}_{D}$ +1.7 (c = 1.5, 1.0 % NaOH); MS (ESI) *m*/*z* 212 ($M+H^+$); ^{1}H-NMR (TFA-d) δ 7.67 (m, 3H), 7.53 (m, 2H), 6.20 (s, 1H), 5.27 (d, *J* = 4.7 Hz, 1H), 4.96 (d, *J* = 4.7 Hz, 1H); ^{1}H-NMR (1.0 % NaOD-$D_2$0) δ 7.38 (m, 5H), 4.69 (d, *J* = 7.7 Hz, 1H), 3.92 (dd, *J* = 7.7 Hz, 4.4 Hz, 1H), 3.46 (d, *J* = 4.4 Hz, 1H); ^{13}C-NMR (1.0 % NaOD-D_2O) δ 182.5, 143.4, 131.0, 130.5, 129.7, 78.8, 77.4, 60.2.

(2*S*,3*R*,4*S*)-1 (1ab): 56 mg; mp 230-232 °C (dec.); $[\alpha]^{20}_{D}$ –2.9 (c = 2.0, 1.0 % NaOH); MS (ESI) *m*/*z* 212 ($M+H^+$); ^{1}H-NMR (TFA-d) δ 7.69 (m, 5H), 5.62 (s, 1H), 5.08 (s, 1H), 5.07 (s, 1H); ^{1}H-NMR (1.0 % NaOD-$D_2$0) δ 7.44 (m, 5H), 4.09 (dd, *J* = 7.0 Hz, 2.7 Hz, 1H), 3.03 (d, *J* = 2.7 Hz, 1H); ^{13}C-NMR (1.0 % NaOD-D_2O) δ 183.0, 142.9, 131.2, 130.6, 129.3, 78.8, 77.7, 59.7.

(2*R*,3*R*,4*S*)-1 (1ac): 30 mg; mp 125-130 °C (dec.); $[\alpha]^{20}_{D}$ –31.2 (c = 0.6, 1.0 % NaOH); MS (ESI) *m*/*z* 212 ($M+H^+$); ^{1}H-NMR (1.0 % NaOD-$D_2$0) δ 7.39 (m, 5H), 4.65 (d, *J* = 8.3 Hz, 1H), 4.12 (dd, *J* = 8.2 Hz, 2.5 Hz, 1H), 3.55 (d, *J* = 2.3 Hz, 1H); ^{13}C-NMR (1.0 % NaOD-D_2O) δ 183.5, 143.7, 131.1, 130.6, 129.7, 77.8, 76.7, 58.9.

2-Hydroxy-2-(2-nitrophenyl)acetonitrile (10). To a solution of 2-nitrobenzaldehyde (15.1 g, 0.10 mol) in glacial acetic acid (58 mL) at 0 °C was added dropwise with stirring a solution of KCN (7.1 g, 0.11 mol) in H_2O (15 mL). The mixture was stirred at rt for 5 h and, at 0 °C, an additional equivalent of KCN in H_2O was added. Stirring was then continued for 18 h, H_2O (400 mL) was added, and the precipitated cyanohydrin (13.6 g) collected by vacuum filtration. The mother liquor was extracted with CH_2Cl_2, the combined extracts were dried ($MgSO_4$) and evaporated to give an additional 3.1 g of **10**. Combined yield: 16.7 g (94 %). ^{1}H-NMR ($CDCl_3$) δ 8.21 (dd, *J* = 7.9 Hz, 1.0 Hz, 1H), 7.97 (dd, *J* = 8.0 Hz, 1.0 Hz, 1H), 7.81 (dt, *J* = 7.6 Hz, 1.1 Hz, 1H), 7.66 (dt, *J* = 8.0 Hz, 1.3 Hz, 1H), 6.18 (d, *J* = 5.3 Hz, 1H), 3.62 (d, *J* = 6.3 Hz, 1H).

Methyl 2-hydroxy-2-(2-nitrophenyl)acetate (11). Dry HCl was passed through a solution of **10** (6.1 g, 34.4 mmol) in MeOH (200 mL) for 3 h and the mixture was stirred for 15 h. The solvent was removed *in vacuo*, H_2O (120 mL) was added, and the resulting mixture was extracted with Et_2O. The combined extracts were washed with saturated $NaHCO_3$ and with brine, dried over Na_2SO_4, filtered, and evaporated. Crude **11** (6.5 g, 89 %) was obtained as yellow oil and used without further purification. ^{1}H-NMR ($CDCl_3$) δ 8.00 (d, *J* = 8.2 Hz, 1H), 7.67 (m, 2H), 7.51 (dt, *J* = 8.4, 2.8 Hz, 1H), 5.83 (s, 1H), 3.75 (s, 3H).

Methyl 2-(tetrahydropyran-2-yloxy)-2-(2-nitrophenyl)acetate (12). 3,4-Dihydro-2*H*-pyran (DHP) (2.02 g, 24 mmol), pyridinium *p*-toluenesulfonate (0.40 g, 1.6 mmol), and **11** (3.38 g, 16 mmol) were dissolved in CH_2Cl_2 (80 mL). After stirring for 16 h, an additional portion of DHP (1.01 g, 12 mmol) was added and stirring was continued for 5 h. The solution was diluted with Et_2O (100 mL), washed with half-saturated brine (2 × 80 mL), dried (Na_2SO_4), and evaporated. Column chromatography (15 % ethyl acetate/hexanes) yielded **12** (4.7 g, 99 %). ^{1}H-NMR ($CDCl_3$) δ 8.05 (d, *J* = 8.1 Hz, 1H), 8.04 (d, *J* = 8.0 Hz, 1H), 7.94 (d, *J* = 7.8 Hz, 1H), 7.77 (d, *J* = 7.3 Hz, 1H), 7.67 (m, 2H), 7.50 (t, *J* = 7.7 Hz, 2H), 5.99 (s, 1H), 5.95 (s, 1H), 5.13 (t, *J* = 3.0 Hz, 1H), 4.60 (t, *J* = 3.0 Hz, 1H), 4.07 (m, 1H), 3.86 (m, 1H), 3.76 (s, 3H), 3.72 (s, 3H), 3.54 (m, 2H), 2.0 – 1.4 (m, 12H).

2-(2-Nitrophenyl)-2-(tetrahydropyran-2-yloxy)ethanal (14). Diisobutylaluminum hydride (21 mL of a 1.0 *M* solution in hexanes) was added dropwise to a solution of **12** (5.64 g, 19.1 mmol) in dry CH_2Cl_2 (50 mL) at –78 °C. After 3 h, sat. NH_4Cl (50 mL) was added, the mixture was extracted with Et_2O (5 × 50 mL), the combined extracts were dried (Na_2SO_4), evaporated. Chromatography (20 % ethyl acetate/hexanes) yielded **14** (2.72 g, 54 %) beside the alcohol **13** (1.48 g, 29 %) and recovered starting material (0.50 g, 10 %). ^{1}H-NMR of **14** ($CDCl_3$) δ 9.87 (s, 1H), 9.81 (s, 1H), 8.07 (t, *J* = 7.4 Hz, 2H), 7.69 (m. 4H), 7.52, t, *J* = 8.3 Hz, 2H), 5.85 (s, 1H), 5.81 (s, 1H), 5.00 (t, *J* = 2.0 Hz, 1H),

4.63 (t, *J* = 3.5 Hz, 1H), 3.97 (m, 1H), 3.46 (m, 3H), 2.0 – 1.4 (m, 12H). ^{1}H-NMR of **13** ($CDCl_3$) δ 8.05 – 7.30 (m, 8H), 5.38 (m, 2H), 4.83 (s, 1H), 4.46 (d, *J* = 5.4 Hz, 1H), 3.93 (m, 3H), 3.73 (m, 2H), 3.49 (m, 2H), 3.28 (m, 1H), 1.95 – 1.35 (m, 12H).

Swern oxidation of 13. To a solution of oxalyl chloride (0.26 mL, 0.38 g, 3.0 mmol) in dry CH_2Cl_2 (5 mL) was added slowly at –78 °C dimethyl sulfoxide (0.43 mL, 0.46 g, 6.0 mmol). After stirring at –78 °C for 5 min, alcohol **13** (0.68 g, 2.5 mmol) in CH_2Cl_2 (2 mL) was added. After an additional 15 min at –78 °C, the mixture was treated with triethylamine (1 mL), allowed to warm to rt, and, after 30 min, sat. NH_4Cl (10 mL) was added. The mixture was extracted with Et_2O, the combined extracts washed with H_2O (5 × 5 mL), dried ($MgSO_4$), and evaporated to give **14** (0.53 g, 80 %).

2-Amino-3,4-dihydroxy-4-(2-nitrophenyl)butanoic acid (2a-d). To a solution of ethyl *N*-(diphenylmethylene)glycinate (**6**) (0.98 g, 3.66 mmol) in dry EtOH (40 mL) was added at 0 °C NaOEt (3.66 mL of a 1 *M* solution in EtOH), followed by a solution of **14** (0.97 g, 3.66 mmol) in EtOH (25 mL). After stirring for 18 h at rt, the solution was poured into ice-cold half–saturated NH_4Cl (100 mL) and extracted with Et_2O (3 × 30 mL). After drying (Na_2SO_4) and evaporation, the condensation product was obtained as a yellow oil which was dissolved in Et_2O (15 mL) and stirred vigorously for 3 h with 1 M HCl (4.0 mL). The layers were separated, the aqueous layer extracted with Et_2O, evaporated to dryness H_2O was added and evaporated again. Low pressure reverse phase chromatography (eluent: H_2O) gave **15a-d** (0.69 g, 59 %) as yellow oil. Ten 20 mg portions of **15a-d** were separated by HPLC (isocratic at 20 % MeOH/TFA(0.1 %)). Two fractions were obtained, fraction **A** (62 mg) and fraction **B** (123 mg). **A** (62 mg, 0.19 mmol) was dissolved in H_2O (2 mL), the solution adjusted to and kept at pH 10 and, after completed reaction neutralized by addition of 1 M HCl. Palladium on charcoal (10 %, 6 mg) was added and the mixture was hydrogenated at rt and atmospheric pressure. Reverse phase column chromatography gave a 3 : 1 mixture of 2,3-*anti*-3,4-*syn*-**2** (**2c**) and 2,3-*syn*-3,4-*syn*-**2** (**2a**) (29 mg, 67 %), mp 209-210 °C. Saponification and hydrogenation of **B** (123

mg, 0.38 mg) gave 2,3-*anti*-3,4-*anti*-**2** (**2b**) and about 15 % **2a**,**c**, and **d** (55 mg, 65 %), mp 197-199 °C. ^{1}H-NMR of **A** (0.1 % NaOD-D_2O) δ 7.33 (m,1.3H, **2a**,**c**), 7.15 (m,1.3H, **2a**,**c**), 6.89 (m, 2.7H, **2a**,**c**), 4.21 (dd, *J* = 8.7 Hz, 2.2 Hz, 1H, **2c**), 3.99 (dd, *J* = 8.2 Hz, 4.2 Hz, **2a**), 3.62 (d, *J* = 2.5 Hz, 1H, **2c**), 3.54 (d, *J* = 4.3 Hz, 1H, **2a**); ^{13}C-NMR of **A** (0.1 % NaOD-D_2O) δ 183.5 (**2a,c**), 146.8 (**2a,c**), 131.2 (**2c**), 130.7 (**2a**), 130.4 (**2c**), 129.5 (**2a**), 122.1 (**2a,c**), 120.2 (**2a,c**), 77.8 (**2a**), 76.6 (**2c**), 74.2 (**2a**), 72.9 (**2c**), 60.5 (**2a**), 58.9 (**2c**); ^{1}H-NMR of **B** (0.1 % NaOD-D_2O) δ 7.33 (m, 1H), 7.20 (m, 1H), 6.89 (m, 2H), 4.18 (dd, *J* = 7.0 Hz, 2.7 Hz, 1H), 3.09 (d, *J* = 2.7 Hz, 1H); ^{13}C-NMR of **B** (0.1 % NaOD-D_2O) δ 182.9, 146.4, 131.3, 130.6, 122.0, 120.2, 77.0, 74.6, 60.1.

(2*S*)-Methyl 2-acetamido-4-(2-acetamidophenyl)-4-oxobutanoate (*N*^{a},*N*-diacetyl-L-kynurenine methyl ester) (16a). A stream of ozone was passed through a solution of N^{α}-acetyl-L-tryptophan methyl ester (2.28 g, 8.76 mmol) in MeOH (100 mL) at –78 °C. After the reaction was completed (10 % KI-trap), excess oxidizing species were destroyed by treatment with sat. $NaHSO_3$ solution (100 mL). The mixture was extracted with CH_2Cl_2, dried ($MgSO_4$), and evaporated. The residue was taken up in MeOH (80 ml) and treated with TFA (5 mL). After stirring at rt overnight, the mixture was evaporated and the resulting oil taken up in $CHCl_3$ (100 mL). Acetic anhydride (10 mL) was added, the solution was stirred for 1 h, washed with saturated $NaHCO_3$ (10 × 30 mL), dried ($MgSO_4$), and evaporated. Recrystallization from ethyl acetate/hexanes gave **16a** (1.36 g, 53 %) as white needles, mp 163-164°C (lit.[18] 134-136 °C); $[\alpha]^{20}_D$ +141 (c = 0.5, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 11.45 (s, 1H), 8.75 (d, *J* = 8.7 Hz, 1H), 7.88 (d, *J* = 7.8 Hz, 1H), 7.57 (t, *J* = 7.8 Hz, 1H), 7.12 (t, *J* = 7.3 Hz, 1H), 6.54 (d, *J* = 7.7 Hz, 1H), 4.97 (m, 1H), 3.76 (s, 3H), 3.75 (m, 2H), 2.23 (s, 3H), 2.04 (s, 3H); Anal. Calcd for $C_{15}H_{18}N_2O_5$: C, 58.82; H, 5.92; N, 9.15. Found: C, 58.86; H, 5.94; N, 8.97.

(2*S*)-Methyl 2-acetamido-4-(2-acetamido-5-bromophenyl)-4-oxobutanoate (*N*^{a},*N*-diacetyl-5-bromo-L-kynurenine methyl ester) (17a). Diacetyl-L-kynurenine methyl ester (**16a**) (1.02 g, 3.32 mmol) was dissolved in acetic acid (20 mL) containing

fused sodium acetate (1.5 g) and treated with bromine (4 mL). After 1 h at rt, the mixture was poured into ice-water, treated with 10 % $NaHSO_3$ (20 mL), extracted with $CHCl_3$, dried ($MgSO_4$), and evaporated. Remaining acetic acid was removed by repeated evaporation with toluene. Recrystallization from MeOH/H_2O gave **17a** (1.03 g, 80 %) as pale yellow needles, mp 176-178 °C (lit.[13] mp 178 °C); $[\alpha]^{20}_D$ +85.6 (c = 0.81, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 11.35 (s, 1H), 8.70 (d, *J* = 9.0 Hz, 1H), 7.98 (d, *J* = 2.3 Hz, 1H), 7.66 (dd, *J* = 9.2 Hz, 1.9 Hz, 1H), 6.49 (d, *J* = 7.6 Hz, 1H), 4.96 (m, 1H), 3.77 (s, 3H), 3.75 (m, 2H), 2.24 (s, 3H), 2.04 (s, 3H).

