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Enzymatic Synthesis of (R) and (S) 1-Deuterohexanol

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ABSTRACT

This paper describes practical enzymatic procedures for the synthesis of (R) and (S) 1-deuterohexanol, a useful building block for chiral poly isocyanated liquid crystals. Alcohol dehydrogenases from horse liver and *Pseudomonas* catalyzed the reduction of hexanal with deuterated NAD (NADD) resulting in 50% and 89% yields of (R) and (S) 1-deuterohexanol, respectively. The deuterated cofactor was regenerated *in situ* by alcohol dehydrogenase catalyzed oxidation of ethanol-d_6 or 2-propanol-d_8. The (S) alcohol was also synthesized by the horse liver alcohol dehydrogenase reduction of 1-deuterohexanal, which was prepared chemically from hexanal. The yields of the reaction were greatly increased by the use of a biphasic system or with the immobilized enzyme in anhydrous organic solvents. Horse liver alcohol dehydrogenase was stabilized by immobilization on PAN or noncovalent entrapment on XAD resin.

INTRODUCTION

Chiral primary alcohols, by virtue of a deuterium at the terminal carbon, are useful as mechanistic probes of chemical and biological processes (*1*). In addition, 1-deuterohexanol is a precursor to the chiral polymer poly ((R)-1-deuterio-*n*-hexyl isocyanate) (Fig. 1; 2). The chirality introduced by the deuterium atom in the polymer resulted in a cooperative deuterium

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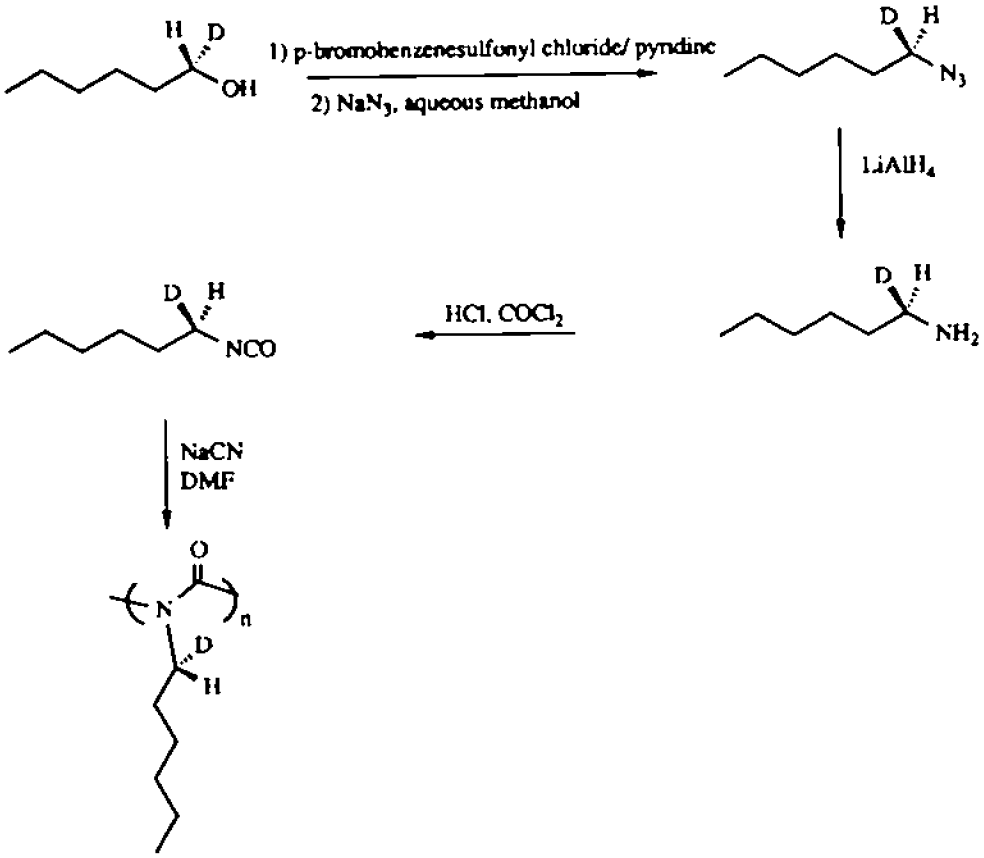

Fig. 1. Synthesis of poly (R)-1-deuterio-*n*-hexyl isocyanate from 1-deuterohexanol.

isotope effect that was manifested by a large optical rotation. We anticipated that the chiral deuteroalcohol could also have an interesting role in liquid crystals.

