INCREASING THE RACEMASE ACTIVITY VERSUS TRANSAMINASE ACTIVITY OF A PYRIDOXAL ENZYME MODEL BY THE ATTACHMENT OF A RIGID BASE‡

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Abstract - A pyridoxal carrying a 2-pyridylacrylate group catalyzes the racemization of L-alanine. Comparison with related compounds shows that this rigid basic sidearm increases the selectivity for racemization relative to transamination.

Pyridoxal phosphate (1) is an ubiquitous coenzyme involved in many transformations at the α, β and γ carbons of amino acids.[1] In a prototypic transamination reaction, pyridoxal phosphate condenses with an amino acid to form the aldimine Schiff base. A basic group within the enzyme catalyzes a 1,3-hydrogen shift to yield the ketimine, which upon hydrolysis gives pyridoxamine phosphate and the corresponding α-keto acid (figure 1). Pyridoxamine phosphate can then react with a different α-keto acid, resulting in a net exchange of functionality between the α-keto acid and an unrelated amino acid.

In pyridoxal phosphate dependent racemases a basic group in the enzyme acts to deprotonate the Schiff base, followed by internal return of the proton to the opposite side of the intermediate (figure 1).[2,3] In this way L-alanine is converted to D-alanine, which is crucial for bacterial cell wall synthesis. The activity and location of the basic groups in racemase enzymes, therefore, is quite distinct from those involved in transamination reactions.

‡ Dedicated to Gilbert Stork on the occasion of his 65th birthday.

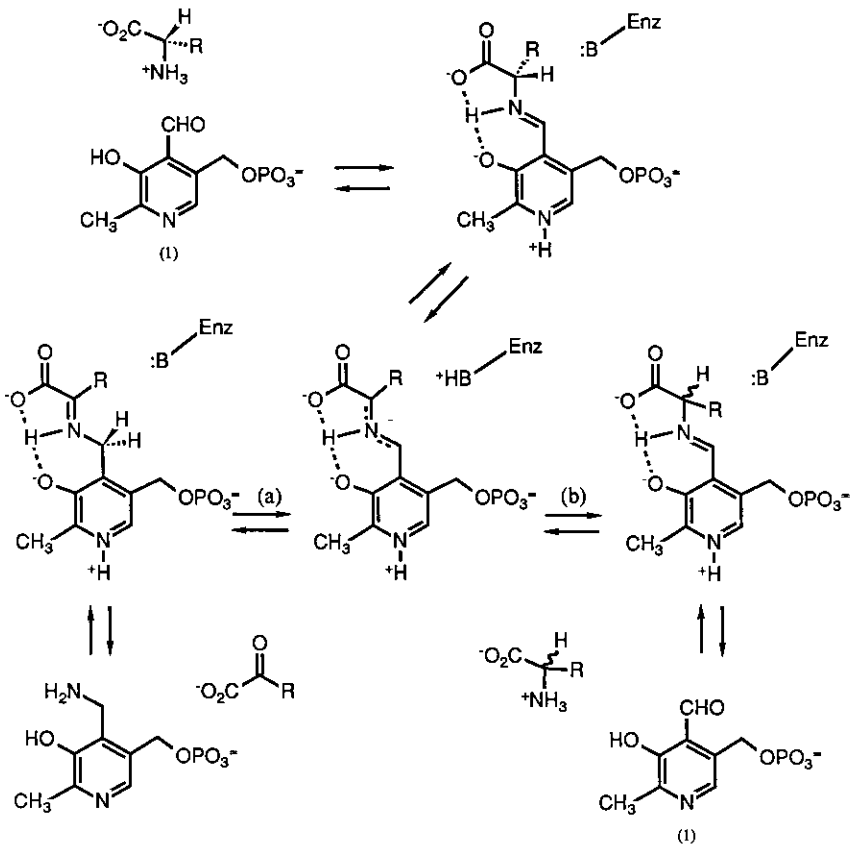
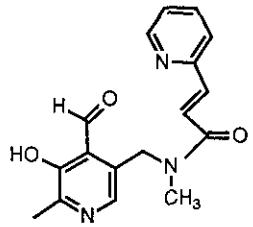


Figure 1. (a) Enzymatic transamination. (b) Enzymatic racemization. The pyridoxal phosphate shown is actually present as an imine with an enzymatic amino group.

Pyridoxal phosphate is also a coenzyme for many other reactions. Serine, for example, can undergo up to five separate reactions involving the aldimine Schiff base, depending on the enzyme involved. The specificity obtained by these enzymes is determined by the correct spatial arrangement of the bond being broken in the substrate, and the catalytic groups within the enzyme.

