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Chemo-Enzymatic Syntheses of Isotopically Labelled L-Amino Acids

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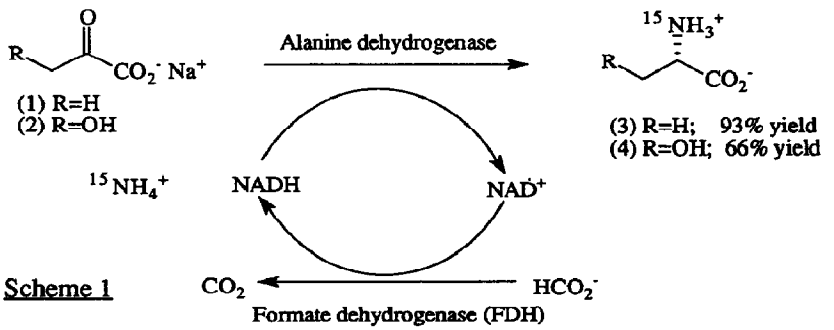
Abstract *: L-Alanine labelled with carbon-13 and nitrogen-15 has been prepared in good yield and high optical purity from achiral precursors using alanine dehydrogenase This versatile approach may be simply adapted for the preparation of a range of isotopically labelled amino acids and efficient syntheses of [^{15}N]-labelled serine, valine, leucine and phenylalanine are described.*

Stable isotope labelled amino acids are required for a variety of bioorganic chemical studies and in particular for examining the tertiary structure of proteins by NMR spectroscopic techniques. These studies are often limited by the cost and availability of appropriately labelled compounds. Various enzymatic methods have been used previously for the synthesis of isotopically labelled amino acids including the use of aspartate transaminase to prepare [^{15}N]-tyrosine,[1] glutamate dehydrogenase to give [^{15}N]-glutamic acid[2] and a coupled glutamate dehydrogenase-amino transferase to prepare [^{15}N]- and [^{13}C]- labelled valine, leucine, methionine and phenylalanine.[3] In this letter we describe a new approach for the preparation of carbon-13 and nitrogen-15-labelled L-alanine from either the sodium salt of pyruvic acid (1, Scheme 1) or its [3-^{13}C]-analogue using alanine dehydrogenase. In addition syntheses of [^{15}N]-labelled serine, valine, leucine and phenylalanine are described (Schemes 1, 2, and 3). The source of ^{15}N label is [^{15}N]ammonium formate.



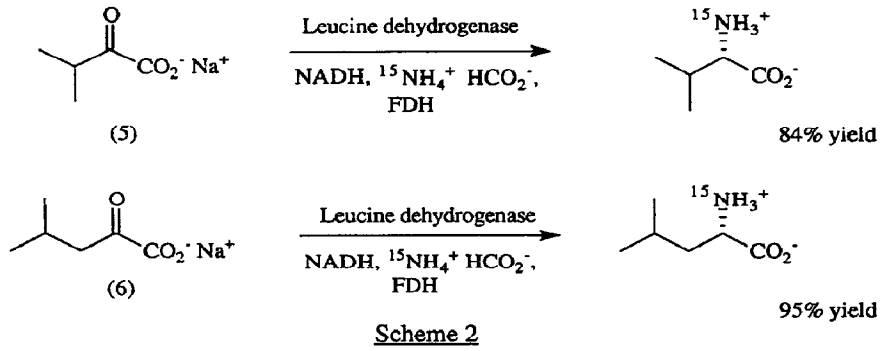
Scheme 1

Alanine dehydrogenase is one of a series of commercially available dehydrogenases which catalyse the reductive amination of a range of α-keto acids to give the corresponding L-amino acids. The natural function of alanine dehydrogenase[4] is to catalyse the reversible conversion of pyruvic acid to alanine. The reductive amination process requires a stoichiometric amount of the co-enzyme NADH. To prepare alanine on a gram scale, we have used alanine dehydrogenase from *Bacillus subtilis* in conjunction with a second enzyme, formate dehydrogenase (FDH)[5] from *Candida boidinii*. Formate dehydrogenase catalyses the oxidation of formate ions to carbon dioxide and releases hydride ion which combines with NAD^+ so enabling the expensive co-factor NADH to be regenerated *in situ*. Ammonium formate is a convenient source of both the requisite ammonium and formate ions, however a combination of sodium formate and ammonium chloride are equally effective. [^{15}N]-Alanine, with 98% incorporation ^{15}N, was efficiently prepared (93% yield) from the sodium salt of pyruvic acid (1) using alanine dehydrogenase in the presence of [^{15}N]-ammonium formate[6] (Scheme 1). The reaction is simple to perform and on a 1 mmole scale is complete within 1 day.[7]

