Artificial Amino Acids Racemase: Stereoselective Deuteration of Amino Acids

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Abstract

An efficient catalytic system is developed for racemization of amino acids. The system consists of a biphasic mixture of water and chloroform. The zwitterionic amino acid in the water layer is catalytically transported to the chloroform layer with a chiral guanidine base. The amino acid salt is then reversibly converted to an imino acid salt with an aldehyde. The guanidine base then racemizes the imino acid salt. The racemized amino acid is released to the water layer upon hydrolysis. The catalytic system is highly efficient at neutral pH and ambient temperature. In addition, similar to racemases in nature, an equilibrium ‘overshoot’ is observed in D$_2$O when the amino acid and the base have the same configurations. Thus $L$-alanine gives enantiomeric excess of deuterated $D$-alanine (20% ee) when $S$ form of the chiral guanidine. The catalytic system can be used to perform crystallization induced diastereomer transformation (CIDT) of amino acids.
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<td>Ar</td>
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<td>CIDT</td>
<td>Crystallization Induced Diastereomer Transformation</td>
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<td>DBU</td>
<td>1,8-Diazabicycloundec-7-ene</td>
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<td>DCS</td>
<td>3,5-dichlorosalicylaldehyde</td>
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<td>Pyridoxal 5′-phosphate</td>
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<tr>
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<tr>
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<td>Resonance Assisted Hydrogen Bond</td>
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Chapter 1
Artificial Amino Acids Racemase: Stereoselective deuteration of Amino Acids

1.1 Introduction

1.1.1 Pyridoxal 5’ phosphate

Pyridoxal 5’ phosphate (PLP) was discovered as one of the active vitamers of Vitamin B6 in 1951, and is one of the essential cofactors in all-living organisms. Pyridoxal along with the other five vitamers of Vitamin B6 namely, pyridoxine, pyridoxamine and their respective phosphate esters, can readily interconvert.

![Figure 1. Structure of pyridoxal 5’-phosphate](image)

PLP-dependent enzymes have been studied extensively since they have important functions in cellular processes. These enzymes are involved in the biosynthesis of amine-containing molecules such as amino acids and amino sugars. These PLP-dependent enzymes are also important since they can serve as drug targets. For example γ-aminobutyric acid aminotransferase (GABA ATase) can be inhibited to treat epilepsy. Inhibitors of Ornithine decarboxylase (ODC) have been developed to treat African sleeping sickness. Defects in PLP function can be linked to some disease pathologies. For example a mutation in cystathionine β-synthase can result in homocystinuria.

Some well known reactions catalyzed by PLP are transamination, racemization, decarboxylation, substitution, and α,β and γ elimination reactions of amino acids. The condensation of the amine containing substrate with PLP results in the formation of an imine called the external aldime. A carbanion is formed upon deprotonation of the alpha proton of the external aldime. This carbanion intermediate is called the quinonoid intermediate (Figure 2).
The amine containing substrate forms an external aldimine with pyridoxal. The carbanion formed by deprotonation of the alpha proton is called quinonid. The negative charge on the carbanion is stabilized by delocalization through the pi system of PLP.

The negative charge of the carbanion is delocalized through the pi system of PLP, which results in the stabilization of the quinonoid intermediate (Figure 2).\(^1\) Hence the major function of PLP, in most reactions, is to act as an electron sink.\(^1\)

The pKa for the alpha proton of the free amino acid is about 30.\(^1\) Therefore the formation of the carbanion in the transition state, which is crucial for all the chemistry catalyzed by PLP, is inaccessible under physiological conditions.\(^1\)

### 1.1.2 Amino Acid Racemization

Amino acids are fundamental building blocks of proteins, and hence key components in living organisms. They are generally found in the optically active \(L\)-form in nature.\(^3\) However there are also examples of \(D\)-amino acids in nature.\(^3,4\) \(D\)-amino acids are an important component of peptidoglycans forming the cell wall of both gram-negative and gram-positive bacteria.\(^3\) In addition \(D\)-amino acids have been found in vertebrates including humans and mammals.\(^3\) For example, \(D\)-serine functions as an endogenous ligand,\(^3,4\) and is selectively concentrated in the mammalian brain.\(^4\) \(D\)-aspartic acid has also a role as a mediator in endocrine system of vertebrates and invertebrates (Figure 3).\(^6\)

Racemases are enzymes responsible for racemization of amino acids in organisms.\(^3\) They work either on their own or with the help of the cofactor, pyridoxal 5'-phosphate (PLP).\(^3\) Alanine
racemase is one of the most important racemases that has been studied extensively. Alanine racemase is a bacterial enzyme that has a fundamental role in growth since it provides D-alanine, an essential factor in peptidoglycan synthesis. The general mechanism starts by amino acid replacing the lysine (Lys) bound to PLP. The alpha proton is abstracted and reprotonation of the amino acid occurs from the opposite face, which results in the formation of the other enantiomer of amino acid.

Studies have revealed that alanine racemases use tyrosine (Tyr) and lysine (Lys) as two catalytic bases. Watanabe et al. proposed a mechanism for racemization of amino acid (Figure 4) where the substrate carboxyl group mediates the proton transfer between the two catalytic bases Lys and Tyr. Tyrosine removes the alpha hydrogen from L-alanine in the external aldimine, while protonating the carboxylic group of the aldimine simultaneously (2, Figure 4). The formation of a sterically favored six-membered ring allows for this proton transfer. The carboxyl group then donates the proton to Lys, which in turn donates a proton simultaneously to form the D-alanyl-PLP-aldimine (4, Figure 4). The OH side chain of Tyr and NH₂ side chain of Lys maintain their non-ionized states in this cycle. In addition stabilization of intermediates (3) and (4) (Figure 4) is achieved by the proton on the carboxylate group.

Lys deprotonates the alpha hydrogen from D-alanine, while Tyr deprotonates the alpha hydrogen from L-alanine. This mechanism has been confirmed by obtaining crystal structures which reveals that the alpha hydrogen of L-Ala Schiff base is near the hydroxyl group of Tyr, whereas the alpha hydrogen of D-Ala Schiff base is near the amino group of Lys.

A competing reaction with racemization is transamination. In transamination the pyridine nitrogen is protonated by the aspartate, and hence pyridoxal acts as an electron sink. However, it has been shown that arginine replaces aspartate for alanine racemases, preventing the protonation of the pyridinium nitrogen. Therefore, pyridine can’t act as an electron sink, hence restricting the transamination reaction. This could impair the function of PLP as an electron sink, but racemization might require less electron delocalization. In fact, some amino acid racemases do not require a cofactor.
Amino acid racemization studies can be useful in many fields such as peptide synthesis, geochronology, geothermometry and nutrition. Amino acid racemization in fossils can also be used to gain information about the temperature history of the sample. In addition racemization can induce structural changes in proteins, which in turn could affect their function. Hence racemization can be related to the aging processes in mammals. Ionic strength, pH, buffer concentration, presence or absence of water are some known factors affecting the rate of racemization.

