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Enzymes as reagents in organic chemistry: transketolase-catalysed synthesis of D-[1,2-$^{13}C_2$]xylulose

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ABSTRACT

Oxidation of commercial DL-[2,3-$^{13}C_2$]serine by D-amino acid oxidase gave [2,3-$^{13}C_2$]hydroxypyruvate, the condenstion of which with D-glyceraldehyde, catalysed by transketolase (partially purified from spinach leaves), gave D-*threo*-[1,2-$^{13}C_2$]pentulose (D-[1,2-$^{13}C_2$]xylulose), which was studied by ^{13}C-n.m.r. spectroscopy.

INTRODUCTION

Studies of the metabolism and structure of sugars often require isotopically labeled compounds. New developments in ^{13}C-n.m.r. spectroscopy, especially *in vivo* studies, are increasing the need for ^{13}C-labeled products. Compounds with two adjacent ^{13}C labels have a particular advantage due to the ^{13}C–^{13}C coupling [1a]. Numerous reports, based on these techniques, have been published that deal with the structure and conformation of sugars in solution[1], their reactivity[2] and metabolism[3], the mechanism of enzymic reactions[4], the structure of polysaccharides and glycoconjugates[5], the biosynthesis of secondary metabolites[6], and medical applications[7]. Although some chemical syntheses of labeled sugars have been published[8], enzymic approaches appear to be particularly suitable. Thus, D-fructose 1,6-diphosphate has been prepared using fructose 1,6-diphosphate aldolase and labeled precursors obtained either chemically[9] or enzymically[10,11]. Labeled D-glucose, D-glucose 6-phosphate, D-fructose 1-phosphate, D-fructose 6-phosphate, D-fructose, and D-galactose have been obtained enzymically from D-fructose 1,6-diphosphate[9]. Sugars with 5, 7, and 8 carbon atoms have also been prepared enzymically[6b,12].

Transketolase (TK) (EC 2.2.1.1) has been recently used to synthesise various natural ketoses and their analogs from hydroxypyruvate[13]. This enzyme offers several advantages for the production of labeled ketoses since C_4, C_5, C_6, and C_7 compounds can be produced and phosphorylated substrates are not required, thus considerably reducing the number of reaction steps. We now report the use of transketolase to prepare [1,2-$^{13}C_2$]ketoses from [2,3-$^{13}C_2$]hydroxypyruvate obtained enzymically by D-amino acid oxidase-mediated oxidation of commercial DL-[2,3-$^{13}C_2$]serine (Scheme 1).

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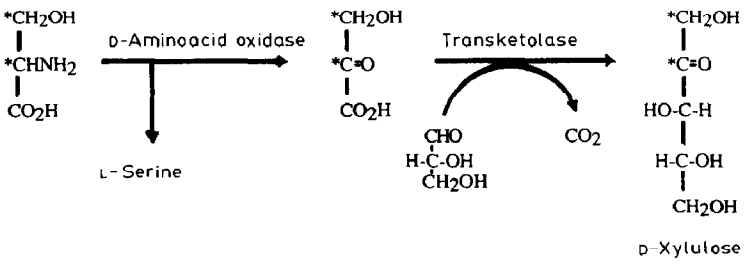

Scheme 1. Conversion of DL-[1,2-$^{13}C_2$]serine into D-[1,2-$^{13}C_2$]xylulose.

RESULTS AND DISCUSSION

Synthesis of hydroxypyruvate. — α-Keto acids may be obtained from amino acids by chemical[14] or enzymic[15] transamination. However, the chemical reaction on serine produces pyruvic acid instead of hydroxypyruvic acid[16], and serine aminotransferases are commercially unavailable. Enzymic oxidation of α-amino acids also leads to α-keto acids. Alanine dehydrogenase oxidises serine, but the activity for this substrate is low[17] and the method requires reoxidation of the cofactor (NAD^+). Thus, amino acid oxidases, which utilise molecular oxygen, are more attractive. Of these enzymes, only D-amino acid oxidase (EC 1.4.3.3) is active on serine[18].

The synthesis of hydroxypyruvate from D-serine with D-amino acid oxidase (7 U/mL) in oxygen-saturated Tris buffer at pH 8.6 was monitored by the lactate dehydrogenase assay[19]. The effect of the concentration of the substrate on the activity of the enzyme is shown in Fig. 1 and maximum activity was reached at 20mM serine. Excess of substrate did not cause inhibition since the rate of reaction was still high at 140 mM. The maximum rate was 57% of that observed for D-alanine, the usual substrate for the D-amino acid oxidase assay. Moreover, at the same concentration, L-serine did not inhibit the reaction.