(2*S*)-Methyl 2-acetamido-4-(2-amino-5-iodophenyl)-4-oxobutanoate (*N*^{a},*N*-diacetyl-5-iodo-L-kynurenine methyl ester) (17b). *N*$^\alpha$-acetyl-L-kynurenine methyl ester (**16b**)[19] (1.5 g, 5.7 mmol) was dissolved in acetic acid (50 mL) containing fused sodium acetate (3.75 g) and treated with iodine (3.6 g, 14.2 mmol). After 1 h at rt, the mixture was poured into ice-water, treated with 10 % $NaHSO_3$ (20 mL), extracted with $CHCl_3$, dried ($MgSO_4$), and evaporated. Remaining acetic acid was removed by repeated evaporation with toluene. Passage of the crude product through a short column of silica gel and elution with ethyl acetate gave **17b** (1.15 g, 52 %) as pale yellow needles, mp 156-157 °C; $[\alpha]^{20}_D$ +131 (c = 0.56, $CHCl_3$); ^{1}H-NMR ($CDCl_3$) δ 7.93 (d, *J* = 1.9 Hz, 1H), 7.49 (dd, *J* = 8.8 Hz, 1.9 Hz, 1H), 6.56 (d, *J* = 7.9 Hz, 1H), 6.46 (d, *J* = 8.8 Hz, 1H), 4.95 (m, 1H), 3.74 (s, 3H), 3.71 (dd, *J* = 3.9 Hz, 1H), 3.52 (dd, J = 18.2 Hz, 3.9 Hz, 1H), 2.02 (s, 3H).

(2*S*)-2-Amino-4-(2-amino-5-bromophenyl)-4-oxobutanoic acid (5-bromo-L-kynurenine) (18a). Compound **17a** (0.96 g, 2.5 mmol) was refluxed for 18 h with 6 *M* HCl (9 mL). The solution was evaporated and the yellow oily residue was purified by reverse phase column chromatography (10 % MeOH/H_2O) to give **18a** (0.57 g, 79 %) as a waxy yellow solid, mp 213-217°C (lit.[20] mp 233 °C for racemate); $[\alpha]^{20}_D$ +62.5 (c = 0.28, dioxane/ H_2O 1: 1); ^{1}H-NMR (1 % DCl-D_2O) δ 8.21 (d, J = 2.1 Hz, 1H), 7.77 (dd, J = 8.6

Hz, 2.1 Hz, 1H), 7.22 (d, J = 8.6 Hz, 1H), 4.48 (t, J = 5.0 Hz, 1H), 3.84 (d, J = 5.1 Hz, 2H).

(2*S*,4*S*)- and (2*S*,4*R*)-2-Amino-4-(2-amino-5-bromophenyl)-4-hydroxybutanoic acid (5-bromodihydro-L-kynurenine) (19a and b). Compound **18a** (143 mg, 0.5 mmol) was suspended in H_2O (50 mL)and treated with $NaBH_4$ (50 mg, 1.3 mmol). The mixture was stirred overnight, brought to pH 3 by addition of 1 *M* HCl, and loaded onto a Dowex-50 ion exchange column. Elution with 1 *M* NH_3 yielded a 2 : 1 di-astereomeric mixture of **19a** and **19b** (100 mg, 69 %); Anal. Calcd for $C_{10}H_{13}BrN_2O_3 \cdot 0.5\ H_2O$: C, 40.29; H, 4.73; N, 9.40. Found: C, 40.04; H, 4.50; N, 9.17. The mixture was separated by HPLC (isocratic at 5 % MeOH/TFA (0.1 %)) in 4 mg portions. **19a**: mp >360 °C; $[\alpha]^{20}_D$ –53.5 (c = 0.40, 1 % NaOH); ^{1}H-NMR (1.0 % NaOD-D_2O) δ 7.36 (d, *J* = 2.1 Hz, 1H), 7.21 (dd, *J* = 8.6 Hz, 2.4 Hz, 1H), 6.69 (d, *J* = 8.6 Hz, 1H), 3.31 (dd, *J* = 8.0 Hz, 5.0 Hz, 1H), 2.07 (m, 1H), 1.75 (m, 1H); ^{13}C-NMR (1.0 % D_2O) δ 185.3, 162.6, 145.0, 133.3, 131.6, 121.7, 113.2, 69.9, 55.9, 43.4. **19b**: mp >360 °C; $[\alpha]^{20}_D$ +8.2 (c = 0.44, 1 % NaOH); ^{1}H-NMR (1.0 % NaOD-D_2O) δ 7.39 (d, *J* = 2.1 Hz, 1H), 7.25 (dd, *J* = 8.6 Hz, 2.3 Hz, 1H), 6.74 (d, *J* = 8.6 Hz, 1H), 3.27 (t, *J* = 6.9 Hz, 1H), 2.00 (m, 1H), 1.89 (m, 1H); ^{13}C-NMR (1.0 % D_2O) δ 185.2, 162.5, 145.1, 133.4, 131.6, 121.6, 113.1, 71.0, 56.4, 43.4.

(2*S*)-2-amino-4-(2-amino-3-bromophenyl)-4-oxobutanoic acid (3-bromo-L-kynurenine) (21a). N^{α}-Acetyl-5-iodo-L-kynurenine methyl ester (**17b**) (400 mg, 1.03 mmol) was dissolved in acetic acid (8 mL) containing fused sodium acetate (600 mg) and treated with bromine (0.051 mL, 160 mg, 1.0 mmol). After 30 min at rt, the mixture was poured into ice-water and the precipitate (380 mg) was filtered and dried. The product was a mixture of **20a** (242 mg, 50 %) and N^{α}-acetyl-3,5-dibromo-L-kynurenine methyl ester (**20b**) (135 mg, 31 %). The mixture (309 mg, 0.42 mmol **20a**, 0.26 mmol **20b**) was refluxed in 6 *M* HCl overnight. Reverse phase chromatography (10-20 % MeOH/H2O) gave **21a** (52 mg, 43 %), mp 210-212 °C (lit.[14] >175 °C); $[\alpha]^{20}_D$ +63.2 (c = 0.5, dioxane/

H_2O, 1 : 1); ^{1}H-NMR (1 % DCl-D_2O) δ 7.85 (d, *J* = 8.1 Hz, 1H), 7.75 (d, *J* = 7.7 Hz, 1H), 6.71 (t, *J* = 7.9 Hz, 1H), 4.34 (t, *J* = 4.9 Hz, 1H), 3.81 (d, J = 5.0 Hz, 1H).

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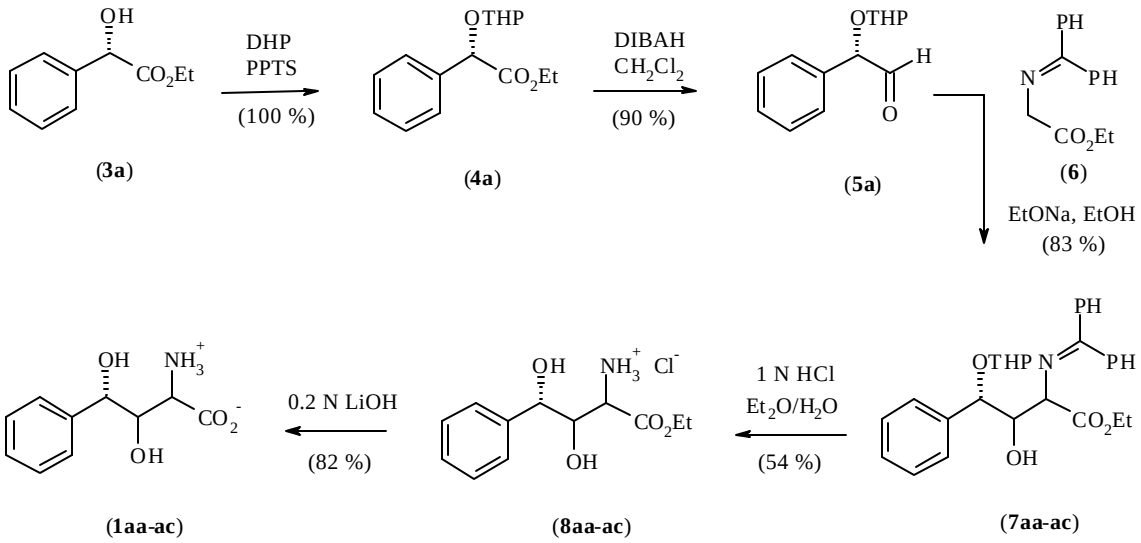
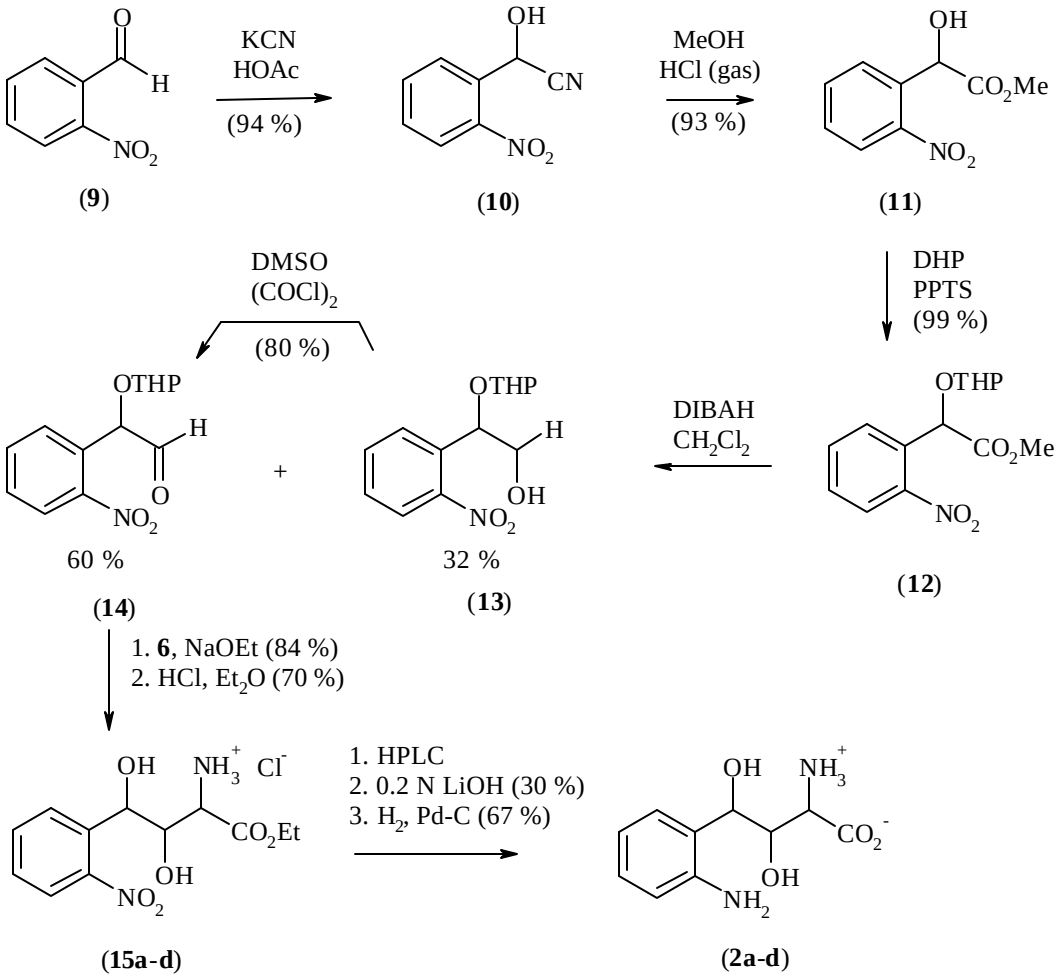


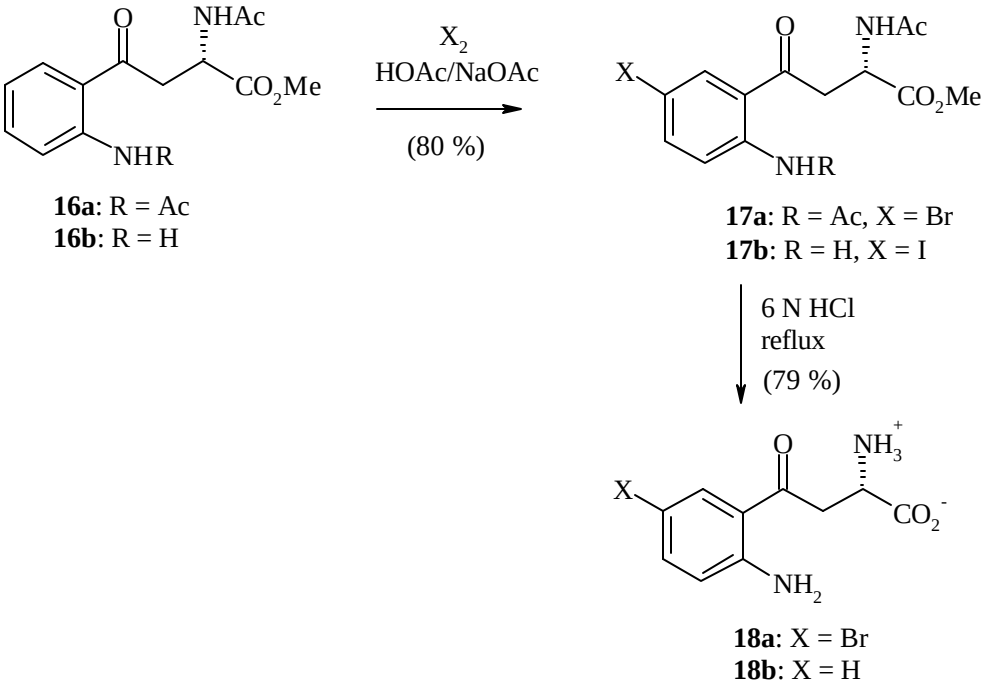
(**3a**) (**4a**) (**5a**) (**6**) (**7aa-ac**) (**8aa-ac**) (**1aa-ac**)

Scheme 1.