We have shown that a basic group attached to pyridoxamine can catalyze transamination reactions by performing the 1,3-proton shift on a ketimine[4], and that the chiral placement of such a group[4,5] permits the synthesis of optically active amino acids. We were interested to see whether a different kind of geometric control of the base position would let us select for racemase activity in preference to transaminase activity: rigid placement of a basic group so that it can reach the α carbon of the amino acid in a pyridoxal Schiff base. Racemization requires simply that the base reach both faces of the Snell-Braunstein intermediate, but rigid placement might prevent the movement toward the pyridoxal ring needed for transamination.

There have been numerous examples of non-enzymatic racemizations of amino acids by pyridoxal and pyridoxal derivatives in metal and metal ion free systems.[6,7] There have also been reports of transamination catalysis by the attachment of basic groups to pyridoxamine.[1] Work has never been described combining these two approaches. This paper reports on the synthesis and kinetic results of pyridoxal derivative **2**, containing a rigidly placed pyridine base to assist in the racemization of L-alanine. This compound should selectively catalyze racemization rather than transamination, due to its lack of flexibility. By comparison the more flexible pyridoxal derivative **3**, should show less selectivity in the above reactions. The pyridoxamine derivatives **4** and **5** were also prepared to allow spectrophotometric measurement of rates of ketimine to aldimine interconversion. We found the hoped for effects, although the preferences were not large.



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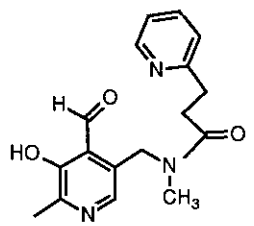

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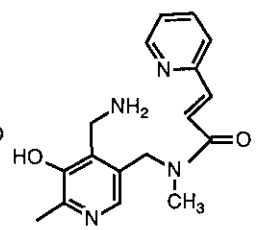

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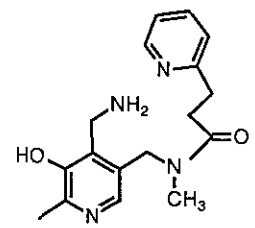

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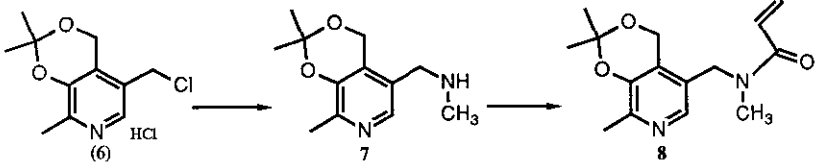
Results and Discussion

1. Synthesis of pyridoxal derivatives 2 and 3. Treatment of the known α[4], 3-O-isopropylidene-5-pyridoxyl chloride·HCl (6)[8] with aqueous CH_3NH_2 gave the secondary amine **7**, which was acylated with the acid chloride of 2-pyridineacrylic acid[9] to give compound **8**. Treatment of compound **8** with HCl removed the acetonide group, affording alcohol **9**, which was converted to pyridoxal derivative **2** with activated MnO_2.



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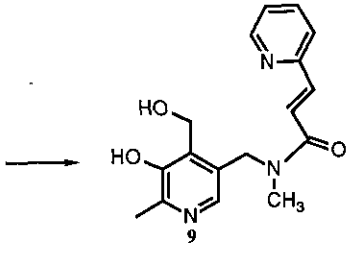

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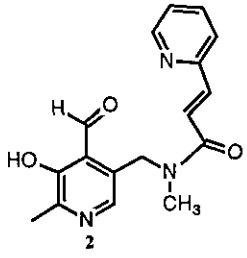