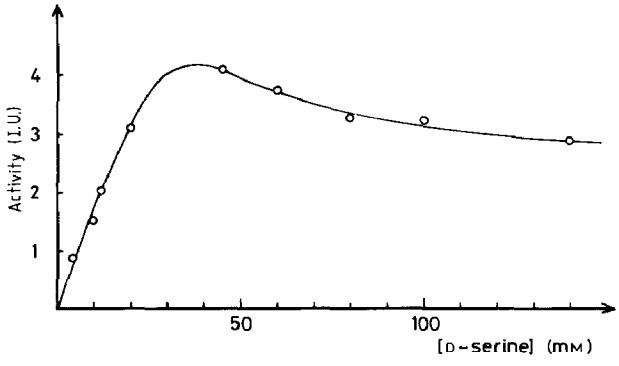

Fig. 1. Dependence of D-amino acid oxidase activity (in μmol of hydroxypyruvate formed per min) on the concentration of D-serine.

Increasing the levels of H_2O_2 during D-serine oxidation inhibits D-amino acid oxidase[18b]. Thus, catalase (EC 1.11.16) must be added to decompose the H_2O_2. Under the experimental conditions used, D-amino acid oxidase remained active over 10 h, whereas additions of catalase were required every 2 h. Hydroxypyruvate is difficult to extract from the aqueous phase and has limited stability (half-life, ~20 h). Therefore, it was trapped directly by the transketolase reaction.

Synthesis of D-*xylulose.* — Transketolase, extracted from spinach leaves, was shown[13] to catalyse the formation of D-xylulose from hydroxypyruvate and D-glyceraldehyde. The enzyme was extracted as described[13] and purified by elution from Sephadex G75. Transketolase was used with D-amino acid oxidase in a one-pot reaction. Under the conditions required (oxygen-saturated Tris buffer) for activity of the latter enzyme, transketolase displayed reduced stability ($t_{1/2}$ 10 h) and was used portionwise in order to maximise the yield.

Synthesis of D-*[1,2-$^{13}C_2$]xylulose from* DL-*[2,3-$^{13}C_2$]serine.* — The synthesis was carried out on a 2 mmolar scale with 7% labeled DL-serine. A large excess of D-glyceraldehyde was used in order to consume the labeled precursor completely. Proteins were then precipitated with methanol, Dowex (H^+) resin was used to retain the L-serine and the Tris buffer, and the labeled D-xylulose (70% recovery) was purified by chromatography on silica gel. L-Serine was eluted from the Dowex (H^+) resin and purified on Dowex (HO^-) resin.

TABLE I

^{13}C-N.m.r. chemical shift data for natural and 1,2-^{13}C-labeled D-xylulose in water and for its acyclic tetra-acetate

Compound	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C=O (acetate)*	*CH_3 (acetate)*
β-D-Xylulose	66.00	105.76	79.23	77.80	72.70		
Labeled β-D-xylulose	65.92 (*J* 41.5 Hz)	105.72 (*J* 41.5 Hz)					
α-D-Xylulose	65.31	108.50	78.70	78.14	74.74		
Labeled α-D-xylulose	65.0 (*J* 41.5 Hz)	108.40 (*J* 41.5 Hz)					
keto-D-Xylulose	68.83	215.70	83.45	74.74	64.75		
Labeled *keto*-D-xylulose	68.78 (*J* 41.5 Hz)	108.40 (*J* 41.5 Hz)					
keto-D-Xylulose tetra-acetate	66.58	197.67	74.45	68.99	61.04	170–169 (1) 169.7 (2) 169.6 (1)	20.42 (2) 20.18 (2)
Labeled *keto*-D-xylulose tetra-acetate	66.70 (*J* 45.3 Hz)	197.73 (*J* 45.3 Hz)					

Acetylation[20] of the labeled D-xylulose gave a mixture of cyclic and acyclic products that was fractionated by chromatography. The acyclic form preponderated, and the ^{13}C-n.m.r. data for the labeled and authentic unlabeled compounds are shown in Table I. The ^{13}C-n.m.r. spectrum of the D-[1,2-$^{13}C_2$]xylulose tetra-acetate is shown in Fig. 2. The structures of the C-1 and C-2 signals are different from those of the unlabeled compounds since each is a doublet (J 0.60 Hz) reflecting the ^{13}C–^{13}C coupling. The ^{13}C-n.m.r. data for solutions of D-xylulose and D-[1,2-$^{13}C_2$]xylulose in D_2O are reported in Table I. The assignment of signals is complicated by the presence of one acyclic and two anomeric cyclic forms, and the signals at 215.70, 108.50, and 105.76 p.p.m. were assigned to C-2 of these forms, respectively. The β configuration was assigned to the preponderant cyclic form by analogy with ^{13}C-n.m.r. data for fructofuranose[21]. The signals for C-1 and C-2 of the three forms of the labeled xylulose were doublets due to ^{13}C–^{13}C coupling.

