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Alanine-dependent reactions of 5′-deoxypyridoxal in water

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ABSTRACT

The non-enzymatic reaction of 5′-deoxypyridoxal (DPL) with L-alanine in water at 25 °C was investigated. DPL reacts with alanine to form an imine, which then undergoes deprotonation at the α-amino carbon of alanine to form a resonance delocalized DPL-stabilized carbanion. At early reaction times the only detectable products are pyruvate and the dimeric species formed by addition of the α-pyridine stabilized carbanion to DPL. No Claisen-type products of addition of the α-amino carbanion to DPL, as was previously reported to form from the reaction between DPL and glycine [K. Toth, T.L. Amyes, J.P. Richard, J.P.G. Malthouse, M.E. NíBeilliu, J. Am. Chem. Soc. 126 (2004) 10538–10539], are observed. The electrophile reacts instead at the α-pyridyl carbon. This dimer is in chemical equilibrium with reactants. At longer reaction times about 50% of DPL is converted to 5′-deoxypyridoxamine, the thermodynamically favored product of formal transamination of DPL.

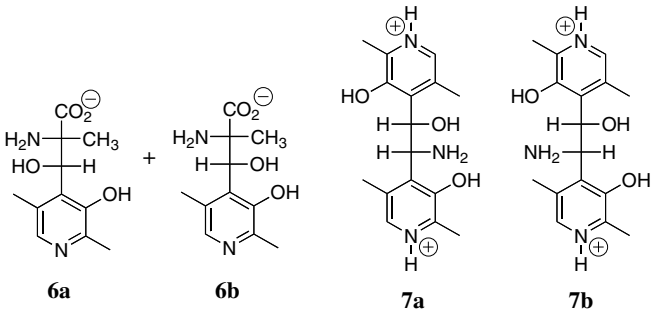
1. Introduction

Pyridoxal 5′-phosphate (PLP) is utilized as a cofactor in a large number of enzymatic reactions [1–3], because of the extreme stabilization it provides for resonance stabilized α-amino carbanion intermediates of these reactions [4–9]. The chemistry of PLP is assumed to be thoroughly characterized and we were therefore surprised to observe that the reaction between the pyridoxal 5′-phosphate (PLP) analog 5′-deoxypyridoxal (DPL, 0.01 M) and glycine (0.10 M) in buffered solutions of D_2O at neutral pD does not give any of the expected products of a PLP-catalyzed reaction, but rather a *quantitative* yield of diastereomers **4a** and **4b** (Scheme 1) is obtained from the formal Claisen-type addition of the DPL-stabilized glycine carbanion **3** to **1** [10]. At pH ⩾ 8.0, where there is essentially quantitative conversion of DPL to the imine **2**, an additional product from addition of the DPL-stabilized glycine carbanion **3** to the electrophilic α-pyridyl carbon of the imine **2** is also observed [11].

The trapping of **1** by **3** (k_{add}[**1**], Scheme 1) is so efficient that formation of **3** by deprotonation of **2** (k_{-p}, Scheme 1) is the rate-determining step for Claisen-type addition to form **4** (k_{add}[**1**] ≫ k_p). This step is rate-determining even when [**1**] < 1 mM). The first-order rate constants for deprotonation of **2** have been determined as the observed first-order rate constants for the overall Claisen-type addition of glycine to **1** to give **4** [5]. A study of the pH dependence of the rate constants for Claisen-type addition has provided a thorough description of the kinetic acidity of the different ionic forms of **2**, along with estimates of the effect of the protonation of basic sites of the pyridoxal cofactor on carbon acid pK_a [5].

An interesting question is whether the Claisen addition reaction observed for glycine is also observed for the reaction of other amino acids in the presence of PLP or DPL. An extremely thorough 40-year-old study of 3-hydroxypyridine 4-aldehyde-catalyzed reactions of D,L-alanine noted only the formation of pyruvate from a transamination reaction of alanine [12,13]. In this study, the concentration of the cofactor-analog was only 0.25 mM. However, the yields of the products of the formally bimolecular addition of the cofactor-stabilized α-amino carbanion to a second molecule of cofactor will increase as the concentration of the cofactor is increased.

We report here an examination of the reaction of **1** (0.01 M) in the presence of alanine (0.10 M). No **6a** and **6b** were detected to form from the Claisen-type addition of alanine to **1**. The major products observed at early reaction times are **7a** and **7b** from addition of the α-pyridyl carbon of the delocalized pyridoxal-stabilized alanine carbanion to a second molecule of pyridoxal.



