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Selective racemization in preference to transamination catalyzed by pyridoxal enzyme analogs

Lei Liu and Ronald Breslow*

Department of Chemistry, Columbia University, New York, NY 10027, USA

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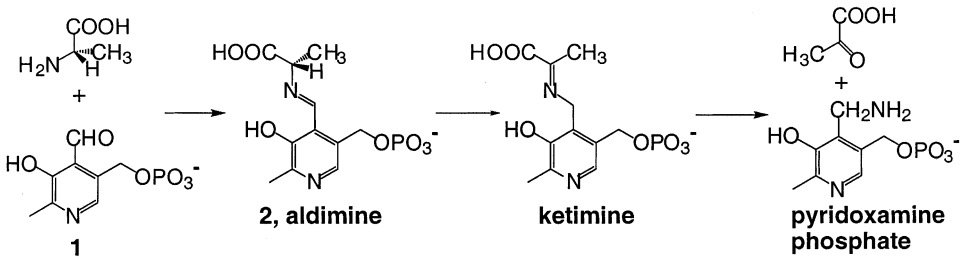
Abstract—The racemase activity of pyridoxal enzyme models is selectively increased in preference to transaminase activity by the attachment of rigid basic groups.

Pyridoxal phosphate **1** is the coenzyme involved in the biochemical reactions that create and transform amino acids.[1,2] In transformations of amino acids a Schiff base aldimine **2** is formed and then a base group of the enzyme performs proton transfers. In the process called transamination the base performs a 1,3-shift of a proton, from C-α of the amino acid to the 4′ position of the pyridoxal unit. Hydrolysis of the ketimine affords an α-ketoacid and pyridoxamine phosphate; reversal of this overall process with a different α-ketoacid achieves transamination.

We have described enzyme mimics based on pyridoxal in which a flexible base is able to perform the 1,3-proton shift leading to transamination, including cases in which a chirally mounted base is able to produce optically active amino acids.[2] However, it is also important to learn how to suppress transamination so as to catalyze other transformations of amino acids with pyridoxal derivatives. In transamination the pyridoxal phosphate is not a catalyst until the second ketoacid restores it, while in other transformations of amino acids the pyridoxal phosphate is normally a true catalyst. Thus, to develop catalysts for amino acid reactions other than transamination, it is necessary to block the transamination process. For instance, pyridoxal phosphate catalyzes the racemization of amino acids, a process in which a base group of the enzyme removes the C-α proton of the amino acid unit in **2** and then reprotonates that same carbon on the opposite face. (In enzymes the reprotonation may be by the same base or by another protonated base group on the opposite face of enzyme-bound **2**, the so-called two-base mechanism).

We earlier described[3] a catalyst **3** with a somewhat rigidly mounted base designed to perform proton transfers at the C-α proton on the amino acid unit in a pyridoxal Schiff base but in which we hoped to suppress proton transfer to the 4′ position of the pyridoxal unit, a process that kills the catalyst. However, the preferences were small, a matter of two-fold or so. Thus, it was clear that a better system was needed. We have now synthesized six pyridoxal-based enzyme models, **4**–**9**.



* Corresponding author.

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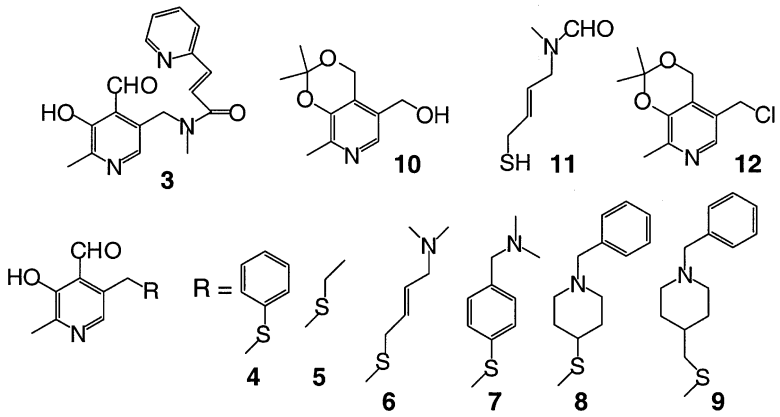


The thiophenyl compound **4** was synthesized from pyridoxine acetonide **10** with diphenyldisulfide and tributylphosphine,[4] then deprotection and oxidation with MnO_2.[5] The *S*-ethyl compound **5** was prepared from diethyldisulfide and **10** by analogy to the preparation of **4**.[5] The butenyl derivative **6** was prepared by alkylation of the thiol **11** with chloride **12**, then Red-Al reduction, deprotection, and then MnO_2 oxidation to the aldehyde.[5] The dimethylamino compound **7** was prepared, in analogy to **4**, from **10** and the corresponding disulfide.[5] The *N*-benzyl compound **8** was prepared from **12** and the *N*-benzoylthiol, by analogy to the preparation of **6**.[5] The *N*-benzyl compound **9** was prepared from the corresponding *N*-benzoylthiol and **12**, by analogy to the preparation of **6**.[5]