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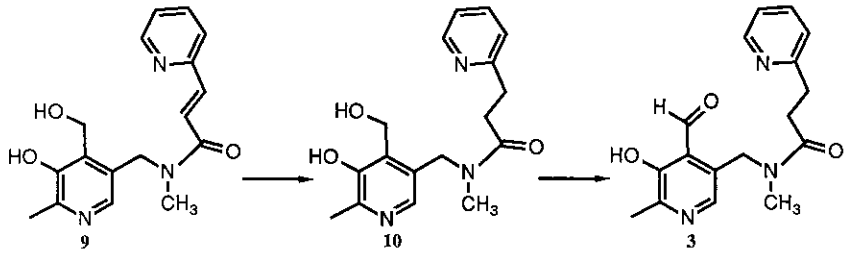
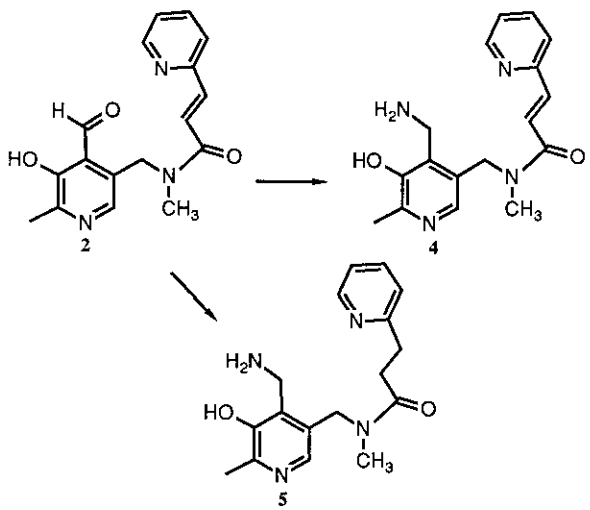
Alcohol **9** was also converted to the dihydro-compound **10** by treatment with hydrogen over Pd/C. Compound **10** was then treated with activated MnO_2 to give pyridoxal derivative **3**. All compounds were purified on silica gel and structures were confirmed by ^1H NMR spectroscopy.



2. Synthesis of pyridoxamine derivatives 4 and 5. Pyridoxal derivative **2** was transaminated to compound **4** using a large excess of D,L-alanine (500 eq) at room temperature. Pyridoxal derivative **2** was also treated with $NH_2OH{\cdot}HCl$ to give an oxime, which was reduced with zinc dust to yield compound **5**. Both pyridoxamine derivatives **4** and **5** were isolated by ion exchange Sephadex chromatography with an aqueous ammonium bicarbonate eluent, and were characterized by ^1H NMR spectroscopy.



3. Racemization and transamination kinetics with compounds 2 - 5. Racemization studies with Al^{3+} metal and an excess of L-alanine were performed with pyridoxal, **2** and **3**. Loss of optical rotation was followed with a polarimeter. As seen in Table I, both **2** and **3** show a two-fold catalytic advantage over pyridoxal for racemization. Thus the pyridine base is having some effect on the deprotonation of the aldimine Schiff base, and the flexibility of this base does not affect the observed rates of racemization.

For comparison, transaminations with **4** and **5** were performed with Zn^{2+} and an excess of pyruvic acid.[4,9] The selectivity of the rigid derivatives **2** and **4** for racemization over transamination, as compared to **3** and **5**, is seen by examining Table II. There is a 2.5 fold preference for the more flexible **5** to promote transamination. Therefore, **2** and **4** containing the rigidly attached pyridine base do show increased

selectivity for racemization over transamination, because of diminished ability to reprotonate at the benzylic position of the Schiff base intermediate. It will be of interest to see whether this selectivity can be increased further by appropriate molecular design.

Table I. Rates of Racemization of L-Alanine

reagent	k_{rac} (min^{-1})[a]	k_{rel}
pyridoxal	1.64	1.0
2	3.37	2.0
3	3.13	1.9

[a] Psuedo-first-order rate constants for the racemization of L-alanine x 10^{-2}.

Table II. Rates of Conversion of Ketimines to Aldimines.

reagent	k_{trans} (min^{-1})[a]	k_{rel}
4	0.48	1.0
5	1.21	2.5

[a]Psuedo-first-order rate constants for transamination x 10^{-3}.

EXPERIMENTAL

Preparation of amine 7. To a rapidly stirred solution of α^4,3-O-isopropylidene-5-pyridoxyl chloride·HCl (6)[8] (2.3g, 8.75 mmol) in a 1:1 mixture of H_2O/MeOH (50 ml) at room temperature was added a 40% by weight aqueous solution of CH_3NH_2 (3.8 ml, 43.7 mmol). After 4 h, the solvent was removed under reduced pressure, and the crude residue was recrystallized from isopropanol giving a 1:1 mixture of **7** and CH_3NH_2·HCl. The mixture was treated with K_2CO_3 in MeOH for 30 min, filtered, and the solvent was removed under reduced pressure. The crude residue was again recrystallized from isopropanol to yield **7** as white needles (1.35g, 71%): mp 191-193°C; ^1H NMR (CD_3OD, 200 MHz) δ 8.06 (s, 1H), 5.03 (s, 2H), 4.12 (s, 2H), 2.79 (s, 3H), 2.39 (s, 3H), 1.57 (s, 6H); MS (DCI/NH_3) 223 (M+1).