(**9**) (**10**) (**11**) (**12**) (**13**) (**14**) (**15a-d**) (**2a-d**)

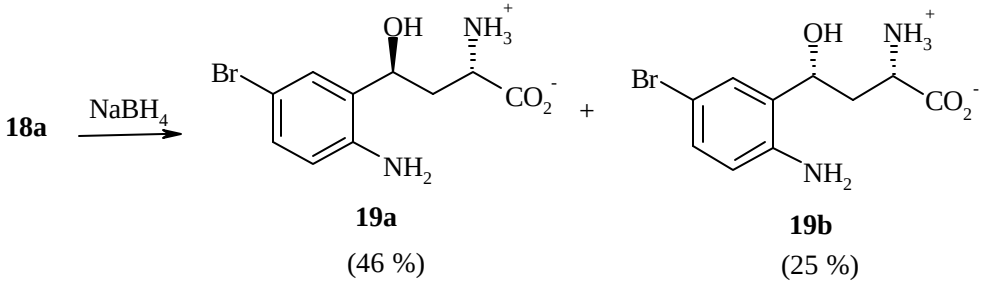
Scheme 2.



16a: R = Ac
16b: R = H

17a: R = Ac, X = Br
17b: R = H, X = I

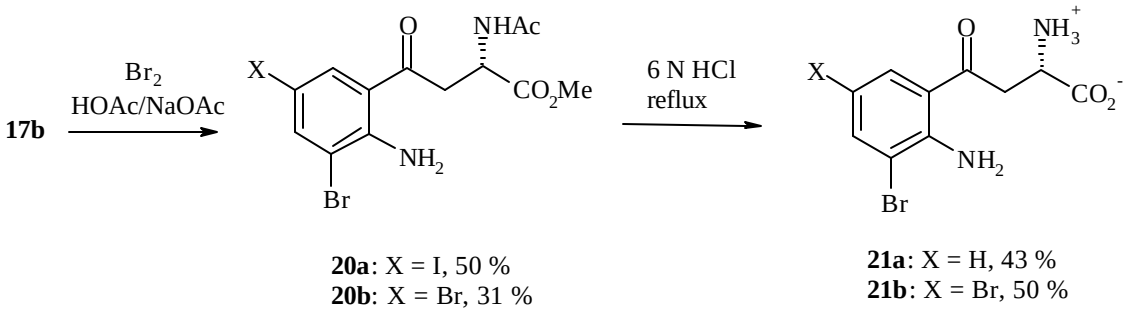
18a: X = Br
18b: X = H



19a
(46 %)

19b
(25 %)

Scheme 3.



20a: X = I, 50 %
20b: X = Br, 31 %

21a: X = H, 43 %
21b: X = Br, 50 %

Scheme 4.

Table 1. Kinetic parameters

Compound	K_i (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ s^{-1}$)
Kynurenine	-	6.9	25	0.28
1aa	8.4×10^{2}	-	-	-
1ab	$> 5 \times 10^{3}$	-	-	-
1bc	$> 5 \times 10^{3}$	-	-	-
2a,c	3.1	8.9×10^{-4}	3.1	2.9×10^{-4}
2b	7.5	2.3×10^{-4}	7.5	3.1×10^{-5}
19a	0.17	-	-	-
19b	5.5×10^{-2}	4.6×10^{-3}	5.2×10^{-2}	8.8×10^{-2}
18a	-	2.1	12	0.18
21a	-	11	2.0×10^{3}	5.5×10^{-3}

CHAPTER II

ASYMMETRIC REDUCTION OF ETHYNYLKETONES AND ETHYNYLKETOESTERS BY SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS*[1]

[1] Heiss, C. and Phillips, R. S. To be submitted to *J. Chem. Soc., Perkin Trans. 1*.

Introduction

Due to their high chemoselectivity, their environmentally benign character, and their increasing availability as a result of rapid progress in genetic engineering, enzymes play an increasingly important role in organic synthesis. Dehydrogenases from various sources have been widely applied to the asymmetric reduction of ketones to give optically active secondary alcohols. Among these enzymes, horse liver alcohol dehydrogenase (HLADH)[1] and yeast alcohol dehydrogenase (YADH)[2] have been the most extensively studied. Unfortunately, both HLADH and YADH exhibit some disadvantages that limit their routine usage in asymmetric synthesis. HLADH gives good results only with cyclic ketones, and YADH reduces ketones with usually low, unreproducible, and hardly predictable enantioselectivity. Furthermore, both enzymes have low thermal stability and tend to lose their activity in the presence of organic solvents or upon immobilization. In contrast, the NADP-dependent alcohol dehydrogenase from *Thermoanaerobium brockii* (TBADH),[3] a highly thermophilic, ethanologenic bacterium, is stable to these conditions and reduces ketones with excellent enantioselectivity.

We have been studying a very similar NADP–dependent enzyme, secondary alcohol dehydrogenase (SADH) from *Thermoanaerobacter ethanolicus*, which was first described by Bryant *et al.*[4] We found that the enantioselectivity with which it reduces ketones depends upon temperature,[5] the nature of the employed cofactor,[6] and substrate structure.[7] The reduction of small ketones like 2-butanone, 3-butyn-2-one (**1** in Scheme 1), and 2-pentanone affords alcohols of low optical purity whereas larger ketones and ketoesters (*e.g.* **3**) give alcohols with ee's ranging from 92 to 99 %.

This behavior of SADH is similar to results obtained from reductions with TBADH. Keinan *et al.*[3] put forth a model of the active site of TBADH proposing a large and a small alkyl binding pocket. They suggested that the substrate binds in an orientation determined by the best fit of the two groups attached to the carbonyl into these pockets. The nicotinamide cofactor then transfers its *pro-R* hydride to the *re* face of the

ketone, producing secondary alcohols with (*S*)-configuration (assuming that the larger substituent has higher Cahn–Ingold–Prelog priority), *i.e*. the enzyme follows Prelog's rule.[8]

Based on the high degree of sequence identity (~99 %) of TBADH and SADH,[9] Keinan's active site model can also be applied to SADH. On this basis, the high enantioselectivity with which SADH reduces the various ketoesters together with the low ee in the case of 3-butyn-2-one led us to believe that if the triple bond in the ketone is "counterbalanced" with an ester function on the opposite side of the carbonyl as in **5** (Scheme 2), it could be forced into the small pocket giving rise to (*R*)-hydroxyalkynoates **6** in high optical purity. These represent useful chiral building blocks for asymmetric syntheses, since they possess three functional groups that can undergo further transformation independently of each other.

In order to obtain a more detailed model and greater predictability of the scope and stereochemistry of SADH-reductions, we also subjected simple alkyl-substituted ethynylketones **7** to the reduction by the enzyme. We were especially interested in the minimum size of the alkyl substituent required to give predominantly (*R*)-alcohols **8**, as well as how branching of the alkyl group would affect the reaction.

Results and Discussion

Synthesis of Ethynylketones and ketoesters. The ethynylketone **7** were prepared by a modification of the method by Walton and Waugh[10] (Scheme 3). *Bis*-(Trimethylsilyl)acetylene (BTMSA) was acylated with the appropriate acyl chloride **9** in the presence of $AlCl_3$ to give the TMS-protected ethynylketone **10**, followed by phase-transfer catalyzed removal of the TMS group with NaF and NBu_4Cl. Essentially the same procedure[11] was employed in the synthesis of the methyl and ethyl esters **5a**,**b**,**d**, and **f**.

The isopropyl esters **5c** and **e** had to be made in a different manner (Scheme 4) because the acylation was accompanied by formal ester hydrolysis (presumably by an

AAL1 type mechanism). The resulting acids **14a** and **b**, however, could easily be re-esterified with 2-propanol yielding the TMS-protected oxoalkynoates **15a** and **b**, and since they are readily prepared from succinic or glutaric anhydride (**13a** and **b**)[12], respectively, this offered a convenient route to the isopropyl oxoalkynoates.

Enzyme reactions. The enzyme reductions were carried out in aqueous buffer containing 15 % isopropanol, which serves as both co–solvent and coenzyme regenerator.

We found that ethynylketones require higher concentrations of SADH for reaction than unconjugated ketones because they are decomposed slowly ($t_{1/2}$ ~ 1d) under the reaction conditions, presumably due to imine formation with TRIS buffer, and, more importantly, they cause irreversible inactivation of the enzyme. We ran the reduction of isopropyl 4-oxo-5-hexynoate (**5c**) and 4-methyl-1-pentyn-3-one (**7c**) with varying amounts of enzyme and found that the minimum concentration of SADH to give reasonable conversions (> 90 %) is 54 U/mL for **5c** and less than 10 U/ml for **7c** (compared to 2 U/mL for unconjugated ketones[7]). Although initially fast, the reactions slowed significantly after 15 – 30 min and stopped after 1 – 2 h at conversions dependent on the enzyme concentration (see the experimental section). The enzyme inactivation is likely caused by conjugate addition of a nucleophilic residue in the active site of the enzyme to the triple bond of the substrate, which is in conjugation with the Zn–coordinated carbonyl. Product inhibition can be excluded because the inactivation takes place even if no product is formed.

Fortunately, the need for large amounts of enzyme poses no major problem since Burdette *et al*. recently cloned SADH and prepared a plasmid to overexpress it in *E. coli.*[9] This new source offers major advantages over cells of *T. ethanolicus*: *E. coli* does not require high temperature anaerobic growth conditions and can therefore be grown easily in large quantities; SADH is produced in larger amounts in the recombinant cells than in *T. ethanolicus*; and, due to the thermal stability of SADH, its purification is greatly simpli-

fied because the *E. coli* proteins can be selectively precipitated by heat treatment of the cell-free extract, resulting in sufficiently pure enzyme for the reductions.

The reduction of the ethynylketones (**7**) are summarized in Table 1. The enantiomeric excess of the alcohols (**8**) was measured by NMR using a lanthanide shift–reagent and their absolute configuration was determined by comparison of the sign of optical rotation with literature values of identical or similar alcohols. Initially (compounds **7a**-**c**), increasing the size of the alkyl substituent from methyl to ethyl to isopropyl leads to higher amounts of the (*S*)-enantiomer with the latter being the only observed product when the alkyl group is isopropyl. Similar behavior was observed in TBADH–reductions of dialkyl ketones by Keinan for TBADH,[3] and was explained by assuming that the small pocket has higher affinity for hydrophobic groups. The observations that 4-methyl-1-pentyn-3-one (**7c**) is reduced with an ee >98 %, that 1-hexyn-3-one (**7e**) prefers the orientation in which the *n*-propyl group, although being substantially larger than the ethynyl group, occupies the small pocket and that 4-methyl-1-heptyn-3-one (**7i**) does not give the reaction indicate that the geometry of the α-carbon partly determines the binding orientation. It seems likely that the sp^3-hybridized α-carbon of the alkyl group suffers some unfavorable interaction with a putative steric restriction in the large pocket close to the catalytic site. If the α-carbon is substituted, this interaction prevents binding in the large pocket completely. With its linear geometry, the sp-carbon of the ethynyl group is less likely to be subject to this kind of steric hindrance. Starting with **7e** substrates substituted with longer alkyl groups give increasingly higher amounts of the (*R*)-enantiomer as is to be expected from Keinan's model.

Whereas secondary carbons are tolerated well (compounds **7c**, **f**, and **k**), tertiary carbons in the substrates result in very slow reaction rates and marginal yields (compounds **7d** and **g**), the ketones being decomposed before significant conversion has taken place. The moderate yields in the other cases can be ascribed to losses due to evaporation and to some decomposition of the substrates under the reaction conditions.

Table 2 lists the results of the SADH-reduction of the ethynylketoesters **5**. The yields are higher the faster the reactions take place, in accordance with both the slow decomposition of the substrates and competition of reduction with inactivation. As expected, the enantioselectivity of the reduction depends on the number of methylenes between the carbonyl and the ester moiety as well as on the size of the latter. Due to the high enzyme concentration employed, the initial rates of reduction were too high to be measured accurately. However, the time required for complete conversion gives some indication of the reaction rates. In contrast to earlier results involving the reduction of aliphatic methylketones, where each additional methylene decreased the reaction rate by a factor of 3,[7] as well as to the observations made with the simple ethynylketones (**7**), increasing the size of the ester function results in faster reaction rates in the 4-oxo-5-hexynoate series (**5a**-**c**) and has no effect on the rates in the 5-oxo-6-heptynoate series (**5d** and **e**). Elongation of the tether also seems to have no marked effect on the rate. The higher enantioselectivities with which the ethynylketoesters are reduced compared to the simple ethynylketones and especially the high enantioselectivity, high yield, and the short reaction time in the case of isopropyl 4-oxo-5-hexynoate (**5c**) cannot be explained by simply invoking the size of the substituents. Apparently, the ester group plays a role in the binding to the active site. This assumption is supported by a revised model of the active site of SADH published by Burdette et al.,[13] which is based on evidence for acetyl-coenzyme A being the physiological substrate for SADH. In this model, the large alkyl-binding pocket is replaced by an open-ended channel that accomodates part of the pantotheine moiety of acetyl-CoA. This part of acetyl-CoA possesses an amide function in the approximate position of the ester group in methyl 6-oxo-7-octynoate. The notable influence of the isopropyl group on the enantioselectivity may be due to favorable hydrophobic interaction with part of the binding channel of the enzyme.

The observation that **7c**, which is reduced to (*S*)-**8c**, inactivates the enzyme much more slowly than **5c**, which yields (*R*)-**6c**, suggests that the putative nucleophilic residue responsible for the inactivation is located in the small alkyl binding pocket.

There is at least one hydroxyalkynoate that is obtained in excellent optical purity for each length of the tether between the carbonyl and ester groups (n = 2,3,4). Therefore, by choosing the appropriate ester, useful chiral building blocks can be readily prepared with this enzymatic reduction methodology. The nature of the ester group should have only minor influence on further transformations. The achiral starting materials are obtained in two (**7** and **5a**,**b**,**d**,**f**) or three (**5c**,**e**) steps from commercially available compounds.

Experimental Section

General. UV-Vis spectra and enzyme assays were recorded on a Varian Cary 1E spectrophotometer equipped with a thermoelectric 6 × 6 cell cuvette changer. Capillary GC was performed on a Varian 3300 gas chromatograph with FI detection (Supelco β-Dex 120 chiral column, 30 m × 0.250 mm i.d., 0.25 μm film thickness) programmed between 40 and 175 °C. ^{1}H and ^{13}C NMR spectra were taken on a Bruker AC-250 or AC-300 spectrometer, respectively, using the residual $CHCl_3$ signal (δ 7.26 ppm) as internal reference. Optical rotations were measured on a Rudolph Autopol IV polarimeter.