6a **6b** **7a** **7b**

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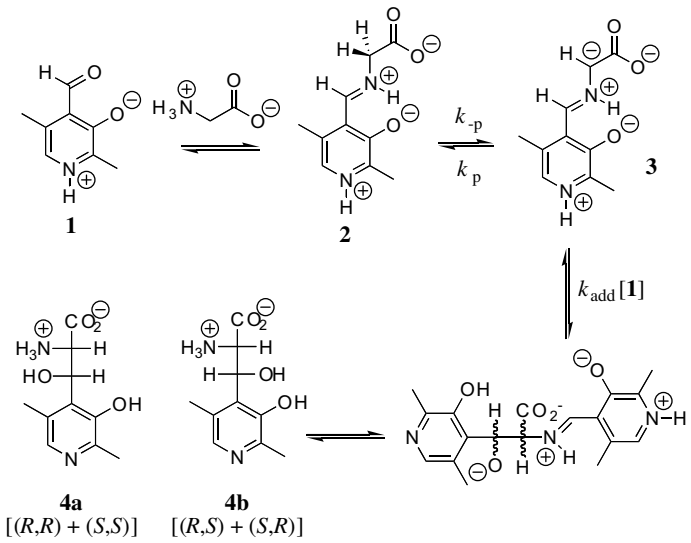


Scheme 1.

2. Experimental

The water used for product studies was first distilled and then passed through a Milli-Q water purification system. Pyridoxal phosphate monohydrate was from Aldrich and was used without further purification. The other organic chemicals used in chemical syntheses were reagent grade from Aldrich. Monobasic and dibasic potassium phosphate, potassium chloride, and hydrochloric acid (37 w/w%) were from J.T. Baker and were used without further purification. Tetramethyl ammonium hydrogen sulfate and Celite 545 diatomaceous earth were from Fisher. Aminomalonic acid and 5′-deoxypyridoxal (**1**) were synthesized by literature procedures [14].

2.1. ^{1}H NMR and mass spectral analyses

^{1}H NMR spectra at 500 MHz in H_2O at 25 °C were obtained using a concentric inner tube that contained D_2O for locking the spectrometer. A 6000 Hz sweep width, 6 s acquisition time and a 90° pulse angle were used. In general, the spectra were determined after 64 transients, using a 70 s relaxation delay, which was at least sevenfold greater than T_1 for the protons of interest. The spectra in H_2O were obtained with suppression of the peak for HOH. The chemical shifts are reported relative to HOH at 4.67 ppm. Molecular weights were determined using a Bruker BioApex 30 FT-MS with electrospray interface and a VG 70-SE double-focusing high resolution mass spectrometer.