Table 1. Racemization and transamination by the pyridoxal enzyme mimics

Compound	Racemization (30.0°C)		Transamination (60.0°C)	
	k_{rac} ($\times 10^{-5}$ min^{-1})[a]	$k_{relative}$	k_{trans} ($\times 10^{-2}$ min^{-1})[b]	$k_{relative}$
4	5.8±0.2	1.00	2.38±0.08	1.00
5	4.6±0.1	0.8	1.72±0.01	0.72
6	34.8±2.4	6.0	4.76±0.09	2.00
7	41.8±1.3	7.2	1.56±0.06	0.66
8	6.1±0.3	1.1	1.72±0.07	0.72
9	9.5±0.4	1.6	1.62±0.10	0.68

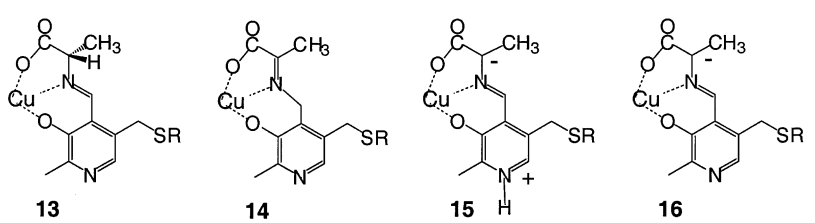
Average of at least three runs, with standard deviations.

[a] Pseudo-first-order rate constant for the conversion of L-alanine to D-alanine at 30.0°C in ethanol/water (7:3, v:v) at pH 9.0. The reaction solution is 1.0×10^{-3} M in pyridoxal derivative, 1.0×10^{-3} M in $CuCl_2$, and 1.0×10^{-2} M in L-alanine. The conversion of L to D was carried to 20%, and the plot took account of the reversibility of the process.

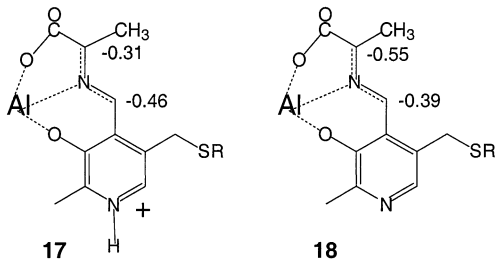
[b] Pseudo-first-order rate constant for conversion of the aldimine to the ketimine, from the decrease in UV at 400 nm, at 60.0°C in water at pH 9.0. The reaction solution is 5.0×10^{-5} M in pyridoxal derivative, 5.0×10^{-5} M in $CuCl_2$, and 1.35 M in L-alanine.

We then examined them, as their Cu(II) complexes, as catalysts for transamination and for racemization of L-alanine (the racemization of L-alanine by bacterial racemases is a principal source of D-alanine for bacterial cell-wall synthesis). Rates of the key step in transamination were followed in the UV at 400 nm, observing the transformation of the Cu(II) complexed aldimine into the ketimine, while racemization of L-alanine to DL-alanine was followed with HPLC, as described previously.[6] The results are listed in Table 1.

Of course racemization of an amino acid could occur by reversible transamination, in which the amino acid aldimine **13** is converted to the optically inactive ketimine **14**, and then back to the racemic aldimine followed by hydrolysis. Olivard, Metzler and Snell studied the relative rates, and concluded that at pH 5 this is actually the mechanism of racemization of amino acids by pyridoxal in solution, since transamination was faster than racemization.[7] However, they showed that at pH 9 racemization is an independent process, faster than transamination. Martell has pointed out[1] that transamination involves preferential protonation of intermediate **15** at the 4′ position, while racemization involves the same nominal intermediate that preferentially protonates at the amino acid α position. However, at pH 5 the pyridine ring in intermediate **15** is protonated, while at pH 9 the intermediate is **16**, with an unprotonated pyridine ring.



As Martell indicates, the change in reaction path reflects the expected difference in electron distribution in intermediates **15** and **16**, from the electrostatic effect of a pyridinium ring on the 4′ carbon. Our Mulliken charge calculations on species **17** and **18**, with the semiempirical PM3 method,[8] indicate that protonation of the pyridine ring significantly changes the electron distributions at the 4′ and amino acid α positions so as to favor transamination in the pyridine-protonated case, and racemization in the unprotonated case. It is interesting that in the alanine racemase[9] from *Bacillus stearothermophilus* the unprotonated pyridine nitrogen is hydrogen bonded to a weakly acidic guanidinium group of Arg219, while in aspartate aminotransferase[10] the pyridine nitrogen is protonated and hydrogen bonded to a weakly basic aspartate carboxylate group.