Enzyme Assay. SADH was assayed following the increase in absorption at 340 nm due to the formation of NADPH ($\Delta\varepsilon_{340}$ = 6.22 mM^{-1} cm^{-1}) at 50 °C. The assay mixture contained 200 mM *i*-PrOH and 1.25 mM NADP in 50 mM TRIS buffer, pH 8.0. One unit (U) is the amount of enzyme required to reduce 1 μmol of NADP per minute.

Purification of SADH. *E. coli* cells expressing SADH were grown as described.[9] The wet cells (150 g) were suspended in 50 mM TRIS buffer, pH 8.0, (375 mL) containing 0.1 mM dithiothreitol, sonicated, and centrifuged (30 min at 10000 × g, all of the following centrifugations were done under these conditions). The supernatant was heated

in a capped bottle at 70 °C for 1 h, 100 mL buffer was added and the thick suspension again centrifuged. $(NH_4)_2SO_4$ (48.0 g, 20 % saturation) was added to the supernatant in portions, and the mixture was stirred for 1 h at rt. After removal of the precipitate by centrifugation, an additional 110 g $(NH_4)_2SO_4$ (60 % saturation) were added and stirring was continued for 90 min. The mixture was centrifuged, the pellet was dissolved in TRIS buffer (50 mL), and the resulting solution was dialyzed against 50 mM TRIS buffer, pH 8.0, (3 × 4 L). The solution was then heated at 70 °C for 1 h, centrifuged, and lyophilized yielding 2.12 g of SADH as a tan powder with the specific activity of 27 U/mg solid.

Determination of minimum enzyme concentration required for complete conversion. SADH (2.0, 13.5, 27, 54, 81, 108 U) and NADP (0.1 mg, 0.12 μmol) were dissolved in 50 mM TRIS buffer, pH 8.0, (0.85 mL). After pre-incubating the solution for 10 min at 50 °C, a solution of **5c** (16.8 mg, 0.10 mmol) or **7c** (9.6 mg, 0.10 mmol) in i-PrOH (0.15 mL) was added in one portion. The reactions were monitored by extracting an aliquot (40 μL) with CH_2Cl_2 (40 μL) followed by GC analysis (40 - 125 °C, 2 °C/min for **7c**, 40 - 175 °C, 2 °C/min for **5c**). The presence or absence of enzyme activity was assessed by incubating an aliquot (0.2 mL) with 2-pentanone (1.7 mg, 0.02 mmol) for 10 min and checking for the presence of 2-pentanol by GC.

Preparation of Ethynylketones (7).[10] A solution of acyl chloride **9** (10.0 mmol) and BTMSA (1.87 g, 11.0 mmol) in dry CH_2Cl_2 (25 mL) was cooled to 0 °C and $AlCl_3$ (1.60 g, 12.0 mmol) was added in portions during 30 min. The mixture was stirred for 2 h at 0 °C and for 2 h at rt and then poured into a 1 : 1 mixture of 1 *M* HCl and ice (50 mL). The layers were separated and the aqueous phase extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried with Na_2SO_4 and the solvent was removed *in vacuo*. The residue was taken up in Et_2O (40 mL) and the resulting solution was stirred vigorously overnight with an aqueous solution of NaF (0.462 g, 11.0 mmol) and NBu_4Cl (0.31 g, 1.1 mmol). The layers were separated and the aqueous layer extracted with Et_2O

(3 × 25 mL). The combined organic extracts were dried with Na_2SO_4, the solvent was removed *in vacuo*, and the residue chromatographed on silica gel.

1-Pentyn-3-one (7b). Yield 0.62 g (75 %), R_f 0.27 (5 % Et_2O/pet. ether), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.21 (s, 1H), 2.62 (q, *J* = 7.4 Hz, 2H), 1.15 (t, *J* = 7.5 Hz, 3H).

4-Methyl-1-pentyn-3-one (7c). Yield 0.62 g (65 %), R_f 0.28 (5 % Et_2O/pet. ether), ^{1}H-NMR (250 MHz, $CDCl_3$) δ 3.23 (s, 1H), 2.68 (m, 1H), 1.21 (d, *J* = 6.8 Hz, 6H).

4,4-Dimethyl-1-pentyn-3-one (7d). Yield 0.73 g (66 %), R_f 0.36 (5 % Et_2O/pet. ether), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.24 (s, 1H), 1.22 (s, 9H).

1-Hexyn-3-one (7e). Yield 0.70 g (73 %), 0.26 (5 % Et_2O/pet. ether), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.20 (s, 1H), 2.57 (t, *J* = 7.4 Hz, 2H), 1.71 (m, 2H), 0.96 (t, *J* = 7.4 Hz, 3H).

5-Methyl-1-hexyn-3-one (7f). Yield 0.51 g (46 %), R_f 0.30 (5 % Et_2O/pet. ether), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.20 (s, 1H), 2.47 (d, *J* = 7.1 Hz, 2H), 2.27 (m, 1H), 0.97 (d, *J* = 6.3 Hz, 6H).

5,5-Dimethyl-1-hexyn-3-one (7g). Yield 0.78 g (63 %). R_f 0.34 (5 % Et_2O/pet. ether), ^{1}H-NMR (250 MHz, $CDCl_3$) δ 3.23 (s, 1H), 2.51 (s, 2H), 1.06 (s, 9H).

1-Heptyn-3-one (7h). Yield 0.63 g (57 %), R_f 0.29 (5 % Et_2O/pet. ether), ^{1}H-NMR (250 MHz, $CDCl_3$) δ 3.20 (s, 1H), 2.59 (t, *J* = 7.4 Hz, 2H), 1.67 (m, 2H), 1.36 (m, 2H), 0.93 (t, *J* = 7.3 Hz, 3H).

4-Methyl-1-heptyn-3-one (7i). Yield 0.72 g (58 %), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 3.22 (s, 1H), 2.59 (m, *J* = 6.8 Hz, 1H), 1.79 (m, 1H), 1.39 (m, 3H), 1.19 (d, *J* = 7.2 Hz, 3H), 0.93 (t, *J* = 7.2 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.1, 80.9, 79.0, 48.1, 34.5, 20.1, 15.5, 13.9.

6-Methyl-1-heptyn-3-one (7k). Yield 0.61 g (49 %), R_f 0.30 (5 % Et_2O/pet. ether), ^{1}H-NMR (250 MHz, $CDCl_3$) δ 3.22 (s, 1H), 2.58 (t, *J* = 7.5 Hz, 2H), 1.57 (m, 3H), 0.89 (d, *J* = 6.0 Hz, 6H).

Preparation of Methyl and Ethyl Ethynylketoesters (5).[11] To a suspension of $AlCl_3$ (4.00 g, 30.0 mmol) in CH_2Cl_2 (40 mL), cooled to 0 °C, was added dropwise over 30 min a solution of the appropriate acyl chloride **11** (10.0 mmol) and BTMSA (1.87 g, 11.0 mmol) in CH_2Cl_2 (15 mL). After the addition was complete, the mixture was stirred 2 h at 0 °C and 2 h at rt. and poured into a 1:1 mixture of 1 *M* HCl and ice (50 mL). The layers were separated and the aqueous phase extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried with Na_2SO_4 and the solvent was removed *in vacuo*. The residue was taken up in Et_2O (40 mL), and desilylated as described above for **7**.

Methyl 4-oxo-5-hexynoate (5a). Yield 1.18 g (84 %), R_f 0.21 (15 % ethyl acetate/hexanes), ^{1}H-NMR (250 MHz, $CDCl_3$) δ 3.70 (s, 3H), 3.27 (s, 1H), 2.95 (t, *J* = 6.6 Hz, 2H), 2.66 (t, *J* = 6.6 Hz 2H).

Ethyl 4-oxo-5-hexynoate (5b). Yield 0.94 g (61 %), R_f 0.24 (15 % ethyl acetate/hexanes), IR (neat) 3255, 2094, 1731, 1686 cm $^{-1}$. ^{1}H-NMR (250 MHz, $CDCl_3$) δ 4.15 (q, *J* = 7.2 Hz, 2H), 3.26 (s, 1H), 2.94 (t, *J* = 6.6 Hz, 2H), 2.65 (t, *J* = 6.7 Hz, 2H), 1.24 (t, *J* = 7.0 Hz, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 184.9, 171.8, 80.9, 79.1, 60.8, 39.9, 27.7, 14.1. Anal. Calcd for $C_8H_{10}O_3$: C, 62.33; H, 6.54. Found: C, 62.23; H, 6.53.

Ethyl 5-oxo-6-heptynoate (5d). Yield 1.09 g (65 %), R_f 0.22 (10 % ethyl acetate/hexanes), IR (neat) 3248, 2092, 1733, 1684 cm $^{-1}$. ^{1}H-NMR (250 MHz, $CDCl_3$) δ 4.14 (q, *J* = 7.1 Hz, 2H), 3.23 (s, 1H), 2.69 (t, *J* = 7.3 Hz, 2H), 2.36 (t, *J* = 7.3 Hz, 2H), 1.99 (m, 2H), 1.26 (t, *J* = 7.2 Hz, 3H).

Methyl 6-oxo-7-octynoate (5f). Yield 1.36 g (81 %), R_f 0.20 (10 % ethyl acetate/hexanes), IR (neat) 3249, 2092, 1735, 1683 cm $^{-1}$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 3.64 (s, 3H), 3.23 (s, 1H), 2.60 (t, *J* = 6.9 Hz, 2H), 2.31 (t, *J* = 6.9 Hz, 2H), 1.66 (m, 4H);

^{13}C-NMR (75 MHz, $CDCl_3$) δ 186.7, 173.6, 81.2, 78.6, 51.5, 44.9, 33.6, 24.0, 23.0. Anal. Calcd for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found: C, 63.74; H, 7.25.

Preparation of Isopropyl Ethynylketoesters. A 25 mL round-bottom flask fitted with an inverse Dean-Stark trap was charged with the appropriate acid **14**[12] (10.0 mmol), *i*-PrOH (3.82 mL, 50.0 mmol), $CHCl_3$ (5.0 mL), and TsOH (50 mg, 0.29 mmol). The solution was heated to reflux for 20 h, and, after cooling, washed with water and dried with Na_2SO_4. The solvent was removed in vacuo and the residue was taken up in Et_2O. Desilylation and workup were performed as described above.

Isopropyl 4-Oxo-5-hexynoate (5c). Yield 1.41 g (84 %), R_f 0.21 (10 % ethyl acetate/hexanes), IR (neat) 3253, 2094, 1729, 1685 cm $^{-1}$. ^{1}H-NMR (250 MHz, $CDCl_3$) δ 5.00 (m, 1H), 3.26 (s, 1H), 2.91 (t, *J* = 6.6 Hz, 2H), 2.61 (t, *J* = 6.6 Hz, 2H), 1.22 (d, *J* = 6.2 Hz, 6H); ^{13}C-NMR (62.5 MHz, $CDCl_3$) δ 185.0, 171.2, 80.9, 78.9, 68.2, 39.9, 28.0, 21.6. Anal. Calcd for $C_9H_{12}O_3$: C, 64.27; H, 7.19. Found: C, 64.29; H, 7.18.

Isopropyl 5-Oxo-6-heptynoate (5e). Yield 1.35 g (74 %), Rf 0.25 (10 % ethyl acetate/hexanes), IR (neat) 3249, 2092, 1728, 1684 cm $^{-1}$. ^{1}H-NMR (300 MHz, $CDCl_3$) δ 5.00 (m, 1H,), 3.23 (s, 1H), 2.67 (t, *J* = 7.1 Hz, 2H), 2.32 (t, *J* = 7.4 Hz, 2H), 1.97 (m, 2H), 1.22 (d, *J* = 6.1 Hz, 6H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 186.4, 172.3, 81.3, 78.7, 67.8, 44.3, 33.3, 21.8, 18.8. Anal. Calcd for $C_{10}H_{14}O_3$: C, 65.92; H, 7.74. Found: C, 65.88; H, 7.72.

Enzymatic Reduction of Ethynylketones and Ethynylketoesters with SADH. SADH (1000 U) and NADP (1 mg, 1.2 μmol) were dissolved in 50 mM TRIS buffer, pH 8.0, (8.5 mL). After preincubation for 10 min at 50 °C, the appropriate ketone **5** or **7** (1.0 mmol) was added in one portion and the mixture was kept at 50 °C. When complete conversion was obtained (GC), the reaction mixture was saturated with NaCl and extracted with Et_2O (3 × 4 mL). Due to their tendency to form emulsions, the extractions had to be centrifuged (10 min at 4000 rpm) prior to separation. The combined extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed.

Determination of Optical Purity of the Alcohols. The alcohol (0.02 mmol) was dissolved in $CDCl_3$ (0.3 mL) in an NMR tube and a 50 mM solution of $Eu(hfc)_3$ in $CDCl_3$ (0.5 mL) was added in portions of 0.1 mL. A ^{1}H-NMR spectrum was taken after each addition, and the two signals corresponding to the diastereotopic carbinol protons were integrated. If only one signal was visible, the enantiomeric excess was assumed to be greater than 98 %.

(*S*)-1-Pentyn-3-ol (8b). Yield 30.3 mg (36 %), 80 % ee, R_f 0.20 (10 % ether/pet. ether), $[\alpha]^{20}_D$ -19.2 (c = 12, dioxane) (lit.[14] $[\alpha]^{25}_D$ +23.15 (c = 2, dioxane), 86 % ee for (R)-enantiomer), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.32 (dt, *J* = 6.5 Hz, 2.0 Hz, 1H), 2.46 (d, *J* = 1.8 Hz, 1H), 1.74 (m, 3H), 1.02 (t, *J* = 7.4 Hz, 3H).

(*S*)-4-Methyl-1-pentyn-3-ol (8c). Yield 49.1 mg (50 %), >98 % ee, R_f 0.22 (10 % ether/pet. ether), $[\alpha]^{20}_D$ -15.7 (c = 13, dioxane) (lit.[14] $[\alpha]^{20}_D$ +13.8 (c = 2, dioxane), 86 % ee for (R)-enantiomer), ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.17 (dd, *J* = 5.7 Hz, 2.0 Hz, 1H), 2.45 (d, *J* = 2.3 Hz, 1H), 1.89 (m, 2H), 1.01 (m, 6H).

(*S*)-1-Hexyn-3-ol (8e). Yield 27.5 mg (28 %), 51 % ee, R_f 0.22 (10 % ether/pet. ether), $[\alpha]^{20}_D$ -4.5 (c = 1.5, $CHCl_3$) (lit.[15] $[\alpha]^{20}_D$ +9.0 (c =1.0, $CHCl_3$), 75 % ee for (R)-enantiomer), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.39 (dt, *J* = 6.5 Hz, 2.2 Hz, 1H), 2.47 (d, *J* = 2.1 Hz, 1H), 1.71 (m, 3H), 1.50 (m, 2H), 0.97 (t, *J* = 7.0 Hz, 3H).