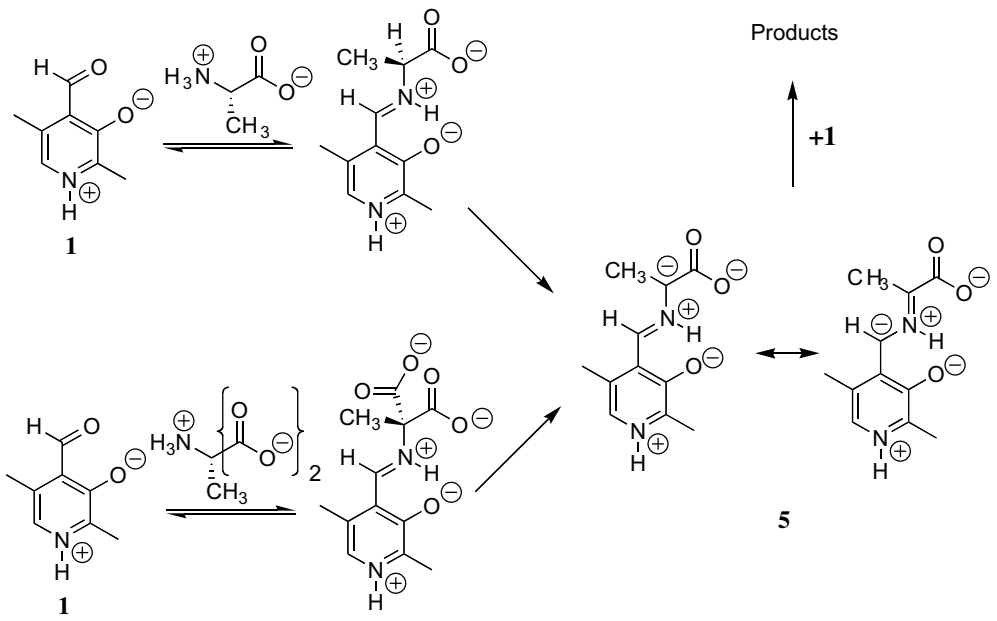
2.2. Reaction of *1* with alanine

A solution (55 mL) in water was prepared to contain 10 mM **1**, 0.10 M L-alanine, and 0.1 M phosphate buffer at pH 6.4. The solution was stirred at 25 °C for 24 h and the reaction was quenched by the slow addition of 1.60 g (0.0426 mol) $NaBH_4$. This removes pyruvate from the reaction, and is necessary to eliminate the pyruvate-catalyzed cleavage of **7a** and **7b** to form **1** and alanine. Five milliliters of concentrated HCl was then added, and the reaction was loaded into an Amberlite AG 50W x 8, 200–400 mesh cation-exchange column, H^+ form. Step elution with several column volumes each of solutions of 2, 2.8 and 3.3 M HCl, and monitoring at 295 mm the eluant from the column, gave a single major UV absorbing peak. The peak fractions were collected and the solvent was evaporated on a rotary evaporator to give a mixture of the diastereomers **7a** and **7b** as a dark yellow solid. ^{1}H NMR in D_2O,: major diastereomer of (87%): δ 1.82, 1.98, 2.48, 2.49 (3H, s, CH_3); 5.15 (1H, d, J = 10 Hz, $CH(ND_3)^+$); 5.89 (1H, d, J = 10 Hz, CH(OD)); 7.57, 7.75 (1H, s, ArH), minor diastereomer (13%): δ 1.62, 1.64, 2.44, 2.53 (3H, s, CH_3); 5.03 (1H, d, J = 10 Hz, $CH(ND_3)^+$); 5.69 (1H, d, J = 10 Hz, CH(OD)); 7.41, 7.71 (1H, s, ArH); exact mass calculated for $C_{16}H_{21}O_3N_3$, 304.1663. Found: 304.1667.

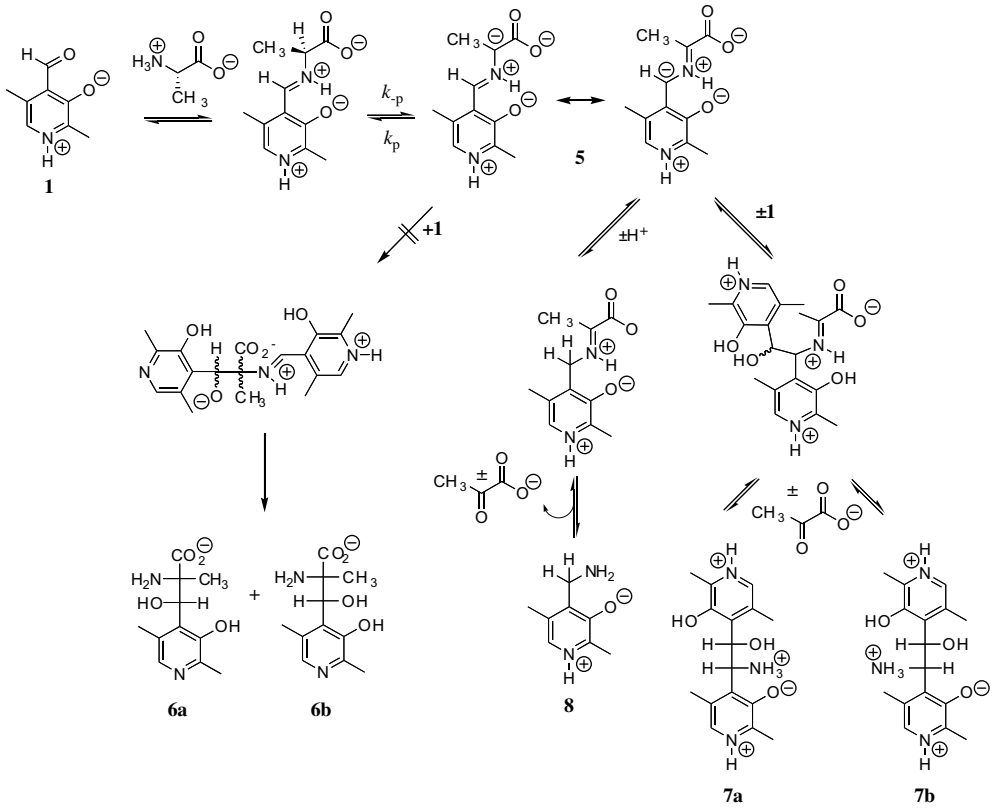
A 2 mL reaction mixture was prepared in water to contain 10 mM **1**, 0.10 M L-alanine, 0.1 M phosphate buffer at pH 6.4 (I = KCl, 1.0 M) and 1 mM tetramethyl ammonium hydrogen sulfate. The progress of the reaction was monitored by ^{1}H NMR over a period of 120 h. ^{1}H NMR spectra were obtained periodically; and, the concentrations of the reactants and products were determined from the area of the relevant reactant or product peak relative to the area for the methyl group of the internal standard tetramethylammonium hydrogen sulfate (1 mM). The following peaks were used for these analysis: the singlet at 2.25 ppm due to the methyl protons of pyruvate; the singlet at 4.09 ppm due to the methylene protons of the pyridoxamine from the transamination reaction; the singlets at 2.49, 2.48, 1.98 and 1.82 ppm for the aromatic methyl groups of the major diastereomer of **7**; and, the singlets at 2.53, 2.44, 1.64 and 1.62 for the aromatic methyl groups of the minor diastereomer of **7**.