In contrast to the commercially available alcohol dehydrogenases from horse liver (3), yeast (3), and *Thermoanaerobium brockii* (4), which transfer the hydride from the nicotinamide cofactor to the *re* face of the carbonyl to give (S) alcohols, the enzymes from *Pseudomonas sp.* (PADH and PED; 5) and *Lactobacillus kefir* (6) transfer the hydride to the *si* face of the carbonyl to give (R) alcohols. With the use of these two classes of enzymes, both optical isomers of an alcohol can be synthesized. Hexanal, for example, is a substrate for both horse liver alcohol dehydrogenase and PADH. Highly enantiomerically pure 1-deuterohexanol can be prepared from 1-deuterohexanal or hexanal by enzymatic reduction coupled with regeneration of the nicotinamide cofactor.

MATERIALS AND METHODS

All chemicals were from Aldrich or Sigma unless otherwise noted. α-Methoxy-α-trifluoromethylphenylacetyl chloride is available from Fluka. The optical purity of 1-deuterohexanol was determined by NMR analysis of the α-methoxy-α-trifluoromethylphenylacetic acid ester (MTPA; *7*). ^{1}H NMR of the MTPA ester of racemic 1-deuterohexanol ($CDCl_3$) δ 0.87 (t, 3H), 1.28 (m, 6H), 1.66 (m, 2H), 3.54 (s, 3H), 4.29 (quartet, 1H), 7.46 (m, 5H). Decoupling of the methylene protons at 1.66 ppm changes the quartet of the deuterated methylene group of the racemic MTPA ester to a doublet, which becomes a singlet for enantiomerially pure alcohol. ^{1}H NMR of the decoupled MTPA ester of racemic 1-deuterohexanol ($CDCl_3$) δ 0.84 (m, 3H), 1.26 (m, 6H), 3.55 (s, 3H), 4.29 (d, 1H), 7.46 (m, 5H). NMR spectra were recorded on a 300 MHz spectrometer. The absolute stereochemistry of (R) and (S) 1-deuterohexanol was determined by comparison of the sign of the optical rotation with literature values for the (S) enantiomer (*8*). Glucose dehydrogenase is available from Amano. *Pseudomonas sp.* strain SBD6 is available from American Type Culture Collection (ATCC 49688).

Enzyme Assays

The change in absorbance of NADH was monitored at 340 nm (ε_{NADH} 622M $^{-1}$mm^{-1}) after addition of the enzyme to a cuvet containing appropriate aliquots of the following stock solutions; 5 m*M* NADH, 20 m*M* freshly purified hexanal, and 20 m*M* freshly purified hexanol in 50 m*M* Tris buffer, pH 8.5. Hexanol was purified by refluxing with sodium metal and storing over magnesium sulfate. Hexanal was purified by mixing with an equal volume of ethyl ether followed by washing with 10% sodium bicarbonate, dilute HCl, and water. After drying over sodium sulfate and evaporating the ethyl ether, the hexanal was distilled and stored over sodium sulfate.

Synthesis of Hexanal-1,3-Dithiane

Propane-1,3-dithiol (75 mL) was added to a solution of hexanal (75 g, 749 mmol) in 500 mL dry chloroform. The solution was cooled to 0°C, saturated with a slow gaseous stream of HCl (20 min), allowed to warm to room temperature, and stirred for a further 30 min. The organic layer was washed with water (100 mL), 1*N* NaOH (2 × 100 mL), saturated brine (100 mL), and dried over sodium sulfate. The solvent was removed under reduced pressure and the oil obtained was purified by vacuum distillation (b.p. 100–105°C at 1.5 mm Hg) to give 111.4 g of a clear, colorless liquid, 78% yield. ^{1}H NMR ($CDCl_3$) δ 0.85 (t, 3H), 1.2 (m, 4H), 1.4 (m, 2H), 1.6–

1.8 (m, 3H), 2.05 (m, 1H), 2.8 (m, 1H), 4.0 (t, 1H). ^{13}C NMR ($CDCl_3$) δ 13.9, 20.05, 22.4, 26.3, 30.5, 31.4, 35.4, 47.6.