There has been much interest in non-enzymatic racemization reactions. Early examples of racemization of amino acids were done by heating in water under strong alkaline or acidic conditions. Heating amino acids at temperatures around 150-250 °C in a sealed vessel is also known to racemize amino acids. Snell showed that heating amino acids with pyridoxal analogues in the presence of metal ions could also result in racemization. However these methods are still unsatisfactory because amino acids can decompose at high temperatures. In addition, the rate of racemization is too slow to be practical. A more recent example of non-enzymatic racemization was reported by Yoshioka et al. Various amino acids are heated at 80-100 °C in acidic medium with catalytic amount of aldehydes.
1.1.3 Crystallization Induced Stereoisomer Transformation

Crystallization induced stereoisomer transformations (CIST) involves crystallization of enantiomerically or diastereomerically pure products by racemization. In other words it involves simultaneous optical resolution and racemization of a racemate mixture. The process could be highly practical since theoretically all the racemate (100%) will be transformed into the desired isomer through racemization. Therefore this technique could theoretically be as practical as asymmetric synthesis. CIST could either involve enantiomers called crystallization induced enantiomer transformations (CIET), or diastereomers called crystallization induced diastereomer transformations (CIDT).

CIDT can be depicted as shown in Figure 5. The two solid diastereomers As and Bs can equilibrate while dissolving in solution (A_L and B_L). If the solubility of the diastereomers are the same, there is no transformation of diastereomers and hence no change. If solubility of B-diastereomer is lower than that of A diastereomers, then the mixture should be transferred to only B diastereomer. The mixture will contain only A diastereomer, if A diastereomer has lower solubility compared to Bs.

![Figure 5](image)

**Figure 5.** A representation of crystallization induced diastereomer transformation (CIDT).

The two diastereomers can be either chiral or achiral. In general, they are made up of 2 separate moieties. The moieties could be held together either electrostatically (salt) or covalently.

The first resolved example of a CIDT dates back to 1846 by Dubrunfunt, where the less soluble alpha-D-glucose hydrate was crystallized from the mixture of anomers (Figure 6).
In 1913, Leuchs also reported an early example of CIDT where (+)-indanone was crystallized using brucine as a monohydrate salt in 93% yield (Figure 7). The interconversion of the keto and enol forms drives the equilibration.

The equilibration can also be driven by epimerization of an acidic carbon-hydrogen bond. Leuchs also reported one of the early examples of this equilibration where diastereomerically pure 1, (Figure 8) was obtained by using quinine.

CIDT is also useful for amino acids since they are important building blocks of life as well as critical precursors of pharmaceutical and other important synthetic products. Resolution of phenylglycine ester using (+)-tartaric acid (TA) by a Glaxo group is one of the revolutionary examples. It was found that when carbonyl additives are added the reaction is facilitated. This is because of formation of an imine intermediate, which results in an increased acidity of the alpha proton of the amino acid. Hence, addition of benzaldehyde to a racemic mixture of methyl phenylglycinate and (+)-TA results in formation of enantiopure (+) phenylglycinate (Figure 9).
To do a CIDT directly on phenylglycine, a harsher reaction condition is needed; (+)-Camphorsulfonic acid in propionic acid was used and the reaction was heated at 100 °C.\(^{15}\)

Yoshioka et al. reported a CIDT on \(\beta\)-methyl ester of aspartic acid (Figure 10).\(^{16}\) The racemic mixture of the aminoester was heated at 80 °C for 6 hours in the presence of (−)phenylethanesulfonic (PESA), a chiral acid, and salicylaldehyde to obtain the diastereomically pure (+)-\(\beta\)-methyl ester aspartic acid salt.\(^{16}\) It was noted that as the temperature increases the solubility difference between the two diastereomers also increases.\(^{10}\)

CDIT has been used to isolate diastereomERICALLY pure precursors for synthesis of many important commercially and pharmaceutically important compounds such as penicillins and cephalosporins such as amoxicillin, cefatrizine and cefadroxil.\(^{17,18}\) A CIDT-based process could be more economically advantageous compared to alternatives, such as purification by chromatographic separation.\(^{10}\) A comprehensive review article on this subject has been written by Brands and Davies.\(^{10}\)
1.2 Research Goals

Alanine racemase is a pyridoxal phosphate dependent enzyme that efficiently catalyzes racemization of alanine. Equilibrium ‘overshoot’ has been reported in D₂O for enzyme catalyzed racemization of alanine.¹⁹ Thus this enzyme catalyzed racemization of L-alanine in D₂O results in initial formation of excess deuterated D-alanine over deuterated L-alanine. Over time the enantiomeric excess of deuterated D-alanine disappears. The purpose of this study is to use a pyridoxal analog (3,5-dichlorosalicylaldehyde) and a chiral guanidine (Figure 12) to obtain a highly efficient non-enzymatic racemization system for amino acids. My aim is to develop an artificial enzyme that closely mimics nature’s racemases including the equilibrium ‘overshoot’. Another goal is to develop a practical method for deracemisation of amino acids using the artificial racemase.
1.3 Experimental

1.3.1 General Information

Commercially available compounds were used without further purification or drying. The $^1$H NMR spectra were recorded on Mercury 300, Mercury 400 or Varian 400 spectrometer and processed on MestReNova. Enantiopure [(4R,5R) or (4S,5S)]-4,5-di(naphthalen-1-yl)-4,5-dihydro-1H-imidazol-2-amine [abbreviated as Naphthyl guanidine, 1, Figure 11], and enantiopure [(4R,5R) or (4S,5S)]-4,5-diphenyl-4,5-dihydro-1H-imidazol-2-amine [abbreviated as DPEN-guanidine, 2, Figure 11] were synthesized by Dr. So, a fellow post doc in the group.

![Figure 11. Structures of naphthyl-guanidine (1) and DPEN-guanidine (2)](image)

1.3.2 Synthesis of (S,S) 4,5-dimesityl-4,5-dihydro-1H-imidazol-2-amine [(S,S) Mesityl-Guanidine]

![Figure 12. Synthesis of (S,S)-mesityl-guanidine.](image)

(S,S)-1,2-bis(2,4,6-trimethylphenyl)-1,2-diaminoethane (TPEN) was made from (R,R) 1,2-bis(2-hydroxyphenyl)-1,2-diaminoethane (HPEN) and mesitaldehyde according to literature procedure.$^{20}$ (S,S)-4,5-dimesityl-4,5-dihydro-1H-imidazol-2-amine (abbreviated as mesityl-guanidine) was prepared from (S,S)-TPEN following the literature procedure with slight modification,$^{21}$ as described below.

CNBr (1.04g, 9.9 mmol) in 10 ml CHCl$_3$ was slowly added to a stirred solution of mesityl diamine (TPEN) (2.25g, 7.6 mmol) in CHCl$_3$ (70 ml) at 0 °C. The solution was stirred at 0 °C for
half an hour. Then the mixture was allowed to come to room temperature, and stirred overnight. The solution was then concentrated under reduced pressure. The guanidinium salt was then dissolved in CHCl$_3$ (30 ml)/H$_2$O (30 ml). NaOH (320 mg) was then added to the solution. The reaction mixture was stirred at room temperature for one hour. The reaction mixture was concentrated under reduced pressure until all the CHCl$_3$ evaporated. The mixture was then filtered and the precipitate was washed with H$_2$O and dried to give (S,S)-mesityl-guanidine in 80% yield.