3. Results and discussion

The major products derived from DPL that were isolated from the large-scale reaction of **1** and L-alanine at pH 6.4 were identified by ^{1}H NMR analyses to be diastereomers **7a** and **7b**. These diastereomers were also reported in earlier work to form as the products of the DPL-catalyzed decarboxylation of α-methyl(aminomalonate) at pH 5.2 [15]. These two studies are consistent with the reaction mechanism shown in Scheme 2. The reaction of **1** with alanine and with α-methyl(aminomalonate) gives the corresponding imines. These then undergo deprotonation or decarboxylation to form the common carbanion intermediate **5**. This carbanion then reacts with a second mole of **1** to form the imine precursors to **7a** and **7b**, or protonation at the α-pyridyl carbon to form the precursor to the pyridoxamine **8** (Scheme 3) [15]. Our ^{1}H NMR analysis of the reaction at pH 6.4 shows that no **6a** or **6b** form from Claisen-type addition of the DPL-stabilized alanine carbanion to **1**. By comparison, these adducts were reported to form as products of DPL-catalyzed decarboxylation of α-methyl(aminomalonate) at



Scheme 2.



Scheme 3.

pH 5.2. The difference between the products observed in these two experiments may be due to the effect of the changing pH on the partitioning of the resonance delocalized carbanion **5** between addition of electrophiles to the α-amino and to the α-pyridyl carbons.

The products of the reaction between L-alanine (0.1 M) and **1** (10 mM) in 0.1 M potassium phosphate buffer (I = 1.0, KCl) at pH 6.4 were monitored by ^{1}H NMR over a period of 120 h. Fig. 1 shows the change in the reactant concentration and product composition during this time. The major products at earliest reaction times are pyruvate and **7**. No pyridoxamine **8** from protonation of the α-pyridyl carbon was observed at early times. However, this product accumulates at later times, and after five days it is the major product. The concentration of **7** increases to a maximum concentration

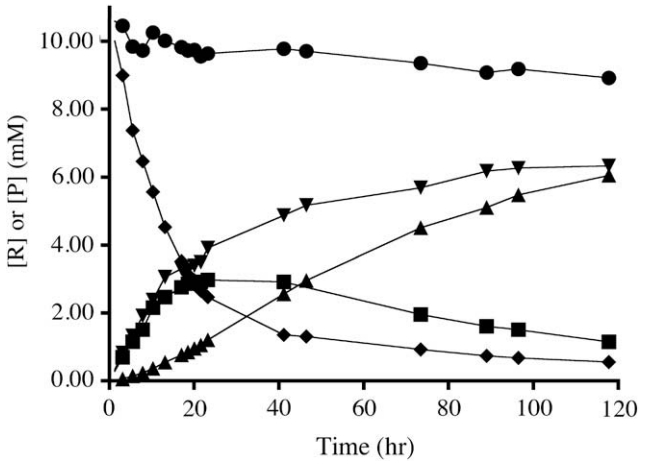


Fig. 1. Time course for reaction of L-alanine (0.1 M) and **1** (10 mM) in 0.1 M potassium phosphate buffer (I = 1.0, KCl) at pH 6.4. Key: (♦), **1**; (■), **7a** + **7b**; (▲), **8**; (▼), pyruvate; (●), the sum of the concentrations of the pyridine cofactor present in reactant **1** and products **8** and **7**, calculated as the sum [**1**] + [**8**] + 2 ∗ [**7**].