Preparation of 1-Deuterohexanal-1,3-Dithiane

A flame-dried round bottom flask was flushed with argon and fitted with a 250 mL addition funnel. Hexanal-1,3-dithiane in 1500 mL tetrahydrofuran (freshly distilled over sodium and benzophenone) was added to the flask, followed by 100 mg triphenylmethane. The reaction mixture was stirred at −20°C in a dry ice/CCl_4 bath for 6 h during which time the solution became a deep red color, typical of the triphenylmethane anion. Deuterium oxide (50 mL, >99% D) was added to quench the reaction and the solution was allowed to warm until the color of the indicator disappeared. After cooling to 0°C, the pH was adjusted to 7 with 1*N* HCl. The tetrahydrofuran was evaporated under reduced pressure and the organic layer was collected. The successive washings of the aqueous layer with chloroform (2×100 mL) and the organic layer were combined and washed with saturated brine, dried over sodium sulfate, and the solvent was removed under reduced pressure. The product as isolated in this manner was pure as determined by gas chromatography and ^1H NMR. ^1H NMR ($CDCl_3$) δ 0.85 (t, 3H), 1.2 (m, 4H), 1.4 (m, 2H), 1.6–1.75 (m, 3H), 2.05 (m, 1H), 2.8 (m, 4H). ^{13}C NMR ($CDCl_3$) δ 13.9, 22.3, 25.9, 26.1, 30.4, 31.3, 35.2, 46.6, 47.1, 47.5.

Preparation of 1-Deuterohexanal

The optimal oxidative cleavage is the claycop procedure (9): 40 g of copper II nitrate was dissolved in 750 mL acetone and 60 g of montmorrillonite K-10 clay was added. The acetone was removed under reduced pressure and the solid crust was broken up and heated at 50°C under reduced pressure for 1 h. The resulting powder was ground in a mortar and pestle. This solid was added to a solution of 1-deuterohexanol-1,3-dithiane (19 g, 0.1 mol) in 750 mL dichloromethane. The solution was stirred until evolution of nitrogen oxides ceased and the starting material was consumed as evidenced by capillary gas chromatography. The solution was then filtered and the spent solid oxidant was washed with dichloromethane (2×75 mL). The filtrate was decolorized by filtration through a maximum of 5 g neutral alumina (larger amounts cause formation of the trimer). The solvent was evaporated under reduced pressure. The 5:1 mixture of aldehyde:trimer was purified by bulb-to-bulb distillation (100°C, 30 mm Hg) to afford 6.5 g of 1-deuterohexanol, 65% yield. ^1H NMR ($CDCl_3$) δ 0.85 (t, 3H), 1.25 (m, 4H), 1.5 (m, 2H), 2.35 (t, 2H). ^{13}C NMR ($CDCl_3$) δ 13.9, 21.8, 22.5, 31.5, 43.85, 68.14, 202.6, 203.1, 203.6. MS mass(% abundance); M^+ 101.0 (2%), 83.0 (10%), 73 (20%), 72 (7%), 58 (53%), 56 (100%). 1-deuterohexanol trimer ^1H NMR ($CDCl_3$) δ 0.85 (t, 9H), 1.2–1.4 (m, 18H), 1.65 (m, 6H). ^{13}C NMR ($CDCl_3$) δ 13.91, 22.55, 23.23, 31.50, 34.22, 100.7, 101.2, 101.6.

Enzymatic Reduction of 1-Deuterohexanal

Horse liver alcohol dehydrogenase (50 mg, 50 U with respect to hexanal) was immobilized on 4 g PAN-700 and glucose dehydrogenase (4 mg, 100 U) immobilized on 1 g PAN-700 (*10*). The immobilization yields were 45% and 80%, respectively. To a suspension of 100 U of PAN-immobilized horse liver alcohol dehydrogenase and 150 U PAN-immobilized glucose dehydrogenase in 1 L 50 m*M* HEPES/10 m*M* magnesium chloride buffer, pH 7.1, was added 20 g glucose and 5 g NAD. This solution was carefully overlaid with hexane (500 mL) and the mixture stirred gently under an argon atmosphere. 1-Deuterohexanal was added periodically until no further conversion was observed (approx 2 g of aldehyde over a period of 12 h), after which time the hexane layer was removed by cannula and the aqueous layer equilibrated with another 500 mL hexanes to remove any hexanol dissolved in the aqueous layer. A further 500 mL of hexane and 2 g of 1-deuterohexanal were added and the protocol was repeated. In this manner, conversions of up to 80% could be realized. The hexane layers were combined and evaporated under reduced pressure to give a mixture of the alcohol and aldehyde. Unreacted aldehyde was readily removed by treatment of an ether solution of the residue with benzylamine in the presence of magnesium sulfate. After the reaction was complete as determined by gas chromatography (1 h), the solvent was extracted successively with water, dilute hydrochloric acid, and water. The organic layer was dried over sodium sulfate and evaporated under reduced pressure. The residue was further purified by bulb-to-bulb distillation to give (S)-1-deuterohexanol in high enantiomeric excess (>97%, *see* Materials and Methods) which was >98% pure as determined by capillary gas chromatography.