$^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 6.83 (br s, 2H), 6.67 (br s 2H), 5.39 (s, 2H), 2.49 (s, 6H), 2.22 (s, 6H), 1.26 (s, 6H).

1.3.3 General Procedures For Amino Acid Racemization System

1.3.3.1 Mesityl Guanidine

Enantiopure mesityl-guanidine ((R,R) or (S,S) ,40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of enantiopure alanine (L- or D-,1000 µmol). The reaction mixture was stirred vigorously at room temperature for times specified in results/discussion section.

Equilibrium Overshoot: To determine the equilibrium overshoot, the D$_2$O layer of the above reaction mixtures were separated after 4 hours. The water layer was extracted with enantiopure mesityl-guanidine (40 µmol) in CDCl$_3$ (0.7 ml). The $^1$H NMR of the solution was taken to determine the ratio of amino acid salts. 3,5-dichlorosalicylaldehyde (20 µmol) was then added to the chloroform layer, and the reaction was followed by $^1$H NMR until equilibrium was reached.

1.3.3.2 Phase Transfer Catalyst

Tetrabutyl ammonium hydroxide (40% in water, 40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature for 9 days.

1.3.3.3 DBU

1,8-Diazabicycloundec-7-ene (40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature for 9 days.
1.3.3.4 Guanidine Hydrochloride

Guanidine hydrochloride (40 µmol), NaOH (40 µmol) and 3,5-dichlorosalicylaldehyde (20 µmol) in D$_2$O (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature for 9 days.

1.3.3.5 Benzaldehyde

(S,S)-mesityl guanidine (40 µmol), and benzaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature for 9 days.

1.3.3.6 2-Pyridinealdehyde

(S,S)-mesityl-guanidine (40 µmol), and 2-pyridinealdehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature for 9 days.

1.3.3.7 Salicylaldehyde

(R,R)-mesityl-guanidine (40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at room temperature. $^1$H NMR of the D$_2$O layer was obtained after 20 min, 24h, 48 h and 72 h. A graph of ln A/A$_0$ vs T (s) was obtained to get the rate constant for the reaction.

1.3.3.8 Effect of Different Guanidines

(R,R)-Naphthyl-guanidine (40 µmol) (1, Figure 11), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine. The reaction mixture was stirred vigorously at room temperature. $^1$H NMR of the D$_2$O layer was obtained at several time points to obtain the rate constant (See Results/Discussion).

(R,R)-DPEN-guanidine (40 µmol) (2, Figure 11), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl$_3$ (0.2 ml) was mixed with a D$_2$O solution (1 ml) of L-alanine. The reaction mixture was stirred vigorously at room temperature. $^1$H NMR of the D$_2$O layer was obtained at several time points to obtain the rate constant (See Results/Discussion).
1.3.3.9 Amino Acids Screening

(R,R)-naphthyl-guanidine (40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in CDCl₃ (0.2 ml) was mixed with a D₂O solution (1 ml) of L- form of following amino acids (2000 µmol).

- Alanine, Glycine, Serine, Histidine, Glutamine (For glutamine, 1 equivalent of NaOH was added) Valine, Iso-Leucine, Threonine, Lysine, Methionine, Arginine, Cysteine, Proline,

(R,R)-naphthyl guanidine (200 µmol), and 3,5-dichlorosalicylaldehyde (100 µmol) in CDCl₃ (0.2ml) was mixed with a D₂O solution (1 ml) of L- form of following amino acids (1000 µmol).

- Phenylalanine, Leucine, Asparagine,

(R,R)-naphthyl guanidine (100 µmol), and 3,5-dichlorosalicylaldehyde (50 µmol) in CDCl₃ (0.2ml) was mixed with a D₂O solution (1 ml) of L- form of following amino acids (500 µmol).

- Tryptophan

1.3.3.10 Rate Constant for DBU

1,8-Diazabicycloundec-7-ene (40 µmol), and 3,5-dichlorosalicylaldehyde (20 µmol) in D₂O (0.2 ml) was mixed with a D₂O solution (1 ml) of L-alanine (1000 µmol). The reaction mixture was stirred vigorously at either 98 °C, 94 °C and 90 °C. After about 40% of deuteration, the pseudo first order rate constants (lnA/A₀= -kt) were determined at each temperature. An Arrhenious plot was obtained to get the rate constant at 25 °C using DBU.

1.3.3.11 Computation

Computational analysis was done using Spartan 2008, version 1.1.1. Global minimum structures for imines and deprotonated imines in Figure 33 were initially obtained using molecular mechanics. Those structures were then further refined using DFT calculations (B3LYP at 6-31G* level). The energy difference between the protonated and deprotonated imines were then obtained and the energy was converted to kcal/mol.
1.3.4  Crystallization Induced Diastereomer Transformation

1.3.4.1  Phenylalanine

3,5-Dichlorosalicylaldehyde (0.66 mmol) and (S,S)-DPEN guanidine (0.9 mmol) were dissolved in CH$_3$CN (3 ml). L-phenylalanine (0.6 mmol) was then added and the reaction mixture was stirred for 5h at 40 °C. The mixture was then cooled to room temperature, stirred for 20 h. The precipitate was filtered and dried to give D-phenylalanine imine salt (1, Figure 13) in 80% yield.

$^1$H NMR (300 MHz, Chloroform-d) δ 10.38 (s, 2H), 8.51-8.44 (br s, 2H), 7.45-7.15 (m, 14H), 7.15-6.90 (m, 3H), 6.59 (d, $J = 2.7$ Hz, 1H), 4.83 (s, 2H), 4.11-4.00 (m, 1H), 3.52 (dd, $J = 14.0$, 3.3 Hz, 1H), 3.00 (dd, $J = 13.9$, 10.6 Hz, 1H).

![Figure 13. CIDT of phenylalanine using DCS and (S,S)-DPEN guanidine.](image-url)

1.3.4.2  Tryptophan

3,5-Dichlorosalicylaldehyde (0.66 mmol) and (S,S)-DPEN guanidine (0.9 mmol) were dissolved in CH$_3$CN (3 ml). L-tryptophan (0.6 mmol) was then added and the reaction mixture was stirred for 5h at 60 °C. The mixture was then cooled to room temperature, stirred for 20 h. The precipitate was filtered to give D-tryptophan imine salt (1, Figure 14) in 90% yield.

$^1$H NMR (400 MHz, Methanol-d$_4$) δ 7.68 (d, $J = 7.9$ Hz, 1H), 7.48-7.27 (m, 13H), 7.17 -7.05 (m, 1H), 7.05-6.96 (m, 2H), 6.76 (d, $J = 2.8$ Hz, 1H), 4.86 (s, 2H), 4.30 (dd, $J = 10.4$, 3.6 Hz, 1H), 3.68 (dd, $J = 14.6$, 3.6 Hz, 1H), 3.14 (dd, $J = 14.6$, 10.4 Hz, 1H).
Figure 14. CIDT of tryptophan using DCS and (S,S)-DPEN-guanidine.
1.4 Results and Discussion

1.4.1 Mechanism of Racemization

Amino acids exist in zwitterionic form, and hence won’t dissolve in chloroform. Mesityl guanidine (Figure 12) is a strong base, and deprotonates the zwitterionic form of amino acids such as alanine. In addition it is hydrophobic enough to extract the amino acids from water to chloroform by forming a doubly hydrogen bonded salt with the carboxyl group (1, Figure 15). In contrast, amines such as triethylamine are too weakly basic to deprotonate the zwitterionic form of amino acids, and hence cannot extract them to the chloroform layer. In another words mesityl guanidine acts as a phase transfer catalyst to transfer the amino acids from the aqueous layer to the chloroform layer. Once in the chloroform layer, the amino acid salt can react rapidly with DCS to form the imino acid salt (2, Figure 15). The deuteration of the iminoacetate salt is efficiently catalyzed by the hydrophobic mesityl guanidine (3 and 4, Figure 15) to give two diastereomers of the amino acid salt (5, Figure 15). Thus mesityl-guanidine, a strong base, is useful for rapid deuteration of the iminoacid salt at the α-carbon in addition to efficient extraction of alanine.