(*R*)-5-Methyl-1-hexyn-3-ol (8f). Yield 22.4 mg (20 %), 50 % ee, R_f 0.20 (10 % ether/pet. ether), $[\alpha]^{20}_D$ +18.3 (c = 1.2, dioxane) (lit.[14] $[\alpha]^{25}_D$ +28.8 (c = 3, dioxane), 88 % ee for (R)-enantiomer), ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.42 (dt, *J* = 7.4 Hz, 2.3 Hz, 1H), 2.46 (d, *J* = 2.1 Hz, 1H), 1.87 (m, 1H), 1.60 (m, 3H), 0.95 (d, *J* = 6.7 Hz, 3H), 0.93 (d, *J* = 6.8 Hz, 3 H).

(*R*)-1-Heptyn-3-ol (8h). Yield 30.3 mg (27 %), 42 % ee, R_f 0.19 (10 % ether/pet. ether), $[\alpha]^{20}_D$ +3.8 (c = 5.8, $CHCl_3$) (lit.[15] $[\alpha]^{20}_D$ -5.5 (c = 0.9, $CHCl_3$), 70 % ee for (S)-enantiomer), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.37 (dt, *J* = 7.3 Hz, 2.1 Hz, 1H), 2.47 (d, *J* = 2.1 Hz, 1H), 1.87 (bs, 1H), 1.72 (m, 2H), 1.41 (m, 4H), 0.92 (t, *J* = 7.0 Hz, 3H).

(*R*)-6-Methyl-1-heptyn-3-ol (8k). Yield 61.8 mg (49 %), 80 % ee, R_f 0.21 (10 % ether/pet. ether), $[\alpha]^{20}_D$ +9.2 (c = 1.2, $CHCl_3$), +13.8 (c = 2.0, dioxane), ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.35 (dt, *J* = 6.6 Hz, 1.9 Hz, 1H), 2.47 (d, *J* = 1.9 Hz, 1H), 2.03 (bs, 1H), 1.71 (m, 2H), 1.57 (m, 1H), 1.33 (m, 2H), 0.89 (d, *J* = 6.5 Hz, 6H).

(*R*)-Methyl 4-hydroxy-5-hexynoate (6a). Yield 49.8 mg (35 %), 82 % ee, R_f 0.20 (20 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +9.9 (c = 1.8, $CHCl_3$), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.49 (dt, *J* = 6.0 Hz, 2.0 Hz, 1H), 3.69 (s, 3H), 2.55 (m, 2H), 2.49 (d, *J* = 2.1 Hz, 1H), 2.21 - 1.98 (m, 3H).

(*R*)-Ethyl 4-hydroxy-5-hexynoate (6b). Yield 79.6 mg (51 %), 90 % ee, R_f 0.18 (15 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +17.9 (c = 2.0, $CHCl_3$), IR (neat) 3443, 3291, 2112, 1732 cm^{-1}; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.49 (dt, *J* = 6.1 Hz, 2.1 Hz, 1H), 4.14 (q, *J* = 7.1 Hz, 2H), 2.52 (m, 2H), 2.48 (d, *J* = 1.7 Hz, 1H), 2.21 (bs, 1H), 2.04 (m, 2H), 1.26 (t, *J* = 7.1 Hz, 3H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.9, 97.6, 84.0, 73.4, 61.3, 60.7, 32.1, 29.8, 14.1.

(*R*)-Isopropyl 4-hydroxy-5-hexynoate (6c). Yield 150 mg (88 %), >98 % ee, R_f 0.21 (15 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +13.1 (c = 2.0, $CHCl_3$), IR (neat) 3442, 3293, 2114, 1731 cm^{-1}; ^{1}H -NMR (250 MHz, $CDCl_3$) δ 5.02 (m, 1H), 4.49 (dt, *J* = 6.1 Hz, 2.0 Hz, 1H), 2.51 (m, 3H), 2.03 (m, 2H), 1.24 (d, *J* = 6.4 Hz, 6H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.2, 84.0, 73.3, 68.1, 61.2, 32.2, 30.2, 21.7.

(*R*)-Ethyl 5-hydroxy-6-heptynoate (6d). Yield 129 mg (76 %), 97 % ee, R_f 0.22 (20 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +16.9 (c = 1.66, CCl_4), +7.8 (c = 3.8, $CHCl_3$) (lit.[16] $[\alpha]^{20}_D$ -16.4 (c = 3.30, CCl_4), 96 % ee for (S)-enantiomer); ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.38 (dt, *J* = 6.2 Hz, 1.8 Hz, 1H), 4.12 (q, *J* = 7.2 Hz, 2H), 2.46 (d, *J* = 2.2 Hz, 1H), 2.35 (t, *J* = 7.0 Hz, 2H), 2.24 (bs, 1H), 1.78 (m, 4H), 1.24 (t, *J* = 7.1 Hz, 3H).

(*R*)-Isopropyl 5-hydroxy-6-heptynoate (6e). Yield 139 mg (76 %), >98 % ee, R_f 0.24 (20 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +10.3 (c = 2.6, $CHCl_3$), IR (neat) 3433, 3291, 2112, 1727 cm^{-1}; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 5.01 (m, 1H), 4.40 (dt, *J* = 6.0 Hz, 2.6

Hz, 1H), 2.47 (d, *J* = 2.6 Hz, 1H), 2.33 (t, *J* = 6.9 Hz, 2H), 2.03 (bs, 1H), 1.78 (m, 4H), 1.23 (d, *J* = 6.2 Hz, 6H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 173.0, 84.5, 73.0, 67.7, 61.7, 36.7, 34.0, 21.8, 20.4.

(*R*)-Methyl 6-hydroxy-7-octynoate (6f). Yield 115 mg (68 %), >98 % ee, R_f 0.22 (20 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +8.8 (c = 2.2, $CHCl_3$), IR (neat) 3434, 3289, 2111, 1734 cm^{-1}; ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.36 (dt, *J* = 6.5 Hz, 2.1 Hz, 1H), 3.65 (s, 3H), 2.45 (d, *J* = 2.5 Hz, 1H), 2.32 (t, *J* = 7.4 Hz, 2H), 2.22 (bs, 1H), 1.79 - 1.60 (m, 4H), 1.50 (m, 2H); ^{13}C-NMR (75 MHz, $CDCl_3$) δ 174.1, 84.8, 73.0, 62.0, 51.6, 37.1, 33.9, 24.5 (2 ×).

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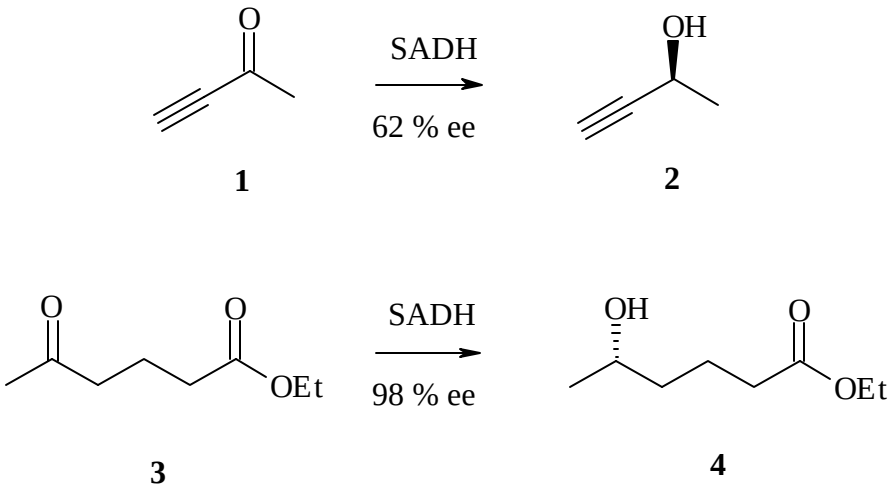
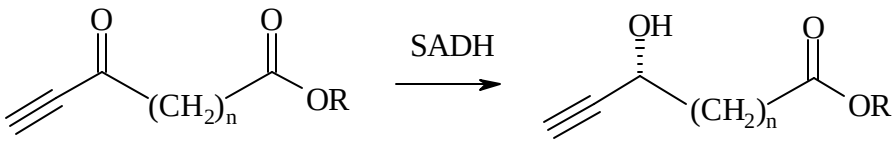
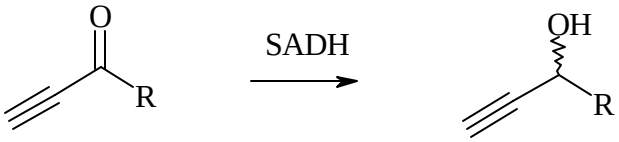


Scheme 1.

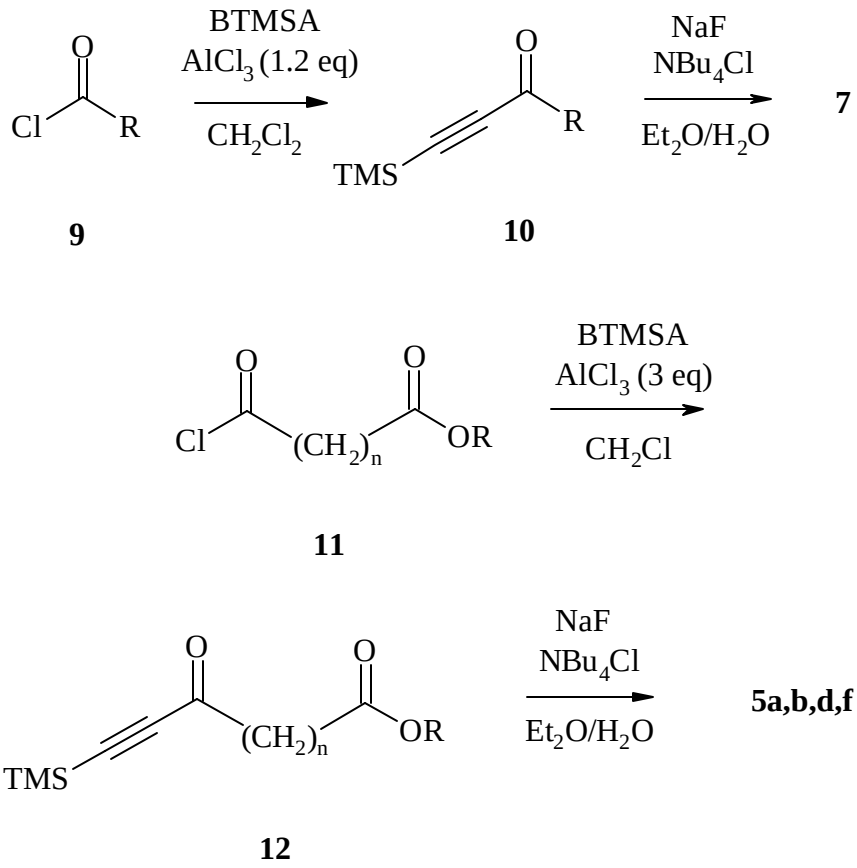


5 **6**

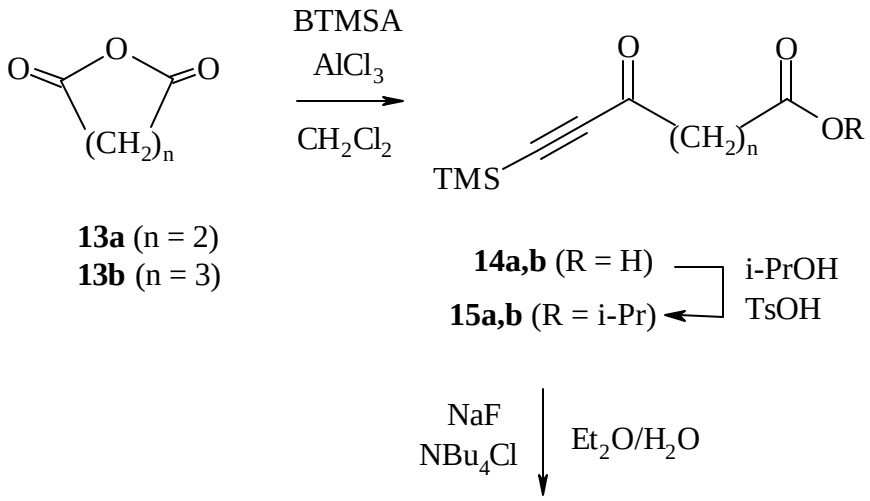


7 **8**

Scheme 2.

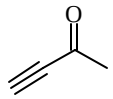
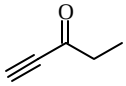
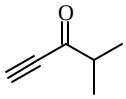
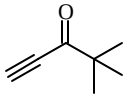
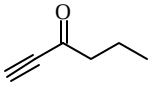
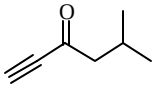
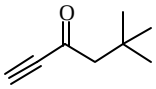
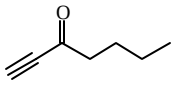
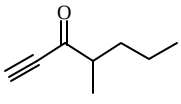
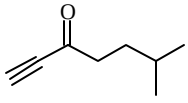

BTMSA
AlCl3 (1.2 eq)
CH2Cl2
NaF
NBu4Cl
Et2O/H2O
7
9
10
BTMSA
AlCl3 (3 eq)
CH2Cl
11
NaF
NBu4Cl
Et2O/H2O
5a,b,d,f
12

Scheme 3.


BTMSA
AlCl3
CH2Cl2
O
O
O
(CH2)n
TMS
O
O
(CH2)n
OR
13a (n = 2)
13b (n = 3)
14a,b (R = H)
15a,b (R = i-Pr)
i-PrOH
TsOH
NaF
NBu4Cl
Et2O/H2O
5c,e

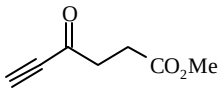
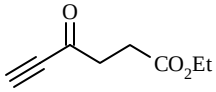
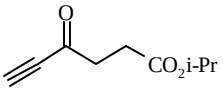
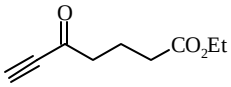
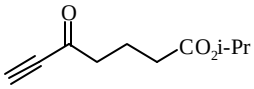
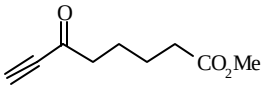
Scheme 4.

Table 1. SADH reduction of ethynylketones.

compd. number	compound	yield (%)	abs. conf.	ee (%)	reaction time (h)
7a[a]		30	S	60	0.5
7b		32	S	80	1
7c		50	S	>98	2
7d		-	S	85[b]	24
7e		28	S	51	1
7f		20	R	50	1
7g		-	R	66[b]	7
7h		32	R	42	4
7i		-	-	-	-
7k		55	R	80	5

[a] From ref. 7. [b] No product was isolated but the absolute configuration and ee were determined by GC.