after a 20-h reaction time and then decreases as **7** is converted to **8**. Once again, No **6** is observed to form at either early or late reaction times. During the 120 h reaction time there is nearly quantitative conversion of **1** to **7** or **8** (♦, Fig. 1) and good conservation of the original cofactor (●, Fig. 1). The concentration of pyruvate observed at long reaction times is ca. 15% smaller than the expected concentration: [Pyr] = [**7**] + [**8**]. This may be due to the slow bimolecular aldol condensation of pyruvate.

The data from Fig. 1 are consistent with the complex set reactions shown in Scheme 3. The negative charge at the DPL-stabilized carbanions is delocalized over the α-amino and the α-pyridyl carbons. The reaction of the pyridoxal-stabilized glycine carbanion **3** is almost exclusively at the α-amino carbon to form **4**, the Claisen-type adduct of **1** to glycine (Scheme 1) [10]. The addition of the methyl group at **3** destabilizes charge at the α-amino carbon and introduces crowding at this site. Both effects favor the reaction of the α-pyridyl carbon as a nucleophile to give the adducts **7a** and **7b**. These are the main products formed at early times when the reaction is under conditions of *kinetic* control. An initial attempt to purify **7** by column chromatography was unsuccessful, presumably because **7** undergoes pyruvate-catalyzed cleavage during the chromatography step. This purification was successful when the pyruvate was reduced to lactate by sodium borohydride prior to the purification of **7**. Fig. 1 confirms that the formation of **7a** and **7b** is reversible by showing that the yields of these products increase to a maximum during the first 20-h of the reaction, and then break down to form the thermodynamically more stable **8**, which is the dominant product at long times.

In conclusion, Claisen-type addition of glycine to form **4** and alanine promoted dimerization to form **6** are each a consequence of the extraordinary, but poorly recognized ability of the pyridoxal-stabilized carbanions such as **3** and **5** to scavenge for the carbonyl electrophile **1** in protic solutions that would otherwise be expected to protonate these carbanions. The physiological significance of this reaction is unclear, or perhaps even doubtful. However, the importance of PLP in biology is so great that there is intrinsic value to thoroughly defining its chemical reactivity, since this knowledge may one day prove useful in ways that are difficult to predict.

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References

[1] J.C. Vederas, H.G. Floss, Acc. Chem. Res. 13 (1980) 455–463.
[2] A.E. Martell, Adv. Enzymol. Relat. Areas Mol. Biol. 53 (1982) 163–199.
[3] M.D. Toney, Arch. Biochem. Biophys. 433 (2005) 279–287.
[4] D.T. Major, J. Gao, J. Am. Chem. Soc. 128 (2006) 16345–16357.
[5] K. Toth, J.P. Richard, J. Am. Chem. Soc. 129 (2007) 3013–3021.
[6] M.A. Spies, J.J. Woodward, M.R. Watnik, M.D. Toney, J. Am. Chem. Soc. 126 (2004) 7464–7475.
[7] A. Rios, J.P. Richard, J. Am. Chem. Soc. 119 (1997) 8375–8376.
[8] A. Rios, J. Crugeiras, T.L. Amyes, J.P. Richard, J. Am. Chem. Soc. 123 (2001) 7949–7950.
[9] A. Rios, T.L. Amyes, J.P. Richard, J. Am. Chem. Soc. 122 (2000) 9373–9385.
[10] K. Toth, T.L. Amyes, J.P. Richard, J.P.G. Malthouse, M.E. NíBeilliu, J. Am. Chem. Soc. 126 (2004) 10538–10539.
[11] K. Toth, L.M. Gaskell, J.P. Richard, J. Org. Chem. 71 (2006) 7094–7096.
[12] D.S. Auld, T.C. Bruice, J. Am. Chem. Soc. 89 (1967) 2098–2106.
[13] D.S. Auld, T.C. Bruice, J. Am. Chem. Soc. 89 (1967) 2090–2097.
[14] W.C. Cunningham, J.W. Thanassi, Experimentia 35 (1979) 451–452.
[15] J.W. Thanassi, Biochemistry 9 (1970) 525–532.