Figure 15. Mechanism of amino acid racemization with DCS and guanidine.
The rate of racemization of zwitterionic alanine (1 M) in D$_2$O with mesityl-guanidine (0.2 M) and 3,5-dichlorosalicylaldehyde (DCS, 0.1 M) in CDCl$_3$ was monitored by following the deuteration of the alpha position by $^1$H NMR. It was found that when mesityl-guanidine and amino acid have opposite configurations, (i.e. L-alanine with (R,R)-mesityl guanidine or D-alanine with (S,S)-mesityl guanidine) the half-life is about 20 min at ambient temperature. This translates to a pseudo first order rate constant of about $5.8 \times 10^{-4}$ s$^{-1}$. Figure 16 shows the $^1$H NMR of the D$_2$O layer when L-alanine is used with (R,R)-mesityl-guanidine. As it is seen, after 20 minutes the methyl signal of alanine has already started to collapse from a doublet to a singlet while the $\alpha$-proton quartet is disappearing (Figure 16). The deuteration is essentially complete in about 5 hours (See Appendix for full NMR).

Figure 16. Partial $^1$H NMR of the D$_2$O layer showing the alpha proton quartet and methyl doublet for L-alanine deuteration after (top) 20 min, (middle) 3 hours and (bottom) 5 hours using DCS and (R,R)-mesityl guanidine

The half-life increases to about 75 min when the amino acid and guanidine have the same configurations. This translates to a pseudo first order rate constant of about $1.5 \times 10^{-4}$ s$^{-1}$. Figure 17 shows the $^1$H NMR of the D$_2$O layer when L-alanine is used with (S,S)-mesityl-guanidine
after 75 minutes.

**Figure 17.** Partial $^1$H NMR of the D$_2$O layer showing the alpha proton quartet and methyl doublet for L-alanine deuteriation after using DCS and (S,S)-mesityl guanidine after 75 min.

Our system is similar in a way to other systems with phase transfer catalysts. O’Donnell, Corey and Maruoka$^{22,23}$ have reported elegant examples of phase transfer catalysts for stereoselective synthesis or alkylation of amino acids. In those systems the carboxylate group of glycine or other amino acids are first protected as an imino ester before alkylation of the $\alpha$-carbon.$^{22,23}$ In our system deuteriation takes place on free amino acids without the need of protecting the carboxyl group. Free alanine is transported catalytically from the aqueous phase to the chloroform droplets as a salt of guanidine base. The amino acid salt is then catalytically activated by reversible imine formation with 3,5-dichlorosalicylaldehyde for deuteriation of the $\alpha$-carbon of the amino acid. It is quite remarkable that the chloroform droplets in our catalytic system provide such a high reactivity for deuteriation and racemization of alanine and other amino acids. Enzymes have remarkable activity partly due to their ability to bind substrates precisely and decreasing the entropy effect. This results in high effective concentrations of the catalytic group. In our system, DCS and mesityl guanidine, which are the catalytic groups, are in high concentrations in the chloroform droplets. Although the amounts of catalysts used in our system are low (2% of DCS and 4% of guanidine relative to alanine) their concentrations in the chloroform droplets are quite high (0.1 M for DCS and 0.2M for guanidine). NMR confirms this as it shows there is almost no leakage of the catalytic group to the water layer. Hence efficient and fast racemization of amino acids is obtained without the need to heat or to use any strong acid or base.
1.4.2 Equilibrium Overshoot

Interestingly, when L-alanine was reacted with \((S,S)\)-mesityl-guanidine for 4h, an excess of deuterated \(D\)-alanine (1.10 ppm) over deuterated \(L\)-alanine (1.12 ppm) was in the \(D_2O\) layer in a ratio of about 2:1 (Figure 18a). This was detected by extracting the amino acid in \(D_2O\) layer with \((S,S)\)-mesityl-guanidine in CDCl₃ where the chiral guanidine acts as a chiral shift reagent. This equilibrium ‘overshoot’ in the \(D_2O\) layer was not observed when \(L\)-alanine was used with \((R,R)\)-mesityl guanidine. This \(D\)-alanine equilibrium overshoot is temporary and the ratio returns to about 1:1 over 2 days. The NMR in Figure 18a shows a small amount of \(L\)-alanine that has not been deuterated as 4h is not enough for complete deuteration. As a control experiment, we checked if there is any stereoselectivity for extraction of alanine with the chiral guanidine and found none. When DCS was added to the above chloroform layer (Figure 18b), the ratio of the diastereomeric amino acid salts reached close to 1:1 in about 3h as the racemization continued. Slight stereoselectivity for imine formation was observed as the ratio of \(D\)-imino acid salt (7.77 ppm) to \(L\)-imino acid salt (7.75 ppm) is about 1.5:1. Thus the \(D\)-iminoacid-(\(S,S\))-guanidinium salt is slightly more stable than the \(L\)-iminoacid-(\(S,S\))-guanidinium salt whereas the two amino acid salts are comparable in energy. This is likely due to interactions (Electronic or steric) between the DCS group of the iminoacid and the mesityl guanidine group.
**Figure 18a.** Partial $^1$H NMR showing the methyl group of amino acid salt for $L$-alanine deuteration when the D$_2$O layer is extracted after 4 hours with (S,S)-mesityl guanidine in CDCl$_3$. The ratio of $D$-deuterated alanine to $L$-deuterated alanine is about 2:1. **Figure 18b.** Partial $^1$H NMR of the above mixture after addition of DCS after 3 hours. The ratio of $D$- and $L$- alanine salt is about 1:1. The ratio of $D$-imino acid to $L$-imino acid is about 1:5: 1.

The chloroform layer was also examined during the initial catalytic racemization. Figure 19 shows the $^1$H NMR of the chloroform layer after 20 min of racemization for $L$-alanine using (S,S)-mesityl-guanidine. It is found that the $D$- (7.78 ppm) and $L$- (7.75 ppm) imino acid salts exist in a ratio of about 3:1. Similarly $D$- (1.09 ppm) and $L$- (1.14 ppm) amino acid salts exist in a ratio of about 2.2:1 in the chloroform layer.