Horse liver alcohol dehydrogenase (30 mg, 60 U), and 1 g NAD was dissolved in 5 mL 300 m*M* HEPES/15 m*M* magnesium chloride buffer, pH 7.5. To that, 5 g XAD 8 resin was added and the mixture stirred at room temperature for 3 h, after which time it was spread on a 15-cm diameter evaporating dish and allowed to dry in air. After the resin appeared to be dry and free-flowing (6 h), it was suspended in 30 mL ethyl acetate containing 1 g 1-deuterohexanal and 2 g absolute ethanol. After 8 h, the enzyme retained 5% of the orginal activity as determined by enzymatic assay in phosphate buffer and the reduction was 70% complete as determined by gas chromatography. A similar trial employing 100 U of enzyme and 5 g of 1-deuterohexanal was only 10% complete after 8 h.

Enzymatic Reduction of Hexanol with NAD(D)H

Horse liver alcohol dehydrogenase (150 U immobilized on 8 g of PAN-750; *10*) was added to a three-necked 1 L flask containing 200 mL 100 m*M* HEPES/10 m*M* magnesium chloride buffer, pH 7.5, 10 mmol hexanal, 38 mmol ethanol-d_6, and 150 mg NAD. The reaction was stirred under nitrogen at room temperature with an overhead stirrer and layered

with 100 mL hexane. After 8 days, the hexane layer was removed by cannula and the aqueous solution layered twice more with 100 mL hexane. The combined organic layers were evaporated to dryness, the excess aldehyde removed as described above, and purified by bulb-to-bulb distillation. Fifty percent yield of (R)-1-deuterohexanol, >97% ee as determined by NMR analysis of the MTPA ester (*see* Materials and Methods).

PADH from *Pseudomonas sp.* strain SBD6 was prepared for synthesis as described previously (5). Hexanal (7.5 mmol) in 50 mL hexane was carefully layered on a solution containing 100 mL 150 m*M* Tris buffer, pH 7.1, 9.1 mmol 2-propanol-d_8, 40 mg NAD, and 25 mL *Pseudomonas sp.* crude cell extract as the source of PADH. After stirring at room temperature for 3 days, the reaction was 89% complete as determined by gas chromatography. The hexane layer was removed and the aqueous layer washed with hexane (2× 50 mL). The combined organic layers were dried over sodium sulfate and evaporated under reduced pressure. The (S)-1-deuterohexanol was purified by silica gel chromatography (5:2, hexane:ethyl ether), 60% yield with >97% ee as determined by NMR analysis of the MTPA ester (*see* Materials and Methods). The unoptimized total turnover number was 120.

RESULTS AND DISCUSSION

1-Deuterohexanal was synthesized according to Fig. 2. Hexanal is quantitatively converted to the dithiane by treatment with propane-1,3-dithiol and hydrochloric acid. Deprotonation of the dithiane in terahydrofuran, although complete at −20°C, is not observed at −78°C as was found for benzaldehyde dithiane. Deuteration using 99% D_2O led to formation of 1-deuterohexanal-1,3-dithiane with >98% deuterium incorporation. Cleavage of the dithiane proved problematic. Standard mercury (II) cleavage in aqueous methanol (*11*) gave the dimethyl acetal as the major product. Acidic deprotection of the acetal resulted in further loss of product owing to the pronounced tendency of the aldehyde to form the trimer. To simplify product isolation, nonaqueous systems were investigated. Alkylative cleavage employing iodomethane and a solid base (*12*) was ineffective and the NBS cleavage (*13*) of the dithiane resulted in rapid oxidation of the product. The use of cupric nitrate absorbed on clay (*9*) was found to be the simplest and cleanest way to cleave the dithiane. Treatment of the dithiane with heterogenous oxidant "claycop," which was easily prepared from copper (II) nitrate and montmorillonite K-10 clay, gives rapid cleavage with no byproduct formation.