The above method opens the way to the synthesis of other labeled ketoses and analogs, using DL-serine as the single commercial precursor, since transketolase enantiospecifically and enantioselectively transfers the hydroxyacetyl group of hydroxypyruvate to many different aldehydes[13]. Moreover, labeled aldoses may also be obtained by this method since some ketoses, such as D-xylulose[22] and D-fructose or some of their analogs[9a,23], can be converted easily with glucose isomerase into their corresponding

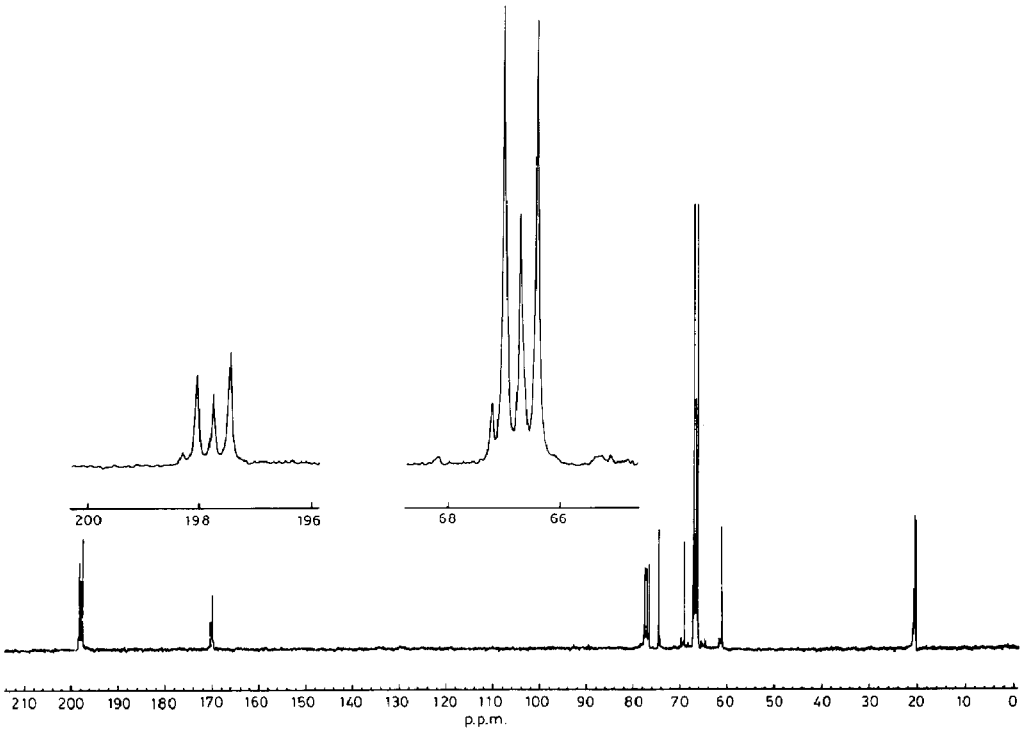

Fig. 2. ^{13}C-N.m.r. spectrum of acetylated D-[1,2-$^{13}C_2$]xylulose.

aldoses. D-Amino acid oxidase leads to DL-serine resolution: only D-serine is oxidised into labeled hydroxypyruvate; the unreacted L isomer is easily isolated and is a convenient source of labeled L-serine.

EXPERIMENTAL

General. — DL-[2,3-$^{13}C_2$]serine (86% isotopic purity) was purchased from Commissariat à l'Energie Atomique. U.v. spectra were obtained with a Beckman DU8 instrument. ^{13}C-N.m.r. spectra were recorded with a Bruker 300 ML spectrometer. Optical rotations were measured on a Perkin–Elmer automatic polarimeter. Flash chromatography was carried out on Silica Gel 60 (Merck). T.l.c. of sugars was performed on silica gel (Merck), using chloroform–1-butanol–ether–pentane (2:1:2:1) and detection by charring with H_2SO_4, and of serine on cellulose, using pyridine–1-butanol–water (6:4:3) and detection with ninhydrin.