**Figure 19.** Partial $^1$H NMR of the chloroform layer for $L$-alanine racmization using DCS and (S,S)-mesityl guanidine after 20 min. The imine peaks exist in a ratio of 3:1 in favor of $D$-imino acid salt. The amino acid salts exist in a ratio of 2.2:1 in favor of $D$-alanine.
The above experiment was also done using H$_2$O in place of D$_2$O. Figure 20 shows the $^1$H NMR of the chloroform layer after 20 min, where it clearly shows the formation of diastereomeric mixtures of the two imino acid and amino acid salts. After 20 min of racemization, the imino acid salt ratio is about 1.5 to 1 in favor of $D$- while the amino acid salt ratio is about 1:1 (Figure 20). This shows that catalytic racemisation is almost complete and the equilibrium for imine formation is slightly stereoselective (1.5 to 1 in favor of $D$).

![Figure 20](image-url)

The H$_2$O layer was also extracted with (S,S)-mesityl guanidine in CDCl$_3$ after 4 hours (Figure 21). The $D$- and $L$-amino acid ratio directly approached 1:1 and hence no equilibrium ‘overshoot’ was observed in the H$_2$O layer unlike that of D$_2$O (Figure 21a). DCS was then added to the above chloroform solution. Similar to the experiment with D$_2$O, The imine formation is slightly stereoselective as the ratio of D-imino acid salt (7.74 ppm) to L-imino acid salt (7.71 ppm) is about 1.5:1 after 3 hours (Figure 21b).
**Figure 21a.** Partial $^1$H NMR showing the methyl group of amino acid salt and the imine peaks of imino acid salt for L-alanine deuteration when the H$_2$O layer is extracted after 4 hours with (S,S)-mesityl guanidine in CDCl$_3$. The ratio of D-deuterated alanine to L-deuterated alanine is about 1:1. **Figure 21b.** Partial $^1$H NMR of the above mixture after addition of DCS after 3 hours. The ratio of D-imino acid to L-imino acid is about 1:5:1.

Similar results were obtained when D-alanine was used with (R,R)-mesityl-guanidine. The D$_2$O layer was extracted with (R,R)-mesityl-guanidine in CDCl$_3$. The ratio of L-alanine salt to D-alanine salt is 2:1 in favor of L (**Figure 22a**). Hence an ‘overshoot’ in racemization equilibrium is observed in the D$_2$O layer. Similarly when DCS was added to the above chloroform layer, the amino acid salts ratio reached 1:1 after about 3 hours (**Figure 22b**). A slight stereoselectivity for imine formation is also observed as the ratio of L-iminoacid-(R,R)-guanidinium salt to D-iminoacid-(R,R)-guanidinium salt is about 1.5:1.
Figure 22a). Partial $^1$H NMR showing the methyl group of amino acid salt for $D$-alanine deuteration when the D$_2$O layer is extracted after 4 hours with $(R,R)$-mesityl guanidine in CDCl$_3$. The ratio of $L$-deuterated alanine to $D$-deuterated alanine is about 2:1. **Figure 22b**. Partial $^1$H NMR of the above mixture after addition of DCS after 3 hours. The ratio of $D$- and $L$-alanine salt is about 1:1. The ratio of $L$-imino acid to $D$-imino acid is about 1:5:1.

Hence when the amino acid and mesityl-guanidine have opposite configurations, the half-life is about 20 min, but no ‘overshoot’ is observed. An equilibrium ‘overshoot’ is observed when the amino acid and mesityl-guanidine have matching configurations. However in this case the reaction is slower and the half-life is about 75 mins. An early example of equilibrium ‘overshoot’ was observed for proline racemase by Cadrinal and Abeles. They observed stereoisomeric equilibrium ‘overshoot’ for enzyme catalyzed racemization in D$_2$O only when $L$-alanine was used as a substrate. It was shown that the extent of overshoot is about 20% by CD spectroscopy, which translates to about a 2:1 ratio in favor of $D$-proline. A similar equilibrium ‘overshoot’ has been observed for alanine racemase in D$_2$O in the $L$- to $D$- direction. Interestingly, our system also shows stereoselective deuteration to give $D$-iminoacid salt preferentially over the $L$-iminoacid salt in a ratio of about 3:1 starting with $L$-alanine and (S,S)-mesityl guanidine (**Figure 19**). This initial ‘overshooting” of racemization equilibrium results in an excess of $D$-alanine over $L$-alanine (2:1) in the D$_2$O layer (**Figure 18**) which returns to a ratio of 1:1 in about 2 days. $L$-alanine is deuterated stereoselectively to give deuterated $D$-alanine in
favor of deuterated L-alanine. However dedeuteration is slower because of a kinetic isotope effect. In other words, initial stereoselective deuteration of alanine is faster than subsequent racemization of deuterated alanine, which would result in formation of more D-deuterated alanine. Similarly, racemization is faster in H2O than in D2O due to a kinetic isotope effect. No overshoot of equilibrium is observed in D-proline to L-proline direction for proline racemase. Similarly, in our system, ‘overshoot’ of equilibrium from D-alanine was observed only with (R,R)-mesityl-guanidine (Figure 22) and not (S,S). Hence one could expect D-proline to L-proline overshoot with the enantiomer of proline racemase.

Figure 23 shows the energy diagram for the racemization system for L-alanine (L-S) and (S,S)-mesityl guanidine. The carbanion intermediate is depicted as ‘I’ which upon deuteration gives deuterated D-imino acid salt (D-P) or deuterated L-imino acid salt (L-P). As Figure 19 shows the D-iminoacid salt and L-iminoacid salt exist in the chloroform layer in a ratio of about 3:1 in favor of D. Hence there is some stereoselectivity and barrier ‘c’ and ‘d’ are different. In addition barrier ‘f’ should be larger than barrier ‘a’ because the product has deuterium at the alpha carbon instead of hydrogen, which results in kinetic isotope effect. The equilibrium stereoselectivity is represented by ‘e’. According to the experimental results the ratio of D-imino acid salt to L-imino acid salt is about 1.5:1 at equilibrium (Figure 18b). Therefore the ‘mismatching iminoacid salt’ (D-P) is slightly more stable as depicted in the energy diagram.

![Figure 23](image)

**Figure 23.** Energy diagram for racemization of L-alanine (L-S) and (S,S)-mesityl guanidine, where I is the intermediate and L-P and D-P are deuterated imino acid salts. Schematic diagram not drawn to scale.

In addition, according to the experimental results, (R,R)-mesityl guanidine deprotonates L-alanine faster than (S,S)-mesityl-guanidine (about 3.5 times). Hence it can be expected that (R,R)-mesityl guanidine would dedeuterate L-P faster than D-P. Thus barrier ‘f’ should be less than ‘g’ when (R,R)-mesityl-guanidine is used with L-alanine, which is in agreement with the
experimental results. Therefore if $(S,S)$-mesityl-guanidine is used with $L$-alanine, barrier ‘f’ should be greater than barrier ‘g’ and will result in an ‘overshoot’ to give more deuterated $D$-imino acid salt. Similarly $(R,R)$-mesityl guanidine will result in an ‘overshoot’ with $D$-alanine.

### 1.4.3 Role of Guanidine

To see the effect of chiral guanidines on our system, control experiments were performed with other compounds in place of mesityl-guanidine. In one control experiment a phase transfer catalyst was used. Phase transfer catalysts (PTC) can be used to transfer one reagent from one phase to another phase containing the second reagent. Tetrabutylammonium hydroxide (40%) was used as a phase transfer catalyst in place of mesityl-guanidine to transfer alanine to the chloroform layer. The $^1$H NMR of the chloroform layer revealed that the PTC transfers the amino acid from the water layer to the chloroform layer where it formed tetrabutylammonium iminoacetate salt. However no trace of deuterium exchange was observed for up to a week (Figure 24a) presumably because of the PTC protonation by the amino acid.