Preparation of 8. To a stirred solution of 2-pyridineacrylic acid[9] (375 mg, 2.52 mmol) in dry DMF (20 ml) under argon at 0°C was added oxalyl chloride (286 μl, 3.37 mmol) over a period of 5 min. The resulting mixture was stirred at 0°C for 15 min and added to a solution of **7** (500 mg, 2.25 mmol) in dry DMF (30 ml) under argon at 0°C, followed by the addition of NEt_3 (1.25 ml, 9.01 mmol). After 1 h at 0°C and 12 h at room temperature, the solvent was removed under reduced pressure and the brown residue obtained was chromatographed on silica gel (5% MeOH-CH_2Cl_2) to give **8** as a white semi-crystalline material (694 mg, 87%): ^1H NMR ($CDCl_3$, 200 MHz) δ 8.63 (d, 1H, J = 4.1 Hz), 7.92 (s, 1H), 7.72 (d, 1H, J = 14.9 Hz), 7.52 (d, 1H, J = 14.9 Hz), 4.77 (s, 2H), 4.64 (s, 2H), 3.07 (s, 3H), 2.40 (s, 3H), 1.52 (s, 6H); MS (DCI/NH_3) 354 (M+1).

Preparation of 9. A solution of **8** (200 mg, 0.57 mmol) in a 1:1 mixture of THF/H_2O (20 ml) and concentrated HCl (6 ml) was stirred at room temperature for 12 h. The THF was removed under reduced pressure and the resulting solution was made basic with satd. $NaHCO_3$. The mixture was extracted with CH_2Cl_2, the organic layer was dried over K_2CO_3 and was filtered. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel (10% MeOH-CH_2Cl_2) to yield **9** (139 mg, 78%) as a white crystalline solid: mp 164-166°C; ^1H NMR ($CDCl_3$, 200 MHz) δ 8.54 (d, 1H, J = 4.2 Hz), 7.72 (s,

1H), 7.66 (t, 1H, J = 8.9 Hz), 7.52 (d, 1H, J = 15.0 Hz), 7.36 (d, 1H, J = 15.0 Hz), 7.30-7.18 (m, 2H), 4.92 (s,2H), 4.54 (s, 2H), 2.92 (s, 3H), 2.40 (s, 3H); MS (DCI/NH_3) 314 (M+1).

Preparation of aldehyde 2. To a stirred solution of **9** (85 mg, 0.27 mmol) in 15:1 $CHCl_3$/MeOH (16 ml) was added activated MnO_2 (355 mg, 4.07 mmol). After 3 h at room temperature the mixture was filtered through celite and the solvent was removed under reduced pressure. The remaining residue was chromatographed on silica gel (10% MeOH-CH_2Cl_2) to yield **2** (74 mg, 88%) which was recrystallized from isopropanol to give a pale yellow solid: mp 124-125.5°C; ^1H NMR ($CDCl_3$, 300MHz) δ 10.46 (s, 1H), 8.59 (d, 1H, J = 4.2 Hz), 8.04 (s, 1H), 7.68 (d, 1H, J = 14.9 Hz), 7.46 (d, 1H, J = 14.9 Hz), 4.96 (s, 2H), 3.08 (s, 3H), 2.52 (s, 3H); MS (DCI/CH_4) 312 (M+1).

Preparation of 10. To a solution of **9** (85 mg, 0.27 mmol) in degassed MeOH (15 ml) was added 10% Pd/C (85 mg). The mixture was kept under a hydrogen balloon for 3.5 h. The catalyst was removed by filtration and the solvent was removed under reduced pressure. The residue obtained was chromatographed on silica gel (10% MeOH-CH_2Cl_2) to yield **10** (80 mg, 93%) as a white solid: mp 136-138°C; ^1H NMR (CD_3OD, 200 MHz) δ 8.44 (d, 1H, J = 4.3 Hz), 7.75 (t, 1H, J = 7.7 Hz), 7.71 (s, 1H), 7.36 (d, 1H, J = 8.1 Hz), 7.26 (t, 1H, J = 4.9 Hz), 4.78 (s, 2H), 4.60 (s, 2H), 3.12 (t, 2H, J = 7.2 Hz), 2.92 (s, 3H), 2.88 (t, 2H, J = 7.3 Hz), 2.40 (s, 3H); MS (DCI/NH_3) 316 (M+1).