Table 2. SADH reduction of ethynylketoesters.

compd. number	compound	yield (%)	abs. conf.	ee (%)	reaction time (h)
5a		35	R	82	2.5
5b		51	R	90	1.5
5c		88	R	>98	1.0
5d		76	R	97	1.5
5e		76	R	>98	1.5
5f		68	R	>98	2.5

CHAPTER III

EFFECT OF THE MUTATION CYSTEINE-295 TO ALANINE ON THE ENANTIOSELECTIVITY OF REDUCTIONS CATALYZED BY SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS*[1]

[1] Heiss, C.; Phillips, R. S. and Zeikus, J. G. To be submitted to *Biochemistry*.

Introduction

Alcohol dehydrogenases (ADH's) have been used extensively for the asymmetric synthesis of chiral building blocks because of their high chemoselectivity, enantioselectivity, tolerance of a wide spectrum of functional groups, and their relative ease of use.[1] In the last few years, a number of ADH's have been isolated from thermophilic organisms, the most prominent one being from *Thermoanaerobacter brockii*.[2] Due to their high stability to heat, organic co–solvents, and immobilisation, these enzymes have received considerable attention. Although thermostable ADH's have been shown to reduce many different ketones enantioselectively bearing a variety of other functionalities to the corresponding secondary alcohols, there is still a need to further explore their synthetic scope and to deepen our knowledge of their stereochemistry.

In an earlier study,[3] we investigated the asymmetric reduction of ethynyl substituted ketones by secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (SADH) and found that trifunctional chiral building blocks are obtained in good yield and excellent enantiomeric excess. We also showed that, although ethynylketones are substrates for SADH and the stereospecificity of their reduction clearly follows the established "pocket"-model,[4] these α,β-unsaturated ketones also irreversibly inactivate the enzyme, which makes necessary the use of relatively large enzyme concentrations. We had postulated that a nucleophilic residue in the active site might cause the inactivation by adding to the electron-deficient triple bond in the ethynylketone substrates.

In the present study, we investigated the effect of the mutation Cys295 → Ala on the inactivation by ethynylketones and on the enantioselectivity of SADH.

Results and Discussion

We examined the X-ray crystal structure[5] of *T. brockii* ADH (1YKF.PDB) to find the location and orientation in which the substrates bind to the enzyme and, if possible, to determine whether there is a nucleophilic active site residue in the active site that could

be responsible for the observed irreversible inactivation of the enzyme by the α,β-unsaturated ketones. Various low–energy conformations (Tripos force-field)[6] of isopropyl 4-oxo-5-hexynoate were docked using the Sybyl[6] program into the active site in such a way that the carbonyl oxygen was in close proximity to the catalytic Zn ion and to the pro-*R* hydrogen in the 4-position of the nicotinamide ring of the NADPH cofactor. The substrate had to be oriented such that hydride transfer would occur on its *si*-face. In order to accommodate the substrate, the nicotinamide ring was turned by rotation about the C-N bond between the pyridine nitrogen and the ribose C1. After thus obtaining reasonable starting structures, these were energy-minimized using the TRIPOS force-field and examined for validity. Only one single structure was found that was free from severe steric overlaps (Fig. 1). In this structure the triple bond protrudes into a region enclosed by the residues His59, Ala85, Ile86, Asp150, Thr153, Cys295. These residues make up the "small alkyl binding pocket". The alkyl chain bearing the ester group occupies space surrounded by Cys37, Ser39, Leu107, Trp110, Tyr267, Gly293, Leu294, and the nicotinamide ring of the cofactor. The nicotinamide ring is also very close in space to the hydrogens on C3 of the substrate which explains that any substitution in this position precludes binding in this orientation as can be seen in the lack of reactivity of 4-methyl-1-heptyn-3-one (entry 8 in Table 1) with wild–type SADH and in the high enantiomeric excess with which 4-methyl-1-pentyn-3-one (entry 2) is reduced. The ester carbonyl oxygen is within hydrogen bonding distance to Ser39 and although no considerable difference in enantiomeric excess or yield between ethynylketoesters and simple ethynyl ketones of comparable size were found, the esters react much faster than the simple ketones.[3] Moreover, hydrogen bonding may take place between Ser39 and the amide nitrogen in SADH's physiological substrate acetylcoenzyme A.[7] That Ser39 is part of the large pocket in SADH is also suggested by the finding that the mutation Ser39 → Thr shifts the enantioselectivity for 2-butanol and 2-pentanol toward the (*R*)-enantiomers.[8] The sulfhydryl group of Cys295 is in close proximity to the acetylenic C1 of the substrate, setting it

up for 1,4-addition to the electrophilic triple bond. Furthermore, this addition would be facilitated by the Zn ion acting as Lewis acid. Therefore, we hypothesized that Cys295 is responsible for the inactivation of the enzyme observed with enones and ynones. Replacement of Cys295 with a non-nucleophilic residue by site-directed mutagenesis should therefore abolish the observed inactivation. We chose the mutation Cys295 → Ala in the hope that enzymatic activity would not be greatly affected by the mutation. The mutation should furthermore result in an enlargement of the small alkyl binding pocket due to the absence of the large sulfur atom, a change which should be reflected in a change in enantioselectivity. Since a larger "small" pocket would accommodate larger substituents, an overall shift of enantioselectivity toward (*S*) (for ethynyl ketones) should occur.

The specific activity of C295A SADH (43 U/(mg protein)) is only slightly lower than that of the wild-type enzyme (54 U/mg).[9] Ethynyl ketones were subjected to the reduction by SADH C295A. The α,β-unsaturated ketones caused inactivation of the mutant enzyme in the same manner and extent as was found with the wild-type. Thus, contrary to our hypothesis, Cys295 is not involved in the mechanism of inactivation. However, we observed a considerable effect of the mutation on the enantioselectivity of the reductions, as can be seen in Table 1. The enantiomeric effect is largely unaffected with the small substrates (entries 1-3) because the outcome of the reactions is governed by the degree of steric interaction with the large pocket (*i.e.* the nicotinamide ring) rather than by the size of the small pocket, which can easily accommodate any of the substituents in this group. The steric interaction with the cofactor is smallest with the ethynyl group due to its linear geometry, hence the preference for the binding orientation that leads to the (*S*)-enantiomers. The substrates of intermediate size, however, experience a large shift of enantioselectivity toward (*S*), indicating that the small pocket in the mutant is able to house significantly larger groups than in the wild-type. There is a reversal of enantioselectivity for entries 5 and 7. Further evidence for the importance of steric interactions with α-substituents by NADPH comes from entry 8: 4-methyl-1-heptyn-3-one, which does not react

with wild-type SADH, is reduced by C295A SADH to give a 2 : 1 mixture of (3*S*,4*S*) and (3*S*,4*R*)-diastereomers with an enantiomeric excess greater than 98 %. The small pocket is now large enough to contain the *sec*-pentyl group whereas binding of this group in the large pocket is still precluded because of steric interactions between NADPH and the α-methyl group in this substrate. As the size of the alkyl group is increased further, the influence of the mutation decreases again (entry 9).

We have previously studied the temperature dependence of the enantiospecificity of wild-type[10] and S39T[8] SADH and found that both enzymes increase in (*R*)-specificity with increasing temperature. In order to find out whether C295A SADH exhibits similar temperature-dependent behavior, we measured k_{cat}/K_m at various temperatures between 288 and 328 K for the oxidation of the (*R*)- and (*S*)-enantiomers of 2-butanol, 2-pentanol, and 2-hexanol by C295A SADH (Fig. 2). The stereospecificity for (*S*)-2-hexanol is reduced considerably with C295A SADH as expected as a reflection of the larger size of the small alkyl binding pocket. The effect is smaller for 2-pentanol, although the enantioselectivity shifts toward a slight preference for (*R*)-2-pentanol. For 2-butanol, finally, C295A SADH is more specific for the (*R*)-enantiomer than wild-type at low temperature and less so above 306 K.

In contrast to the observed temperature–dependent behaviour of wild-type and S39T SADH, C295A SADH shows no temperature dependence on stereospecificity. We have previously related the stereospecificity to the difference in change of entropy of activation between (*R*)- and (*S*)-alcohols according to $-RT\ln E = \Delta\Delta H^{\ddagger} - T\Delta\Delta S^{\ddagger}$.[10] Since with C295A SADH there is no temperature dependence of $-RT\ln E$, $\Delta\Delta S^{\ddagger} = 0$. Thus, $\Delta\Delta S^{\ddagger}$ in the wild-type SADH is apparently associated with the rotational entropy of the Cys295 sulfhydryl group. Although this connection seems to be evident, it is difficult to rationalize in terms of molecular interactions. It would be expected that binding of a larger group rather than a smaller one would restrict the freedom of motion of the sulfhy-

dryl group. However, the data suggest that the loss of entropy is greater upon binding of the smaller group in the small pocket.

Burdette *et al*. found that SADH from *T. ethanolicus* acts as a thioesterase, reducing the acetyl group of thioacetates to give the thiol and acetaldehyde, which is further reduced to ethanol. It was suggested that acetylcoenzyme A is the physiological substrate for SADH.[7] Within this context, we examined whether Cys295 is involved in the thioesterase mechanism of SADH. If so, thioesterase acvtivity should be greatly reduced or abolished in C295A SADH. The kinetic parameters for ethylthioacetate and acetylcoenzyme A (Table 2) show only a small difference between wild–type and C295A SADH. These differences are not significant enough to indicate any involvement of Cys295 in the chemical mechanism of thioester cleavage.

The results obtained from the mutation Cys295 → Ala presented here give a good indication of the location and orientation of the ethynyl ketone substrates in the enzyme–substrate complex. The residues that comprise the large and small alkyl binding pockets were clearly identified. The modification of the small alkyl binding pocket by site-directed mutagenesis resulted in a significant change in enantioselectivity to give more of the anti-Prelog product.

Starting with this active–site model, together with our previous results from the mutation Ser39 → Thr, further mutations could result in even higher specificity for (R)-alcohols. Although the reason for the observed enzyme inactivation by enones and ynones and possible remedies thereof have not yet been found, and although, for this reason large amounts of enzyme are required for these particular substrates, the ease of isolation, and thermal and solvent stability, make SADH a useful catalyst for the preparation of chiral building blocks. The increasing knowledge of its active site should also help make it a valuable synthetic tool. Studies to uncover the mechanism of inactivation, as well as investigations on different mutants are underway in our laboratories.

Experimental

General. Enzyme assays and kinetic experiments were recorded on a Varian Cary 1E UV/visible spectrophotometer equipped with a Peltier thermoelectric temperature-controlled 6 × 6 cell cuvette changer. Capillary GC was performed on a Varian 3300 gas chromatograph with FI detection (Supelco β-Dex 120 chiral column, 30 m × 0.250 mm i.d., 0.25 μm film thickness) programmed between 40 and 175 °C. ^{1}H and ^{13}C NMR spectra were taken on a Bruker AC-250 or AC-300 spectrometer, respectively, using the residual $CHCl_3$ signal (δ 7.26 ppm) as internal reference. Optical rotations were measured on a Rudolph Autopol IV polarimeter.

Enzyme Assay. SADH was assayed following the increase in absorption at 340 nm due to the formation of NADPH ($\Delta\varepsilon_{340}$ = 6.22 mM^{-1} cm^{-1}) at 50 °C. The assay mixture contained 200 mM i-PrOH and 1.25 mM NADP in 50 mM TRIS buffer, pH 8.0. One unit (U) is the amount of enzyme required to reduce 1 μmol of NADP per minute.

Purification of SADH. (a) For asymmetric reductions. *E. coli* cells expressing SADH were grown as described.[9] The wet cells (12 g) were suspended in 50 mM TRIS buffer, pH 8.0, (50 mL) containing 0.1 mM dithiothreitol, sonicated, and centrifuged (30 min at 10000 × g, all of the following centrifugations were done under these conditions). The supernatant was heated in a capped bottle at 70 °C for 1 h, and the thick suspension was centrifuged. This heat treatment was repeated once, whereupon the supernatant had an activity of 92 U/ml and a specific activity of 27 U/mg protein.

(b) For determination of kinetic parameters. The enzyme solution obtained under (a) was further purified by affinity chromatography as previously described[8] and gave C295A SADH with a specific activity of 43 U/mg protein.

Kinetic experiments. Determination of k_{cat}/K_m values for the enantiomers of 2-butanol, 2-pentanol, and 2-hexanol was carried out at five different temperatures between 15 and 55 °C as described previously.[8]

Preparation of Ethynylketones (7).[11] A solution of acyl chloride **9** (10.0 mmol) and BTMSA (1.87 g, 11.0 mmol) in dry CH_2Cl_2 (25 mL) was cooled to 0 °C and $AlCl_3$ (1.60 g, 12.0 mmol) was added in portions during 30 min. The mixture was stirred for 2 h at 0 °C and for 2 h at rt and then poured into a 1 : 1 mixture of 1 N HCl and ice (50 mL). The layers were separated and the aqueous phase extracted with CH_2Cl_2 (3 × 30 mL). The combined organic extracts were dried with Na_2SO_4 and the solvent was removed *in vacuo*. The residue was taken up in Et_2O (40 mL) and the resulting solution was stirred vigorously overnight with an aqueous solution of NaF (0.462 g, 11.0 mmol) and NBu_4Cl (0.31 g, 1.1 mmol). The layers were separated and the aqueous layer extracted with Et_2O (3 × 25 mL). The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed on silica gel.

Enzymatic Reduction of Ethynylketones with SADH C295A. SADH C295A (600 U) and NADP (1 mg, 1.2 μmol) were dissolved in 50 mM TRIS buffer, pH 8.0, (8.5 mL). After preincubation for 10 min at 50 °C, the appropriate ketone (1.0 mmol) was added in one portion and the mixture was kept at 50 °C. When complete conversion was obtained (GC), the reaction mixture was saturated with NaCl and extracted with Et_2O (3 × 4 mL). Due to their tendency to form emulsions, the extractions had to be centrifuged (10 min at 4000 rpm) prior to separation. The combined extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed.

Determination of Optical Purity of the Alcohols. The alcohol (0.02 mmol) was dissolved in $CDCl_3$ (0.3 mL) in an NMR tube and a 50 mM solution of $Eu(hfc)_3$ in $CDCl_3$ (0.5 mL) was added in portions of 0.1 mL. A ^{1}H-NMR spectrum was taken after each addition, and the two signals corresponding to the diastereotopic carbinol protons were integrated. If only one signal was visible, the enantiomeric excess was assumed to be greater than 98 %.