In another control experiment, mesityl guanidine was replaced with 1,8-diazabicycloundecane (DBU). As with PTC, no deuteration was observed for up to a week (Figure 24b). $^1$H NMR of the D$_2$O layer reveals that about 90% of DBU “leaks” to the water layer, while 10% of it remains in the chloroform layer. In another words DBU gets protonated by zwitterionic alanine and is extracted from the chloroform layer to the aqueous layer as the amino acid salt. Thus DBU cannot act as a base catalyst for racemization.
Figure 24. Partial $^1$H NMR of D$_2$O layer showing the alpha proton quartet and methyl group doublet for L-alanine deuteration using (a) DCS and tetrabutylammonium hydroxide or (b) DCS and DBU after 9 days.

Different guanidines such as DPEN-guanidine and naphthyl-guanidine (Figure 11) were examined to determine their effect on the rate of deuteration. Figure 25 shows the NMR of the D$_2$O layer for alanine deuteration using ($R$, $R$)-mesityl guanidine (a), ($R$, $R$)-naphthyl-guanidine (b) or ($R$, $R$)-DPEN-guanidine (c) after 5 hours. As it can be seen, half-lives have not been reached even after 5 hours with either naphthyl guanidine or DPEN-guanidine, whereas the reaction is complete with mesityl guanidine after 5 hours.
Figure 25. Partial $^1$H NMR of D$_2$O layer showing the alpha proton quartet and methyl group doublet for alanine deuteration using (a) DCS and (R,R)-Mesityl-guanidine, (b) DCS and (R,R)-Naphthyl-guanidine and (c) DCS and (R,R)-DPEN-guanidine after 5 hours.

Table 1 shows the $^1$H NMR integration of methyl group to the alpha hydrogen of alanine using the mentioned guanidines. Using these data the pseudo first order rate constant for (R,R)-naphthyl-guanidine was calculated to be $2 \times 10^{-5}$ s$^{-1}$ and $1 \times 10^{-5}$ s$^{-1}$ for (R,R)-DPEN-guanidine (Figure 26). Hence mesityl guanidine (k = 5.8x10$^{-4}$ s$^{-1}$) accelerates the rate of deuteration by a factor of 29 and 58 for naphthyl and DPEN guanidines respectively.
Figure 26. Graph of ln A/A₀ Vs. time (s) for L-alanine deuteration using DCS and (left) (R,R)-Naphthyl-guanidine or (right) (R,R)-DPEN-guanidine. The rate constants (the slop) are 2x10⁻⁵ s⁻¹ and 1x10⁻⁵ s⁻¹ respectively.

Hence, the best base for our system should be hydrophobic and should extract amino acids from water to the chloroform layer and not the other way around. DPEN-guanidine extracts amino
acids into the desired phase but is not as efficient as mesityl guanidine since it is less hydrophobic. Unlike other bases, such as DBU, protonated mesityl-guanidine is insoluble in water and hence can form imino acid salt by extracting amino acids to the chloroform layer. The hydrophobic guanidine is trapped in chloroform, making it strongly basic while the water layer remains neutral. In addition, since the extraction is not 100% efficient, the remaining neutral form can act as a base catalyst.

Another control experiment was done in the absence of chloroform. Guanidine hydrochloride was used in place of mesityl-guanidine. No deuterium exchange of alanine was observed when DCS and neutral guanidine were mixed with L-alanine in water without chloroform for up to a week (Figure 27).

![Figure 27](image_url)

**Figure 27.** Partial $^1$H NMR of D$_2$O layer showing the alpha proton quartet and methyl group doublet for alanine deuteration using DCS and guanidine hydrochloride with out chloroform after 9 days.

The rate of deuteration of our system was compared with that of the DBU. Table 2 shows the pseudo first order rate constants obtained for heating alanine, DBU and DCS dissolved in water at different temperatures. Figure 28 shows the Arrhenius plot obtained using these rate constants. The rate constant at 25 °C was determined to be $9 \times 10^{-9}$ s$^{-1}$ using the equation. This translates to a half-life of about 890 days for deuteration of alanine at 25 °C using DBU. Hence there is a $10^4$-rate acceleration for the amino acid racemization when DCS and mesityl guanidine are used in our system.
<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Pseudo first order rate constant (s⁻¹)</th>
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<tr>
<td>98</td>
<td>1.97x10⁻⁶</td>
</tr>
<tr>
<td>94</td>
<td>1.48x10⁻⁶</td>
</tr>
<tr>
<td>90</td>
<td>1.18x10⁻⁶</td>
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</table>

Table 2. Rate constants for L-alanine deuteration using DBU and DCS at 98, 94 and 90 °C.

Figure 28. Arrhenius plot for L-alanine deuteration using DBU and DCS at 98 °C, 94 °C and 90 °C.

Thus hydrophobic guanidine and chloroform are all essential for highly efficient catalytic racemisation of alanine in water.

1.4.4 Role of DCS

The rate of deuteration was also compared by using other aldehydes in place of DCS. Deuteration of alanine did not take place at an appreciable rate when DCS was replaced with benzaldehyde or 2-pyridine aldehyde (Figure 29).
When salicylaldehyde was used in place of DCS, the half-life was reached in almost 48 hours ($K = 3\times10^{-6} \text{ s}^{-1}$) instead of 75 min (Figure 30,31). Hence, the rate of deuteration of the alpha proton decreased by about a factor of 50 compared to that of DCS.

Figure 29. Partial $^1\text{H}$ NMR of D2O layer for deuteration of $L$-alanine using ($S,S$)-mesityl guanidine and (a) benzaldehyde or (b) 2-pyridinealdehyde after 9 days.
Figure 30. Partial $^1$H NMR of D$_2$O layer showing the alpha proton quartet and methyl group doublet for $L$-alanine deuteration using salicylaldehyde and $(S,S)$-Mesityl-guanidine after (a) 20 min and (b) 48 hours.

Figure 31. Graph of ln $A/A_0$ Vs. time (s) for $L$-alanine deuteration using salicylaldehyde and $(S,S)$-Mesityl-guanidine. The rate constant (the slope) is $3 \times 10^{-6}$ s$^{-1}$. 

$y = -3E-06x - 0.2549$
$R^2 = 0.98264$
Other electron withdrawing aldehydes such as 4-nitrobenzaldehyde or 4-pyridine aldehyde could not be tested because of their instability in the presence of mesityl guanidine.

The trend of aldehyde in terms of reactivity is shown in figure. DCS, an electron deficient aldehyde should favour the formation of the Schiff base. In addition the carbanion, which forms upon deprotonation of the alpha proton is stabilized by DCS. Salicylaldehyde, was observed to be more reactive than benzaldehyde or 2-pyridine aldehyde, even though the hydroxyl group is electron donating. The hydroxyl group can participate in a resonance-assisted hydrogen bond (RAHB), which could overcome the electronic effect. Quantitative dissection of the electronic and hydrogen bond effect on the rate of racemization of amino acids would be interesting for future studies.