The large-scale reduction of 1-deuterohexanal by PAN-immobilized horse liver alcohol dehydrogenase is illustrated in Fig. 3. When the reaction was performed under totally aqueous conditions, conversions of

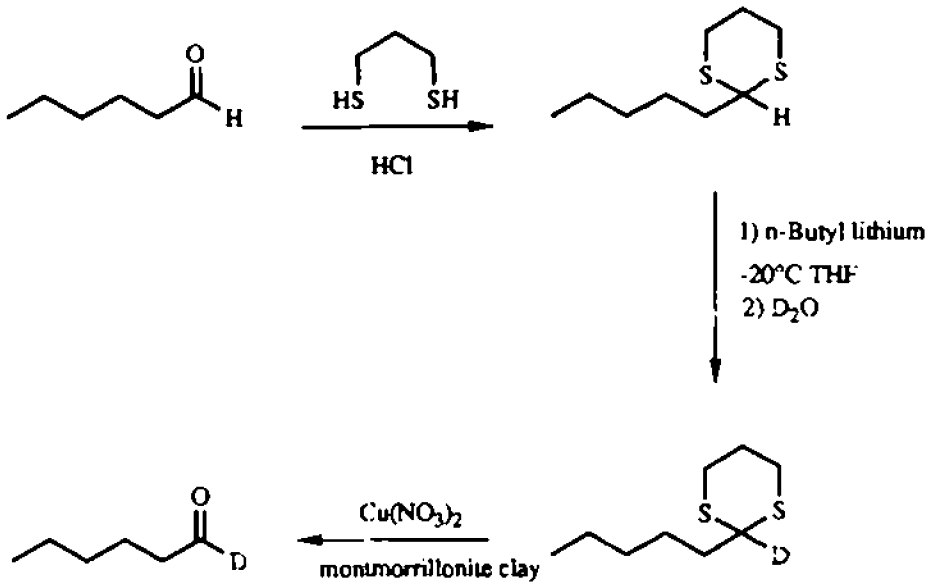

Fig. 2. Synthesis of 1-deuterohexanal.

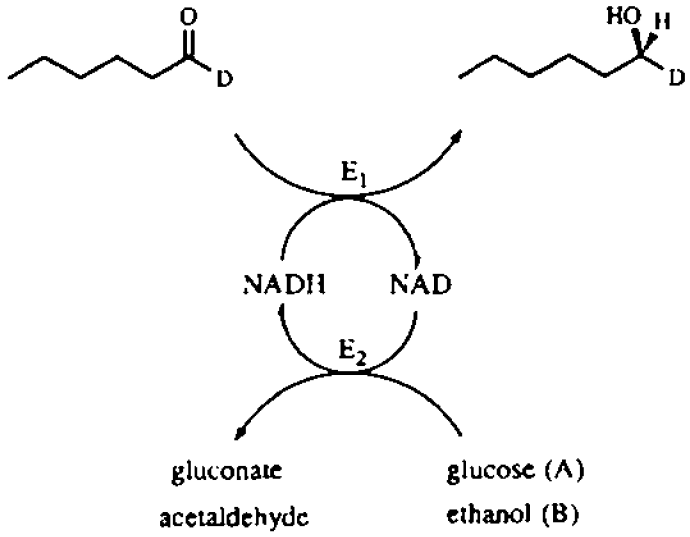

Fig. 3. Horse liver alcohol dehydrogenase catalyzed reduction of 1-deuterohexanal. (A) E_1 = horse liver alcohol dehydrogenase, E_2 = glucose dehydrogenase coimmobilized on PAN gel; (B) $E_1 = E_2$ = horse liver alcohol dehydrogenase immobilized on XAD resin.

only 20% could be realized and were further complicated by the decomposition of the aldehyde to a trimer and to aldol condensation products. Previous studies with horse liver alcohol dehydrogenase have shown significant substrate inhibition owing to formation of an abortive ternary complex of NAD, aldehyde, and enzyme in the presence of limiting amounts of coenzyme (*14*). Using a biphasic system with hydrophobic cosolvents such as hexane and pentane allows conversions of up to 80% and alleviates problems of product and substrate inhibition. Product isolation is simplified by transfer of the hexane layer with a cannula. The nature of the organic layer is important, as no reduction was observed with ethyl ether. The regeneration of NADH was accomplished with PAN-immobilized glucose dehydrogenase catalyzed oxidation of glucose (*15*).