(*S*)-1-Pentyn-3-ol. Yield 32.7 mg (39 %), 76 % ee, R_f 0.20 (10 % ether/pet. ether), ^{1}H-NMR (300 MHz, $CDCl_3$) δ 4.32 (dt, *J* = 6.5 Hz, 2.0 Hz, 1H), 2.46 (d, *J* = 1.8 Hz, 1H), 1.74 (m, 3H), 1.02 (t, *J* = 7.4 Hz, 3H).

(*S*)-4-Methyl-1-pentyn-3-ol. Yield 86.3 mg (88 %), >98 % ee, R_f 0.22 (10 % ether/pet. ether), ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.17 (dd, *J* = 5.7 Hz, 2.0 Hz, 1H), 2.45 (d, *J* = 2.3 Hz, 1H), 1.89 (m, 2H), 1.01 (m, 6H).

(*S*)-4,4-Dimethyl-1-pentyn-3-ol. Yield 13.2 mg (22 %, based on 53 % conversion), 85 % ee, R_f 0.22 (10 % ether/pet. ether), $[\alpha]^{20}_D$ –10.0 (c = 1.5, dioxane) (lit. $[\alpha]^{20}_D$ +35.6 (c =3, dioxane), 90 % ee for (R)-enantiomer), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.02 (d, *J* = 2.1 Hz, 1H), 2.45 (d, *J* = 2.2 Hz, 1H), 1.8 (bs, 1H), 1.00 (s, 9H).

(*S*)-1-Hexyn-3-ol. Yield 48.1 mg (51 %), 76 % ee, R_f 0.22 (10 % ether/pet. ether), $[\alpha]^{20}_D$ –9.9 (c = 2.5, $CHCl_3$) (lit. $[\alpha]^{20}_D$ +9.0 (c =1.0, $CHCl_3$), 75 % ee for (R)-enantiomer), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.39 (dt, *J* = 6.5 Hz, 2.2 Hz, 1H), 2.47 (d, *J* = 2.1 Hz, 1H), 1.71 (m, 3H), 1.50 (m, 2H), 0.97 (t, *J* = 7.0 Hz, 3H).

(*S*)-5-Methyl-1-hexyn-3-ol. Yield 47.3 mg (42 %), 56 % ee, R_f 0.20 (10 % ether/pet. ether), $[\alpha]^{20}_D$ –16.1 (c = 2.3, dioxane) (lit. $[\alpha]^{25}_D$ +28.8 (c = 3, dioxane), 88 % ee for (R)-enantiomer), ^{1}H -NMR (300 MHz, $CDCl_3$) δ 4.42 (dt, *J* = 7.4 Hz, 2.3 Hz, 1H), 2.46 (d, *J* = 2.1 Hz, 1H), 1.87 (m, 1H), 1.60 (m, 3H), 0.95 (d, *J* = 6.7 Hz, 3H), 0.93 (d, *J* = 6.8 Hz, 3 H).

(*S*)-1-Heptyn-3-ol. Yield 67.8 mg (60 %), 67 % ee, R_f 0.19 (10 % ether/pet. ether), $[\alpha]^{20}_D$ –8.5 (c = 2.6, $CHCl_3$) (lit. $[\alpha]^{20}_D$ -5.5 (c = 0.9, $CHCl_3$), 70 % ee for (S)-enantiomer), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.37 (dt, *J* = 7.3 Hz, 2.1 Hz, 1H), 2.47 (d, *J* = 2.1 Hz, 1H), 1.87 (bs, 1H), 1.72 (m, 2H), 1.41 (m, 4H), 0.92 (t, *J* = 7.0 Hz, 3H).

(*R*)-Methyl 4-Hydroxy-5-hexynoate. Yield 15.8 mg (23 %, based on 48 % conversion), 60 % ee, R_f 0.20 (20 % ethyl acetate/hexanes), $[\alpha]^{20}_D$ +7.0 (c = 1.8, $CHCl_3$), ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.49 (dt, *J* = 6.0 Hz, 2.0 Hz, 1H), 3.69 (s, 3H), 2.55 (m, 2H), 2.49 (d, *J* = 2.1 Hz, 1H), 2.21 - 1.98 (m, 3H).

(3*S*,4*S*)- and (3*S*,4*R*)-4-Methyl-1-heptyn-3-ol. (±)-4-Methyl-1-heptyn-3-one (100 mg, 0.81 mmol) were subjected to reduction by C295A SADH. The reaction was monitored by GC and worked up after 70 % conversion. The product was a 2 : 1 mixture of diastereomers. 17 mg (17 %) of the starting material were recovered and found by GC to be identical to (*R*)-4-methyl-1-heptyn-3-one (see below). Therefore the major product of the enzyme reaction was (3*S*,4*S*)-4-methyl-1-heptyn-3-ol. Yield 44 mg (43 %), >98 % ee, R_f 0.19 (10 % ether/pet. ether), $[\alpha]^{20}_D$ –10.2 (c = 2.75, dioxane) ^{1}H -NMR (250 MHz, $CDCl_3$) δ 4.28 (dt, *J* = 5.5 Hz, 2.1 Hz, 1H), 2.46 (d, *J* = 2.2 Hz, 1H), 1.85 – 1.2 (m, 6H), 1.02 and 1.00 (2 : 1)(d, *J* = 6.7 Hz, 3H), 0.92 (t, *J* = 6.9 Hz, 3H).

Resolution of 2-methylpentanoic acid.[12] Lipase from *Candida rugosa* (Sigma L1745, 750 units/mg,1.0 g), 1-octanol (130 mg, 1.0 mmol), 2-methylpentanoic acid (116 mg, 1.0 mmol), and heptane (10 mL) were combined and shaken at rt for 14 h. The reaction progress was followed by TLC (5 % ethyl acetate/ hexanes containing 0.25 % acetic acid, detection by vanillin) R_f (ester) 0.53, (acid) 0.08, (alcohol) 0.03. The enzyme was filtered off, the filtrate evaporated, and the components separated by chromatography. Yield of (*S*)-ester 73.6 mg (64 %), Yield of (*R*)-acid 55.0 mg (93 %).

(*R*)-4-Methyl-1-heptyn-3-one. (*R*)-2-Methylpentanoic acid (55.0 mg, 0.47 mmol) and KOH (31 mg) were dissolved in MeOH (1.0 mL), allowed to stand for 90 min, and evaporated to dryness. Benzene (2 mL) was added and the mixture stirred for 60 min. At 0 °C, DMF (1 µl) and oxalyl chloride (250 mg, 2 mmol) were added and stirring was continued at rt for 2 h. The excess of oxalyl chloride was removed by evaporation with $CHCl_3$. The residue was taken up in CH_2Cl_2, filtered, and treated with *bis*-(trimethylsilyl)acetylene (80 mg, 0.47 mmol) and, at 0 °C, $AlCl_3$. (80 mg) The mixture was stirred for 2 h at 0 °C and for 2 h at rt and then poured into a 1 : 1 mixture of 1 *M* HCl and ice. The layers were separated and the aqueous phase extracted with CH_2Cl_2. The combined organic extracts were dried with Na_2SO_4 and the solvent was removed *in vacuo*. The residue was taken up in Et_2O and the resulting solution was stirred vigorously

overnight with an aqueous solution of NaF (20 mg, 0.5 mmol) and NBu_4Cl (14 mg, 0.05 mmol) in H_2O (2 mL). The layers were separated and the aqueous layer extracted with Et_2O. The combined organic extracts were dried with Na_2SO_4, the solvent was removed in vacuo, and the residue chromatographed on silica gel. Yield 28.2 mg (48 %); ee 78 %; ^{1}H -NMR (250 MHz, $CDCl_3$) δ 3.22 (s, 1H), 2.59 (m, *J* = 6.8 Hz, 1H), 1.79 (m, 1H), 1.39 (m, 3H), 1.19 (d, *J* = 7.2 Hz, 3H), 0.93 (t, *J* = 7.2 Hz, 3H). ^{13}C-NMR (75 MHz, $CDCl_3$) δ 191.1, 80.9, 79.0, 48.1, 34.5, 20.1, 15.5, 13.9.

Preparation of ethyl thioacetate.[13] A mixture of NaOH (4.0 g, 0.1 mol), H_2O (7.5 mL), and ethanethiol (8.9 mL, 0.12 mol) was poured onto cracked ice (50 g). Acetic anhydride (11.8 mL, 0.125 mol) was added with vigorous stirring which was continued for 5 min. whereby the thioester separated immediately. The product was washed with H_2O (5 × 5 mL), dried with $MgSO_4$, and distilled, bp 112-114 °C (lit.[13] bp 114-116 °C). Yield 5.8 g (56 %).

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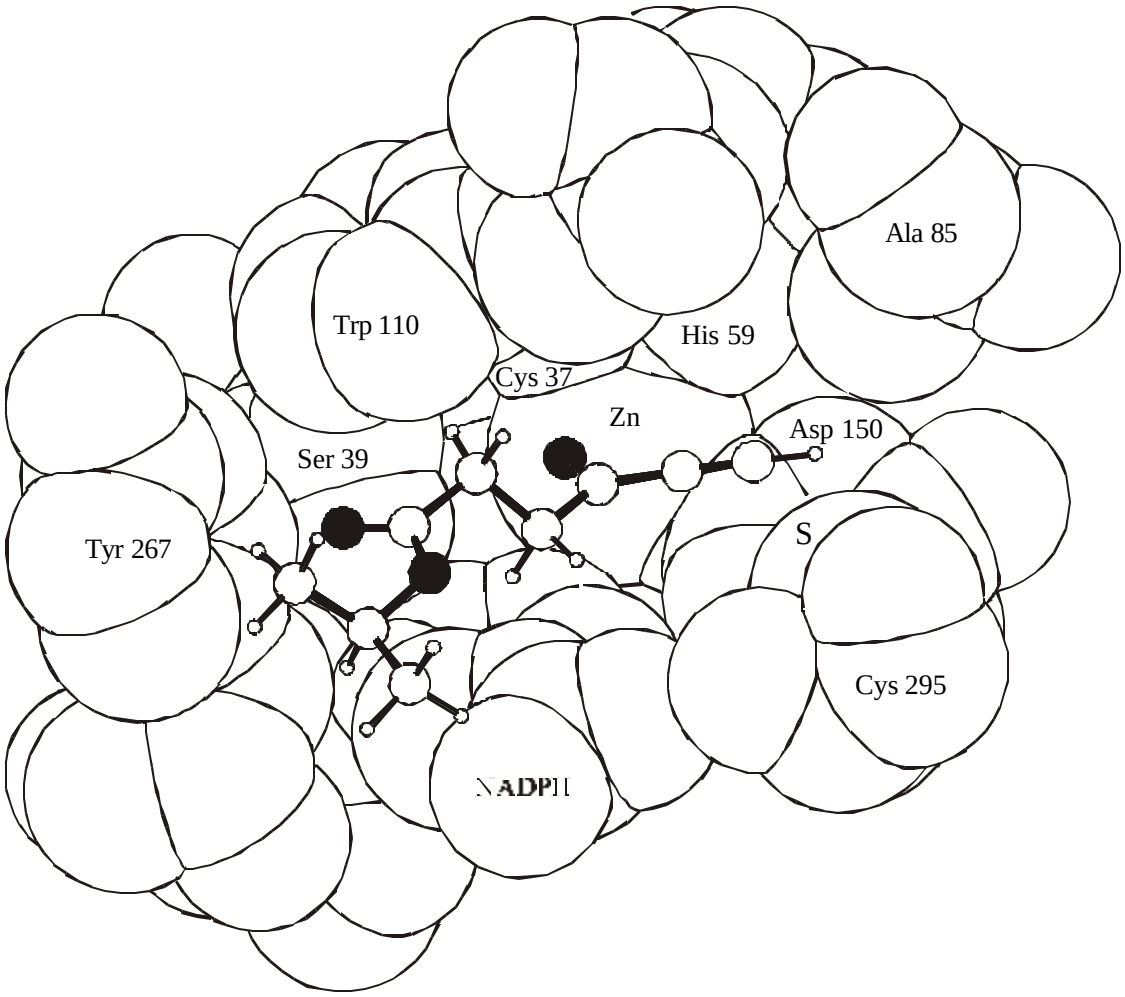
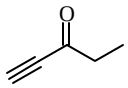
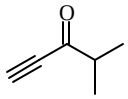
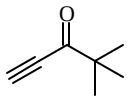
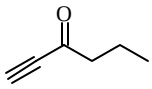
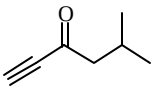
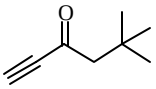
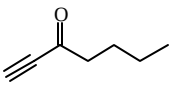
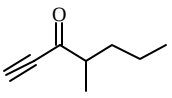
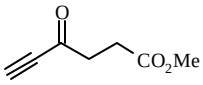


Figure 1. Active site of wild-type SADH with substrate isopropyl 4-oxo-5-hexynoate

Table 1. C295A SADH reductions of ethynylketones

(values of wild-type reductions in parentheses)

entry	substrate	yield (%)	abs. conf.	ee (%)
1		39 (32)	S (S)	76 (80)
2		88 (50)	S (S)	>98 (>98)
3		39 (0)	S (S)	85 (85)
4		51 (28)	S (S)	76 (51)
5		42 (20)	S (R)	56 (50)
6		0 (0)	- (R)	- (66)
7		60 (32)	S (R)	67 (42)
8		43 (0)	S (-)	>98 (-)
9		23 (35)	R (R)	60 (82)

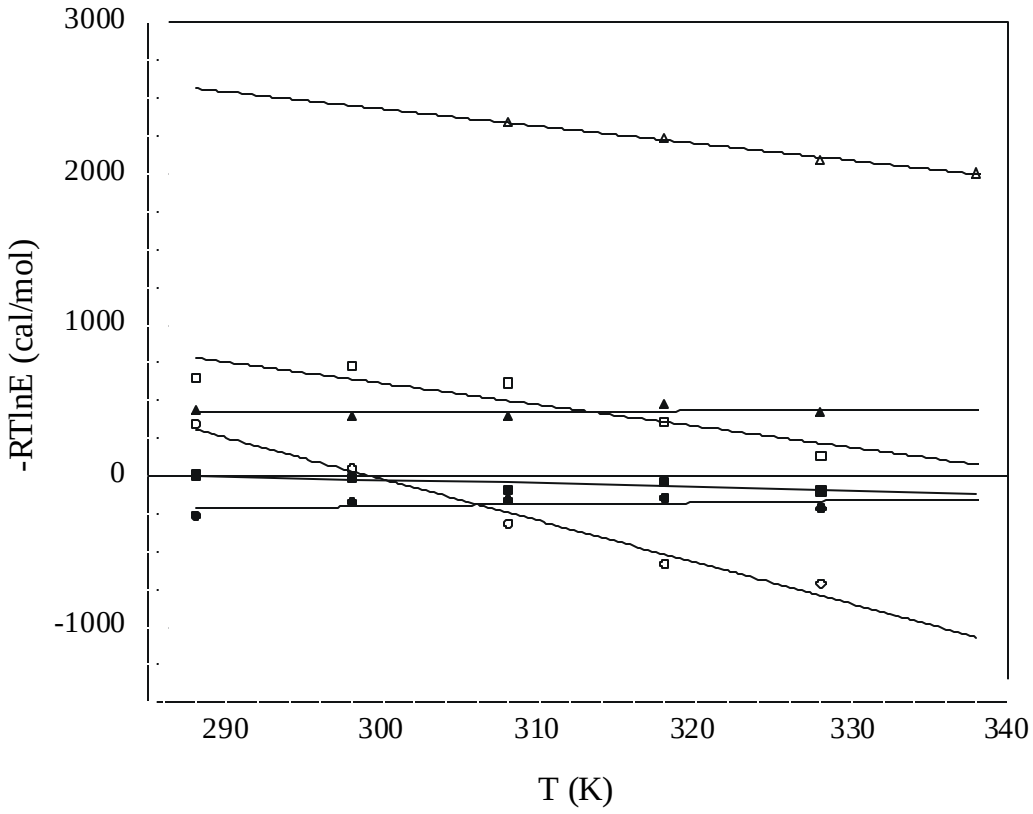


Figure 2. Temperature dependence of enantiospecificity ($E = (k_{cat}/K_m)_R/(k_{cat}/K_m)_S$) for wild-type and C295A SADH for 2-butanol, 2-pentanol, and 2-hexanol. Open shapes: wild-type SADH. Filled shapes: C295A SADH. Circles: 2-butanol. Squares: 2-pentanol. Triangles: 2-hexanol.