![Figure 32. Trend of aldehydes for deuteration of alanine from most reactive to least reactive.](image-url)

When the amino acids or amino esters form an imine with the aldehydes, the most reactive aldehyde should acidify the alpha hydrogen more readily. DFT computation was used to obtain the trend for the most acidic alpha hydrogen (Table 3) using the aldehydes in Figure 32. The alpha hydrogen is found to be the least acidic when benzaldehyde is used to form the imine, while the alpha hydrogen is the most acidic when DCS is used to form the imine (Figure 33). This is in agreement with the experimental results obtained. However the imine formed with salicylaldehyde was found to have a slightly more acidic alpha hydrogen than the one with 2-pyridinealdehyde (by 0.00015 kcal/mol), which is negligible. This is opposite of the experimental results, which suggests that it might be hard to sort out the electronic effect and hydrogen bond effect by computation. pKa values of various compounds such as amines,\(^{25}\) alcohols,\(^{25}\) thiols\(^{25}\) and pyridoxal imine derivatives\(^{26}\) has been obtained by DFT computation. These values are in good agreements with the experimental pKa values. The computation in our case was done in the gas phase, to obtain relative trends in acidities of structurally related compounds rather than to get absolute pKa values. A more detailed computation involving solvent effects and counter ion effects would be required to obtain absolute pKa values.\(^{25,26}\)
<table>
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<th>Imine formed with</th>
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<td>359.329572</td>
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<tr>
<td>Salicylaldehyde</td>
<td>355.25201</td>
</tr>
<tr>
<td>2-pyridinealdehyde</td>
<td>355.155378</td>
</tr>
<tr>
<td>3,5-dichlorosalicylaldehyde</td>
<td>344.857318</td>
</tr>
</tbody>
</table>

Table 3. The energy obtained by DFT computation for the acidity of the alpha hydrogen of glycine methyl ester with 4 different aldehydes.

Figure 33. Trend of the most acidic alpha hydrogen of amino esters upon formation of imine with aldehydes.

DCS, an electron deficient aldehyde with the ability to make RAHB, seems to be the most efficient aldehyde for racemization of amino acids. DCS mimics pyridoxal phosphate, the coenzyme involved in the racemization of amino acids in nature.

1.4.5 Racemization by Computation

Efficiency of the racemization system might in part be due to proton transfer from the guanidinium to the carboxylate group of amino acid. Once the amino acid is deprotonated, the carboxylate group becomes more basic and abstracts the proton from guanidinium (Figure 34). The deprotonation of the alpha hydrogen is expected to be concerted with the proton transfer to the carboxylate group.
Figure 34. Loss of the proton by guanidinium group to the carboxylate group of amino acid upon deprotonating the alpha carbon.

DFT computation was used to obtain the lowest conformation of the deprotonated imine using glycine. Guanidine was used instead of mesityl guanidine to simplify the calculation. As it is shown in Figure 35, computation also showed the loss of proton by guanidine to the carboxylate group of amino acid, upon deprotonation of the alpha carbon.

Figure 35. Optimized structure of the deprotonated imino-acid with guanidine by DFT computation.
1.4.6  Amino Acid Screening

In order to study the scope of our racemization reaction, we investigated 18 natural amino acids. The concentration of the amino acids was lowered to 0.2 M, 0.1 M or 0.05 M in D₂O (1 ml) to accommodate the solubility of all amino acids. Most amino acids (thr, trp, phe, met, glu, gly, gln, asn) exchanged within a day.

**Figure 36.** Partial \(^1\)H NMR of D₂O layer for racemization of (a) glutamine, (b) asparagine and (c) phenylalanine using DCS and \((R,R)\)-Naphthyl guanidine. The top NMR is after 4 hours (Black) and the bottom NMR is after 23 hours (Red).
Serine, lysine and leucine showed >95% deuterium exchange in about a day. Interestingly, no deuteration was observed for valine and isoleucine presumably due to steric effects. Proline has a secondary amine and does not form a Schiff base with DCS. Hence no deuteration was observed. A side reaction takes place with cysteine; the side chain thiol group adds to the imine and hence no deuteration is observed. Arginine and histidine did not extract well and did not deuterate appreciably. Tyrosine has a solubility that is too low to allow it to be tested in our system (See Appendix for NMRs of all the amino acids).
1.4.7 An Application: Crystallization Induced Diastereomer Transformation

Recently, a similar strategy to the racemization system was employed by our group for stereoselective recognition of amino acids based on supramolecular self-assembly.\textsuperscript{27} A bi-phasic solution containing water and chloroform is employed where a racemic mixture of amino acid is dissolved in the water layer. Similar to racemization system, chiral DPEN-guanidine is used to extract amino acids from the water layer to the chloroform layer by deprotonating the zwitterion amino acids, where it forms a Schiff base with the chiral binol aldehyde. The aldehyde has a choice of forming a Schiff base with either the $D$- or $L$ form of amino acid guanidinium salt to form a ternary complex (Figure 37).\textsuperscript{27} When the $(R,R)$ form of guanidine with $R$-binol is used, $L$-amino acid recognition takes place in about 2 hours with great stereoselectivity. $D$-amino acid recognition takes place with the $(S,S)$ form of guanidine and $S$-binol. Thermodynamic equilibrium is reached by imine exchange in about 2h.\textsuperscript{27}

Based on this system and the racemization system, a modified method was used for crystallization induced diastereomer transformation (CIDT) of amino acids. For CIDT to be useful, there has to be a mechanism to facilitate the equilibration.\textsuperscript{10} Rotation around hindered bonds or bond cleavage and formation are mechanisms that could facilitate the equilibration.\textsuperscript{10} Some examples include forming acidic carbon-hydrogen bonds by proton transfer, formation of
carbocations or addition and elimination reactions.\textsuperscript{10} One other way to achieve equilibrium is to use high temperatures. However one downside to this is that decomposition might occur at high temperatures.\textsuperscript{10} One solution is to use additives instead of high temperatures. Examples include aldehydes and ketones, which can facilitate equilibration of amino acids and similar compounds by forming imines.\textsuperscript{10}

The Shiraiwa group described a CIDT for converting $S$-proline to the more expensive $R$-proline (\textbf{Figure 38}).\textsuperscript{15} $S$-proline was heated in the presence of an aldehyde, butanal, and (-)-TA for 3-4 hours.\textsuperscript{15} The $R$-entantiomer was obtained in a form of salt in high yields.

![Figure 38. Conversion of $S$-proline to $R$-proline using (-)-TA and butanal.](image)

The rate of CIDT depends on the rate of racemization/epimerization and the rate of crystallization.\textsuperscript{8} Hence the racemization/epimerization step is a key factor for success of this method. In general, it is recognized that finding a simple and mild condition for racemization is difficult and decomposition can occur.\textsuperscript{8} However our mild and simple condition for amino acid racemization can be employed to do a CIDT directly on amino acids.