Preparation of aldehyde 3. A stirred solution of **10** (80 mg, 0.25 mmol) in 15:1 $CHCl_3$/MeOH (16 ml) was treated with activated MnO_2 (331 mg, 3.81 mmol) at room temperature for 7 h. The mixture was filtered through celite and the solvents were removed under reduced pressure. Chromatography on silica gel (5% MeOH-CH_2Cl_2) gave **3** (59 mg, 74%) as a pale yellow oil: ^1H NMR ($CDCl_3$, 200 MHz) δ 11.80 (bs, 1H), 10.33 (s, 1H), 8.47 (d, 1H, J = 4.7 Hz), 8.03 (s, 1H), 7.61 (t, 1H, J = 7.7 Hz), 7.23 (d, 1H, J = 7.8 Hz), 7.13 (t, 1H, J = 4.8 Hz), 4.87 (s, 2H), 3.19 (t, 2H, J = 6.4 Hz), 2.95 (s, 3H), 2.87 (t, 2H, J = 6.6 Hz), 2.55 (s, 3H); MS (DCI/NH_3) 314 (M+1).

Preparation of amine 4. A solution of **2** (39 mg, 0.125 mmol) and D,L-alanine (5.57 g, 0.63 mol) in 40:1 H_2O/DMSO (200 ml) at pH 4.0 was stirred at room temperature for 48 h. The mixture was acidified to pH 2.5 and applied to a CM-25 ion exchange Sephadex column with an ammonium bicarbonate buffer gradient (0 - 0.5 M) to give **4** (8.0 mg, 20%) as a white solid: ^1H NMR (CD_3OD, 200 MHz) δ 8.64 (d, 1H, J = 4.3 Hz), 7.94 (t, 1H, J = 7.6 Hz), 7.8-7.4 (m, 5H), 4.78 (s, 2H), 4.18 (s, 2H), 3.17 (s, 3H), 2.53 (s, 3H); MS (DCI/NH_3) 313 (M+1).

Preparation of amine 5. To a stirred solution of **2** (40 mg, 0.13 mmol) in 3:1 H_2O/MeOH (20 ml) was added NaOAc (13 mg, 0.19 mmol) and NH_2OH·HCl (32 mg, 0.39 mmol). After 1h at room temperature, the reaction mixture was filtered and the solid obtained was dissolved in HOAc (1 ml) and treated with zinc dust (30 mg, 0.45 mmol) for 0.5 h at room temperature. The mixture was filtered through celite and the solvent was removed under reduced pressure. The residue obtained was chromatographed on

CM-25 ion exchange Sephadex with an ammonium bicarbonate buffer gradient (0 - 0.5 M) to yield **5** (19.8 mg, 50%) as a white solid: mp 138-140°C; ^{1}H NMR (CD_3OD, 200 MHz) δ 8.42 (d, 1H, J = 4.3 Hz), 7.75 (t, 1H, J = 7.6 Hz), 7.52 (s, 1H), 7.36 (d, 1H, J = 7.7 Hz), 7.25 (t, 1H, J = 5.1 Hz), 4.55 (s, 2H), 4.05 (s, 2H), 3.11 (t, 2H, J = 7.2 Hz), 2.90-2.75 (m, 5H), 2.35 (s, 3H); MS (DCI/NH_3) 315 (M+1).

Racemizations by aldehydes 2 and 3. A solution which was 25.2 mM in pyridoxal derivative, 12.6 mM in $Al_2(SO_4)_3$, and 252.5 mM in L-alanine was prepared in a mixed 10:7:3 H_2O/MeOH/DMSO solvent system and the pH was immediately adjusted to 4.0. This mixture was filtered and placed in a polarimeter cell with a path length of 1 dm and volume of 1 ml. Optical rotations were recorded using a Perkin Elmer Model 141 polarimeter at 578 nm and 30 ± 1°C. Good first order plots were linear from 25-95% completion with repeated runs in agreement within 11%.

Transaminations by amines 4 and 5. A kinetic system related to that of Breslow[4] and Martell[10] was used. Methanol solutions 0.16 mM in pyridoxamine derivative, 0.16 mM in $Zn(OAc)_2$, and 1.6 mM in pyruvic acid were brought to "pH" 4.00. The "pH" was read with a glass electrode calibrated against aqueous buffer and is not corrected for solvent change. The kinetics were monitored on a Beckman DU-8 spectrophotometer and rates of conversion of ketimine to aldimine were followed at 383 nm and 30 ± .1°C. Good first order plots were linear from 10- 95% completion with repeated runs in agreement within 7%.

ACKNOWLEDGEMENT

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