Table 2. Kinetic parameters of thioester cleavage by SADH.

Compound	SADH	K_m ($\times 10^{-3}$ M)	k_{cat} (s^{-1})	k_{cat}/K_m ($\times 10^{3}$ M^{-1} s^{-1})
Ethylthioacetate	Wild–type	6.0	37	6.2
Ethylthioacetate	C295A	14	38	2.6
Acetyl-CoA	Wild–type	0.6	73	13
Acetyl-CoA	C295A	1.2	50	40

CONCLUSIONS

In this dissertation, two different enzymes were studied, kynureninase from Pseudomonas fluorescens and secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (SADH). While both cases have in common that the primary focus of our research was the elucidation of enzyme specificity and mechanism, they differ in their possible applications; kynureninase is of interest as target for the treatment of a number of neurological disorders, whereas secondary alcohol dehydrogenase shows promise for use as catalyst in the preparation of synthetically valuable chiral building blocks.

In our investigations toward the synthesis of mechanism based inhibitors of kynureninase, we found that β-hydroxy substituted dihydro-L-kynurenines **2a-c** are bound about one order of magnitude more weakly than the unsubstituted dihydro-L-kynurenines. We observed extremely low substrate activity of **2a** and **2b** and explained it by invoking an unfavorable enolamine intermediate on the basis of the presently accepted catalytic mechanism of kynureninase. In contrast to the diols **2a-c**, the 5-bromodihydro-L-kynurenines **19a** and **b** exhibit very high inhibitory activity, **19a** with a K_i value of 52 nM being the strongest kynureninase inhibitor found to date.

In our studies of asymmetric reductions by SADH, we could show that ethynylketoesters are reduced with high enantioselectivity although these α,β-unsaturated substrates inactivate the enzyme by a yet unknown mechanism. We were able to expand the existing model for the stereoselectivity of SADH reductions by looking at the enantioselectivities of reductions of various alkyl substituted ethynylketones. In this context, the mutation Cys295 → Ala enabled us to determine the location of the binding pockets and the amino acid residues involved and the orientation in which the ketone substrates bind to the enzyme. This mutation resulted in a significant shift in enantiospecificity toward the *anti*-Prelog products.

APPENDIX

FORMATION OF A BENZISOXAZOLE DERIVATIVE DURING THE ACIDIC HYDROLYSIS OF THE AMINONITRILE OBTAINED FROM STRECKER REACTION OF 2-NITROCINNAMALDEHYDE[1]

[1] Heiss, C.; Phillips, R. S. and Newton, G. M. To be submitted to *Tetrahedron Lett*.

A few examples have appeared in the literature of the acid-catalyzed cyclization of nitroaromatic compounds to form benzisoxazoles. Thus, Van Allan and Reynolds reported the formation of a pyrylium-substituted benzisoxazole in the attempted acid hydrolysis of 2,6-diphenyl-4-(α-cyano-2-nitrobenzylidene)-4*H*-pyran.[1] He *et al.* detected benzisoxazole as an intermediate in the decomposition of 2-nitrotoluene in a shock tube at 100 °C.[2] Bullen et al. found that 2-nitroethylbenzene is converted quantitatively to 3-methylanthranil by heating it in trifluoroacetic acid for 24 h.[3] According to work by Kutateladze and coworkers, 3-methylanthranil is also formed as the major product when 2-nitrocyclopropylbenzene is treated with concentrated sulfuric acid followed by hydrobromic acid.[4]

In the course of our studies toward the synthesis of inhibitors of kynureninase, we attempted the acid hydrolysis of the cyano group in *N*-benzyl-2-amino-4-(2-nitrophenyl)-3-butenonitrile hydrochloride[5] (**1** in Scheme 1) as part of a Strecker reaction to obtain 2-nitrostyrylglycine. When a solution of **1** in methanol was treated with a slow stream of HCl at 0 °C for only 5 minutes, and, after 30 minutes, quenched with water, a considerable amount of a yellow-orange solid precipitated. This was tentatively identified by NMR as benzisoxazole **2**. NMR did not allow assignment of the double bond as *E*- or *Z*-alkene. However, X-ray crystallography (Fig. 1) revealed the *Z*-configuration of the olefin. **2** was not formed to any appreciable extent if the introduction of gaseous HCl was faster and continued for more than 30 minutes.

A mechanism was proposed for anthranil formation from 2-nitroethylbenzene,[6] in which the rate-limiting step is the intramolecular transfer of the α-proton to one of the oxygens of the nitro group. In **1** the proton that is to be removed is too far from the nitro group to be transferred intramolecularly. More likely, the reaction proceeds by the mechanism shown in Scheme 2. The reaction can be viewed as the vinylogous analog of the one reported by Allan and Reynolds up to the cyclization. However, in order to form the benzisoxazole, their reaction proceeds by hydrolysis of the cyano group followed by

decarboxylation, whereas in our example, aromatization is accomplished by a simple dehydration. In contrast to the cyclizations of 2-nitroalkylbenzenes, which are accomplished by refluxing in acid, the presence of the cyano group in **1** facilitates the initial proton transfer to give the *aci*-form of the nitroaromatic and thus allows the reaction to proceed under mild conditions.

Experimental

N-Benzyl-2-amino-4-(2-nitrophenyl)-3-butenonitrile hydrochloride (1).

2-Nitrocinnamaldehyde (1.77 g, 10 mmol) was dissolved in dry CH_2Cl_2 (20 mL), finely powdered Linde-type 4Å molecular sieves (3 g) were added, the mixture was cooled to 0 °C and treated with benzylamine (1.09 mL, 10 mmol). After stirring for a 30-min period, TMSCN (1.47 mL, 11 mmol) was added and stirring continued until ^{1}H-NMR showed almost complete disappearance of 2-nitrocinnamaldehyde. (~ 90 min). When the reaction was allowed to proceed longer, formation of *N*-benzyl-2-amino-4-(2-nitrophenyl)-2-butenonitrile by double bond migration was observed. The mixture was therefore quickly filtered, the filtrate evaporated, the resulting yellow oil taken up in ether (30 ml), pentane (15 ml) was added, and the product was precipitated as the hydrochloride salt by passing gaseous HCl through the solution. The resulting tan solid was filtered, washed with ether, and dried. Yield 3.20 g (97 %). ^{1}H-NMR (CD_3OD) δ 8.09 (d, J = 8.1 Hz, 1H), 7.75 (d, J = 4.5 Hz, 2H), 7.54 (m, 7H), 6.31 (dd, J = 15.4 Hz, 7.3 Hz, 1H), 5.55 (dd, J = 7.3 Hz, 1.1 Hz, 1H), 4.41 (s, 2H).

3-(2,1-Benzisoxazol-3-yl)-2-(*N*-benzylamino)-2-propenonitrile (2).

Compound **1** (0.33 g, 1.0 mmol) was dissolved in MeOH (30 ml) and dry HCl (~ 20 ml/min) was introduced at 0 °C for 5 min. After 30 min at 0 – 20 °C, the solution was poured onto ice-water (50 ml) and stirred overnight. The yellow-orange precipitate which had formed was filtered off and recrystallized from EtOH. Yield 80 mg (29 %), mp 173 – 175 °C; UV (MeOH) λ_{max} = 405 nm (log ε = 3.31); IR (KBr) ν 3385, 2231 cm^{-1};

^{1}H-NMR ($CDCl_3$) δ 7.50 (m, 2H), 7.35 (m, 6H), 7.00 (dd, *J* = 9.0 Hz, 6.7 Hz, 1H), 6.65 (t, *J* = 6.6 Hz, 1H), 6.10 (s, 1H), 4.66 (d, *J* = 6.5 Hz, 2H); ^{13}C-NMR ($CDCl_3$) δ 162.8, 156.5, 137.4, 131.5, 129.0, 128.1, 127.4, 123.6, 123.0, 119.4, 115.2, 115.1, 114.3, 93.9, 50.3. Anal. Calcd for $C_{17}H_{13}N_3O$: C, 74.17; H, 4.76; N, 15.26. Found: C, 74.18; H, 4.81; N, 15.33.

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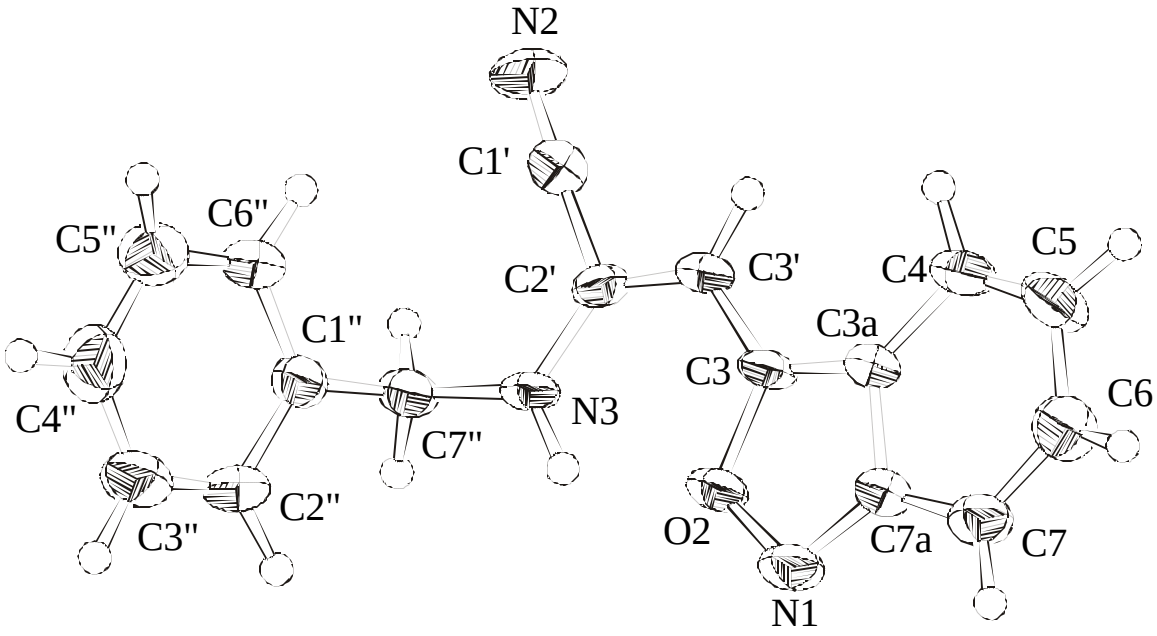
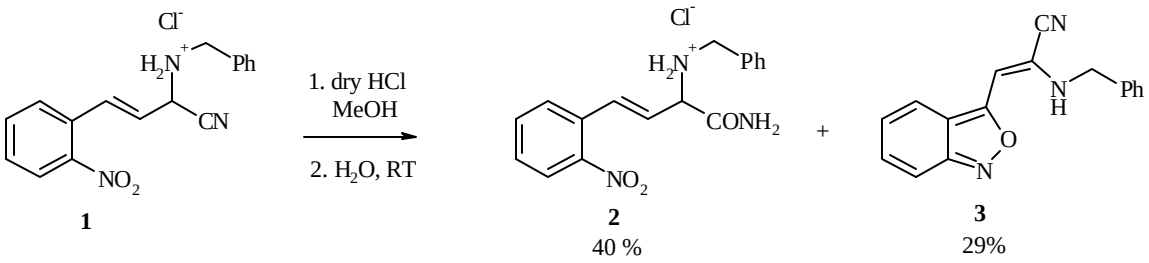
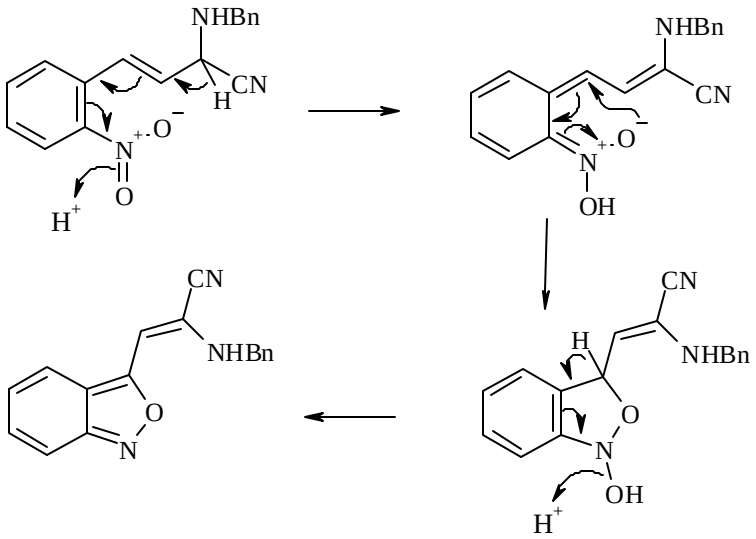


Figure 1. ORTEP-plot of **2**


Cl-
H2N+
Ph
CN
NO2
1
1. dry HCl
MeOH
2. H2O, RT
Cl-
H2N+
Ph
CONH2
NO2
2
40 %
+
CN
N
H
Ph
O
N
3
29%

Scheme 1.



Scheme 2.