Purification and assay of transketolase. — The procedure was based on that described by Villafranca and Axelrod[24]. Spinach leaves (300 g from a local market) were homogenised at 0° in 0.01M K_2HPO_4 buffer (500 mL, pH 9), then centrifuged for 10 min at 10 000*g*. The supernatant solution was filtered, $(NH_4)_2SO_4$ (89.5 g) was added, and the mixture was stirred, and then centrifuged in order to remove the precipitate. The operation was repeated with further portions (70 and 36 g) of $(NH_4)_2SO_4$. To the last supernatant solution, $(NH_4)_2SO_4$ (61 g) was added, and the resulting precipitate was separated by centrifugation, resuspended in distilled water (10 mL), and eluted from Sephadex G75 gel with 0.01M glycylglycine buffer (pH 7.5). Fractions showing transketolase activity were combined to give 200 U of the enzyme.

The transketolase (TK) assay was based on the following reactions:
D-xylulose 5-phosphate + D-ribose 5-phosphate $\overset{TK}{\rightleftarrows}$ D-sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate;
glyceraldehyde 3-phosphate $\overset{TPI}{\rightleftarrows}$ dihydroxyacetone phosphate;
dihydroxyacetone phosphate + NADH + H^+ $\overset{GDH}{\rightarrow}$glycerol 1-phosphate + NAD^+.

TK was assayed in a spectrophotometer cuvette containing 0.1M glycylglycine buffer (1 mL, pH 7.5), 9mM D-xylulose 5-phosphate, 15mM D-ribose 5-phosphate, 1.5 mM thiamine pyrophosphate, 8mM $MgCl_2$, 0.42 mM NADH, 1 U of triose phosphate isomerase (TPI), and 10 U of glycerophosphate dehydrogenase (GDH). The reaction was started by the addition of 10 μL of the enzymic fraction, and the decrease in absorbance at 340 nm was monitored: 1 U of enzyme catalysed the formation of 1 μmol of glyceraldehyde 3-phosphate per min.

Synthesis of D-*[1,2-$^{13}C_2$]xylulose.* — To 0.02M Tris buffer (30 mL, O_2-saturated, pH 8) were added transketolase (100 U), D-amino acid oxidase (150 U), 110mM DL-serine (3.3 mmol), 9mM DL-[2,3-$^{13}C_2$]serine (0.27 mmol), 120mM D-glyceraldehyde (3.6 mmol), 1.25mM thiamine pyrophosphate (0.037 mmol), and 3mM $MgCl_2$ (0.09 mmol). Catalase (20 U) was added every 2 h. The mixture was stirred under oxygen at room temperature and, after 6 h, transketolase (100 U) was added. After reaction for 12 h, methanol (3 vol.) was added, the precipitate was removed by centrifugation, and the

methanol was evaporated under reduced pressure. The treatment with methanol was repeated twice. The resulting solution was eluted from a column of Dowex (H^+) resin with water. The fractions that contained D-xylulose were combined, adjusted to pH 6, and concentrated. Flash chromatography (chloroform–methanol, 4:1) of the residue gave D-xylulose (100 mg, 37%), as a syrup that was identified by comparison (See Table I) with an authentic sample.

Acetylation of D-*[1,2-$^{13}C_2$]xylulose.* — To a solution of xylulose (80 mg) in cool pyridine (10 mL) was added acetic anhydride (2 mL). The mixture was maintained at room temperature for 24 h, aqueous 2% $NaHCO_3$ (100 mmol) was added, the product was extracted with dichloromethane (3 × 50 mL), and the combined extracts were washed with water, dried (Na_2SO_4), and concentrated. The major product (32 mg, 38%), isolated by flash chromatography (ether–pentane, 2:1), had $[\alpha]_D^{20}$ +14° (*c* 1, water), and was identified as peracetylated D-[1,2-$^{13}C_2$]xylulose by comparison with an authentic sample[13].

Purification of L-*[2,3-$^{13}C_2$]serine.* — The serine and Tris buffer retained on the above column of Dowex (H^+) resin were eluted with 0.5M NH_4OH. The serine-containing fractions were combined, adjusted to pH 3, and eluted from a column of Dowex (HO^-) resin with M HCl to yield L-[2,3-$^{13}C_2$]serine (220 mg), $[\alpha]_D^{20}$ +8° (*c* 1, 2M HCl), with 54% enantiomeric excess.

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