Typical procedure: *Tris buffer (25ml, 5mM) and NH_4^+ HCO_2^- (0.126g, 2mmol) were degassed for 1 hour. The α–keto acid (1mmol), dithiothreitol (2μl of 1M solution), FDH (7.8mg, 5eU), NADH (8mg, 0.1 mmol) and alanine dehydrogenase (5eU)[8] were added. The reaction was stirred at room temperature under nitrogen maintaining the pH between 6.5 and 7.0 by the addition of HCl (0.1M). Once no further change in pH was observed the reaction mixture was concentrated* in vacuo *and purified by ion exchange chromatography (using Dowex 50X8-100).*[9]

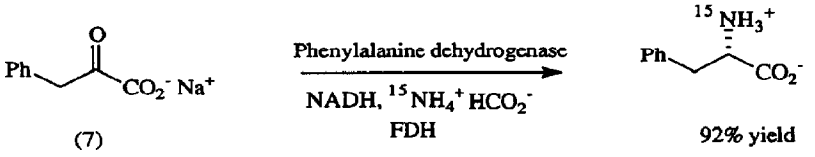
It has been reported[10] that alanine dehydrogenase has a very limited substrate specificity range. However we have found that it will efficiently catalyse the reductive amination of the sodium salt of β-hydroxypyruvic acid (2) to give L-serine. Addition of [^{15}N]-ammonium formate to the reaction mixture gave homochiral [^{15}N]-serine in 66% yield from (2) after ion exchange chromatography.[7]

This enzyme-based approach for the preparation of [^{15}N]-alanine and [^{15}N]-serine is versatile and has potential for the efficient synthesis of a wide range of isotopically labelled amino acids. For example we have found that although the α–keto acids with branched hydrocarbon side-chains (5) and (6) are not suitable substrates for alanine dehydrogenase, another enzyme, leucine dehydrogenase[11] will efficiently catalyse the reductive amination of (5) and (6) in the presence of [^{15}N]-ammonium formate to give homochiral [^{15}N]-valine and [^{15}N]-leucine respectively in excellent yields (Scheme 2). The reaction is performed using an analogous protocol to that used for alanine dehydrogenase. Interestingly the sodium salts of both pyruvic acid (1) and 3-hydroxy-pyruvic acid (2) are also good substrates for the enzyme leucine dehydrogenase.



Scheme 2

In contrast, incubation of the sodium salt of 3-phenyl-pyruvic acid (7) with either alanine dehydrogenase or leucine dehydrogenase simply returned starting material (7). However [^{15}N]-phenylalanine was prepared in 92% yield from (7) using an alternative enzyme, phenylalanine dehydrogenase[12] (Scheme 3).



Scheme 3

A further advantage of this enzyme-based approach for the preparation of isotopically labelled amino acids from α-keto acids is that it may be simply adapted for the synthesis of [^{13}C]-labelled amino acids. For example treatment of diethyl oxalate with [^{13}C]methylmagnesium iodide at -78°C in a mixture of THF and diethyl ether gave [3-^{13}C]ethyl pyruvate in 85% yield. None of the bis-addition product was observed. Saponification of the ester with sodium hydroxide gave the sodium salt of [3-^{13}C]pyruvic acid. Incubation of the salt with alanine dehydrogenase in the presence of [^{15}N]ammonium formate gave homochiral [^{15}N, 3-^{13}C]-alanine in 58% overall yield from [3-^{13}C]ethyl pyruvate.

In conclusion, the chemo-enzymatic approach outlined in this paper, for the synthesis of isotopically labelled amino acids from α-keto acids is versatile and may be applied to the preparation a range of amino acids incorporating nitrogen-15 and/or carbon-13 labels. The reactions are economical and simple to perform on a synthetically useful scale (1- 50 mmol) giving high yields of homochiral amino acids.

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6. $^{15}NH_4{}^+HCO_2{}^-$ is not commercially available but may be simply prepared by bubbling $^{15}NH_3$ through HCO_2H.
7. The amino acids were pure by chiral tlc and hplc and gave the expected NMR and mass spectral data and the optical rotations were in good agreement with literature values on unlabelled material. ^{15}N and ^{13}C incorporations were determined by mass spectrometry.
8. Alanine dehydrogenase is available in glycerol/ phosphate buffer. Removal of the glycerol is achieved by passing the alanine dehydrogenase through an HPLC column pre-charged with Sephadex G-25 Superfine and eluting with tris buffer (20mM).
9. When the reaction is conducted on a larger scale it is advisable to add the substrate in portions to avoid substrate inhibition of the enzyme.
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