Noncovalent entrapment of horse liver alcohol dehydrogenase on XAD beads proved to be a much simpler, but less rugged, immobilized enzyme preparation. By simply dissolving the enzyme and cofactor in buffer, followed by addition of the resin and evaporaton of the bulk water, one can entrap the enzyme in a microenvironment of water. The free-flowing resin was suspended in organic solvent giving a biphasic system in which the aqueous portion is very small. The use of a minimum of water is advantageous in cases where the enzyme is subject to product or substrate inhibition, when the substrate and/or product are sparingly soluble in water, or with moisture sensitive substrates. The use of noncovalent entrapment also circumvents enzyme deactivation that can occur in covalent entrapment. Thus, entrapment of 50 U of horse liver alcohol dehydrogenase and suspension of the beads in ethyl acetate lead to a system in which 1 g of 1-deuterohexanal could be reduced to 70% conversion after 8 h. The enzyme was completely deactivated after this time. This entrapment system is amenable to rapid, small-scale reductions. Cofactor regeneration is achieved by inclusion of ethanol, thus constituting a one enzyme system where an enzyme simultaneously catalyzes oxidation and reduction of the cofactor.

In order to circumvent the synthesis of 1-deuterohexanal, another strategy could be used to synthesize both (R) and (S) 1-deuterohexanol, relying on the opposite stereospecificity of horse liver alcohol dehydrogenase and PADH. As shown in Fig. 4, the deuterated cofactor is synthesized in a one enzyme system where the deuterium is transferred to the cofactor from an appropriate deuterated substrate. With this method, conversions of 50% and 89% were seen for PAN-immobilized horse liver alcohol dehydrogenase and soluble PADH, respectively. PADH is very stable under the reaction conditions, obviating the need for immobilization (*4*). Thus, PADH reduction results in the (S) alcohol and horse liver alcohol dehydrogenase yields (R) 1-deuterohexanol. The K_m values for PADH in phosphate buffer were determined to be 170 μM and 91 μM for hexanal and NADH, respectively. The higher yields for PADH may be attributable to a lack of substrate or product inhibition as observed under assay conditions. Concentrations of up to 2*M* hexanol did not inhibit the

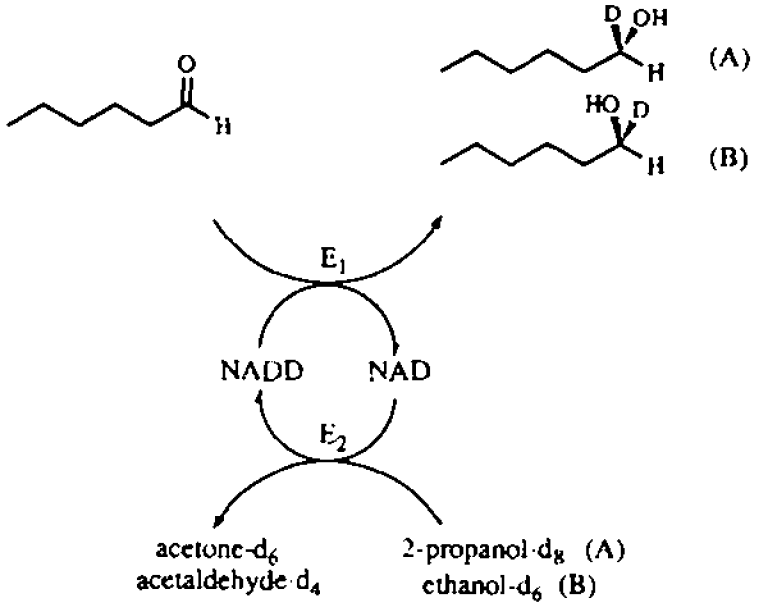

Fig. 4. (A) Synthesis of (S)-1-deuterohexanol, $E_1 = E_2 =$ PADH. (B) Synthesis of (R)-1-deuterohexanol, $E_1 = E_2 =$ horse liver alcohol dehydrogenase

reduction of hexanal. Substrate inhibition was observed for NADH above 100 μM. By regenerating NADH, the concentration is kept low, thereby avoiding substrate inhibition.

The general methods described here are useful for the synthesis of a range of isotopically labeled chiral primary alcohols, limited only by the substrate specificity of the particular enzyme. As demonstrated with horse liver alcohol dehydrogenase, a single enzyme can be utilized for the synthesis of both enantiomers of a chiral primary alcohol. Alternatively, two enzymes with differing stereoselectivity separately acting on the same substrate can also yield two desired enantiomers. These methods are applicable to the other synthetically useful alcohol dehydrogenases (3–6).

ACKNOWLEDGMENT

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