We performed our studies at pH 9, where racemization is a process independent of transamination. As described in Table 1, the racemization study was done with a low concentration of L-alanine, while the UV study of the conversion of aldimine **13** to ketimine **14** required a very large concentration of L-alanine to produce sufficient aldimine, and the media and temperatures also differ; thus the rate constants for the two processes are not directly comparable. To suppress extraneous buffer catalysis of the process, the reactants themselves were the only buffers. Measurements showed that the pH was unchanged at the end of the reactions.

The data show that the flexibly mounted base in **6** was able to catalyze both processes, and models show that it has available conformations to permit the base to deprotonate the aldimine **13** at the amino acid α-carbon and deliver the proton either to that carbon—for racemization—or to the 4′ carbon to form ketimine **14**. However, the rigidly mounted base groups in **7**, **8** and **9** do not catalyze transamination, but suppress it somewhat, perhaps by steric effects or by diverting a common intermediate to racemization. The base group in **7** is able to catalyze racemization—models show that the base can reach the α-carbon of the amino acid unit in **16**, but not the 4′ carbon. The more hindered base groups in **8** and **9** are not significantly catalytic in racemization, but better than in transamination. As expected from these results, with 0.05 M L-alanine, 0.001 M **7** and 0.001 M $CuCl_2$ in 7:3 ethanol:water at 30°C and pH 9.0 we saw 25 turnovers in the racemization of the alanine in 72 hours. Thus, the behavior of compound **7** is an important lead for the production of pyridoxal-based catalysts that suppress transamination. Such extensions will be described elsewhere.

Acknowledgements

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4. Typical experimental procedure: 0.21 g of **10** and 0.22 g of diphenyldisulfide are dissolved in 3 ml of anhydrous THF and 0.3 ml of tributylphosphine is added. The reaction is stirred at room temperature overnight. Chromatography with 2/1 hexane/ethyl acetate provides 0.29 g (95%) of the desired product.
5. **4**: ^{1}H NMR (CD_3COCO_3): δ 2.47 (s, 3H), 2.81 (s, 2H), 4.59 (s, 2H), 7.35 (m, 5H), 7.99 (s, 1H), 10.58 (s, 1H). CI MS: 2600 (M+1).
5: ^{1}H NMR (CD_3COCO_3): δ 1.22 (t, J=7.3 Hz, 2H), 2.47 (s, 3H), 2.54 (q, J=7.3 Hz, 2H), 4.17 (s, 2H), 8.08 (s, 1H), 10.64 (s, 1H). CI MS: 212 (M+1).
6: ^{1}H NMR (CD_3CN): δ 2.30 (s, 6H), 2.50 (s, 3H), 2.95 (d, J=4.7 Hz, 2H), 3.15 (d, J=4.7 Hz, 2H), 3.78 (s, 2H), 5.62 (m, 2H), 7.02 (s, 1H), 10.10 (s, 1H). CI MS: 281 (M+1).
7: ^{1}H NMR (THF-d_8): δ 2.15 (s, 6H), 2.38 (s, 3H), 3.38 (s, 2H), 4.36 (s, 2H), 7.25 (m, 4H), 7.72 (s, 1H), 10.30 (s, 1H). CI MS: 317 (M+1).
8: ^{1}H NMR (CD_3Cl): δ 1.70 (m, 2H), 1.90 (d, J=13.0 Hz, 2H), 2.08 (t, J=10.8 Hz, 2H). 2.53 (s, 3H), 2.61 (m, 1H), 2.85 (d, J=11.8 Hz, 2H), 3.50 (s, 2H), 3.98 (s, 2H), 7.30 (m, 5H), 7.95 (s, 1H), 10.52 (s, 1H). CI MS: 357 (M+1).
9: ^{1}H NMR (CD_3Cl): δ 1.30 (m, 2H), 1.45 (m, 1H), 1.75 (d, J=12.6 Hz, 2H), 1.95 (t, J=11.2 Hz, 2H), 2.40 (d, J=6.8 Hz, 2H), 2.52 (s, 3H), 2.90 (d, J=11.6 Hz, 2H), 3.50 (s, 2H), 3.90 (s, 2H), 7.30 (m, 5H), 7.95 (s, 1H), 10.52 (s, 1H). CI MS: 371 (M+1).
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