Similar to the racemization system, the chiral base DPEN-guanidine, was used to deprotonate $L$-amino acid dissolved in acetonitrile, which can form an imine salt with DCS (1, \textbf{Figure 39}). Free DPEN-guanidine then deprotonates the alpha hydrogen and racemizes the amino acid, forming two diastereomer of the iminoacetate salt (2, \textbf{Figure 39}). The $L$- and $D$- diastereomers have different solubilities in acetonitrile with the $D$ – diastereomer being less soluble (3, \textbf{Figure 39}). Hence the equilibrium shifts towards the formation of the $D$-diastereomer as it precipitates out of the solution.
**Figure 39.** *L*-imino acid guanidinium salt (1) is deprotonated by excess guanidine to give two diastereomers (2). *D*-imino acid guanidinium salt (3) is crystallized out preferentially. 

(S,S)-DPEN-Guanidine was used with DCS and either phenyalanine or tryptophan. Figure 40a shows partial $^1$H NMR of the solution after 5 hours using *L*-phenylalanine. As it is seen there is a 1:1 ratio of *L*- (6.51 ppm) and *D*- (6.62 ppm) iminoacetate salt diastereomers. Figure 40b shows the partial $^1$H NMR of the precipitate which is formed after 20 hours. The precipitate only contains the *D* diastereomer.

**Figure 40a.** $^1$H NMR showing the aromatic peaks of two diastereomeric phenylalanine imine guanidinium salt (2, Figure 39), in solution after 5 hours. The purple arrow belongs to *D*-phenylalanine. **Figure 40b.** $^1$H NMR of the precipitate forming after 20 hours (**b**).
Similar results were obtained for tryptophan. Figure 41a shows the $^1$H NMR of the solution after 5 hours. The ratio of $L$-diastomer to $D$-diastomer salt is about 1:1. Figure 41b shows the $^1$H NMR of the precipitate forming after 23 hours, which only contains the $D$-diastereomer salt.

Figure 41a. $^1$H NMR showing the aromatic peaks of two diastereomeric tryptophan imine guanidinium salt (2, Figure 40) in solution after 5 hours. The purple arrow belongs to $D$-tryptophan. Figure 41b. $^1$H NMR of the precipitate forming after 20 hours.

A successful CIDT of tyrosine and serine has also been obtained by Dr. So in our group. Hence our mild and fast racemization system could be used to convert amino acids enantiomers. This could be industrially useful as amino acids are building blocks of life as well as precursors for many important synthetic chemicals.
1.5 Conclusions and Future Work

In conclusion, a fast and mild system was developed for racemization of amino acids. A biphasic mixture of water and chloroform is used where the amino acid is dissolved in the water layer. A hydrophobic chiral guanidine base is used to extract the amino acid from the water layer to the chloroform layer by deprotonating the zwitterionic amino acid. Other bases such as triethylamine or DBU cannot be used since they are either too weak or they get protonated by the amino acid. The amino acid salt in the chloroform layer can form an imine with a catalytic amount of DCS. DCS is an electron withdrawing aldehyde with the ability to form RAHB. Hence it can stabilize the negative charge of the carbanion developed upon deprotonating of the alpha proton. Free guanidine base can then effectively catalyze deuteration of the imino acid. Combination of two weak forces-electronic and H-bonding- for the aldehyde, together with a strongly basic and hydrophobic guanidine provides enormous rate-acceleration for the racemisation reaction at neutral pH and ambient temperature. In addition amino acids need not be protected as, for example, esters. This artificial racemase represents the most reactive system reported to date for racemisation of amino acids.

Interestingly, it was found that when the amino acid and guanidine have the same configurations, an equilibrium ‘overshoot’ in the racemization reaction is observed (20%), similar to that observed with proline and alanine racemases. This represents the first non-enzymic system for obtaining equilibrium ‘overshoot’. Unlike with the enzymic ‘overshoot’, $^1$H NMR can be conveniently used to study the overshoot in detail. The combination of guanidine base and DCS efficiently catalyze stereoselective deuteration of unprotected and unactivated amino acids under mild conditions by reversible formation of imine intermediates.

Our artificial racemase was used to deracemise amino acids. Dynamic kinetic resolution was used to rapidly deracemise amino acids with the chiral guanidine and the aldehyde. It has been shown in this thesis that developing artificial alaninase is of both academic and practical interest. On the one hand it can provide detailed insights into to the mechanism of racemization including the equilibrium ‘overshoot’. On the other hand, fundamental knowledge gained from such studies can be used to develop useful methods for converting natural amino acids ($L$ forms) to unnatural amino acids ($D$ forms).
Future work would involve catalytic alkylation of amino acids. It would be interesting if our system could also be used for catalytic alkylation of amino acids. The deuteration stereoselectivity observed in our system is relatively low. However, the stereoselectivity could increase as the size of the reactant is increased from deuterium-based electrophiles for deuteration to carbon-based electrophiles for alkylation.
References or Bibliography

Appendices

Appendix A:

Figure S1. $^1$H NMR of (S,S)-mesityl guanidine.
Figure S2. $^1$H NMR of the D$_2$O layer showing the alpha proton quartet and methyl doublet for $L$-alanine deuteration after (top) 20 min, (middle) 3 hours and (bottom) 5 hours using DCS and $(R,R)$-mesityl-guanidine.
Figure S3a). $^1$H NMR of chloroform layer after 20 min for racemization of L-Alanine using DCS and (S,S)-mesityl guanidine in D$_2$O. (b). $^1$H NMR of extraction of D$_2$O layer after 4 hours with (S,S)-mesityl guanidine in CDCl$_3$ for racemization of L-Alanine using DCS and (S,S)-mesityl guanidine. (c). $^1$H NMR of the middle NMR after addition of DCS for 3 hours.
**Figure S4a.** $^1$H NMR of chloroform layer after 20 min for racemization of $L$-Alanine using DCS and (S,S)-mesityl guanidine in H$_2$O. (b). $^1$H NMR of extraction of H$_2$O layer after 4 hours with (S,S)-mesityl guanidine in CDCl$_3$ for racemization of $L$-Alanine using DCS and (S,S)-mesityl guanidine. (c). $^1$H NMR of the middle NMR after addition of DCS for 3 hours.
Figure S5a. $^1$H NMR of extraction of D$_2$O layer after 4 hours with (R,R)-mesityl guanidine in CDCl$_3$ for racemization of D-Alanine using DCS and (R,R)-mesityl guanidine. (b). $^1$H NMR of the top NMR after addition of DCS for 3 hours.
**Amino Acids screening:** $^1$H NMR of D$_2$O layer for racemization of amino acids using DCS and $(R,R)$-Naphthyl Guanidine. For detailed procedures see experimental section.

**Serine**

![Figure S6.](image)

Figure S6.
Tryptophan

Figure S7.
Threonine

Figure S8.
Lysine

Figure S9.
Phenylalanine

Figure S10.
Figure S11.

Asparagine
Methionine

Figure S12.
Glutamine

Figure S13.
Glycine

Figure S14.
Glutamic Acid

Figure S15.
Leucine

Figure S16.
Proline

Figure S17.
Cysteine

Figure S18.
Arginine

Figure S19.
Iso-Leucine

Figure S19.
Valine

Figure S20.
Histidine

Figure S21.
Figure S22. $^1$H NMR of D-imino acid guanidinium salt for phenylalanine using L-phenylalanine and DCS.

Figure S23. $^1$H NMR of D-imino acid guanidinium salt for tryptophan using L-phenylalanine and DCS.