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Enantioselectivity of Alcohol Dehydrogenase-Catalyzed Oxidation of 1,2-Diols and Aminoalcohols[1]

JOSE R. MATOS,[2] MARY B. SMITH, AND CHI-HUEY WONG[3]

Department of Chemistry, Texas A&M University, College Station, Texas 77843

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The alcohol dehydrogenase from horse liver is able to catalyze the oxidation of a number of 1,2-diols and α-aminoalcohols enantioselectively to L-α-hydroxyaldehydes and L-α-amino aldehydes. A decrease of enantioselectivity was found in reactions with 1,3-diols and substrates with hydrophobic substituent at position 3. α-Aminoalcohols are not substrates for yeast alcohol dehydrogenase, but the enzyme can catalyze the oxidation of most of the diols to L-hydroxyaldehydes. New methods for determination of the optical purity of α-hydroxy- and α-aminoaldehydes via converting them *in situ* to the corresponding acids, catalyzed by the aldehyde dehydrogenase from yeast, have been developed. The coupled alcohol dehydrogenase/aldehyde dehydrogenase has been extended to preparatory scale synthesis of optically pure L-α-hydroxyacids in the presence of a cofactor regeneration system. The active-site cubic-space section model has been shown not to be applicable to all substrates.

INTRODUCTION

One of the most remarkable properties of alcohol dehydrogenases, particularly that of horse liver, is that they are able to catalyze the stereospecific oxidoreductions of a broad spectrum of substrates (*1*). This unique property has been employed in a variety of synthetic applications (*2–3*) and in studies of the orientation of the enzyme–substrate–coenzyme complex (*1–4*). Of the compounds tested as substrates for the oxidative reactions catalyzed by horse liver alcohol dehydrogenase, most of them have been cyclic diols. Information regarding the stereospecificity of the enzyme in the oxidation of acyclic prochiral 1,3-, 1,4-, and 1,5-diols is available (*5*); however, little information has been found using nonprochiral acyclic diols or α-aminoalcohols as substrates. In connection with our interest in the aldolase-catalyzed synthesis of unusual L sugars and amino sugars, optically pure L-α-hydroxyaldehydes and aminoaldehydes for use as substrates are needed. Methods for the preparation of these two classes of compounds from readily available substances such as 1,2-diols and amino acids are available but have problems of overoxidation (*6*) and racemization (*7*).

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[2] NSF Predoctoral Fellow.

[3] To whom correspondence should be addressed.

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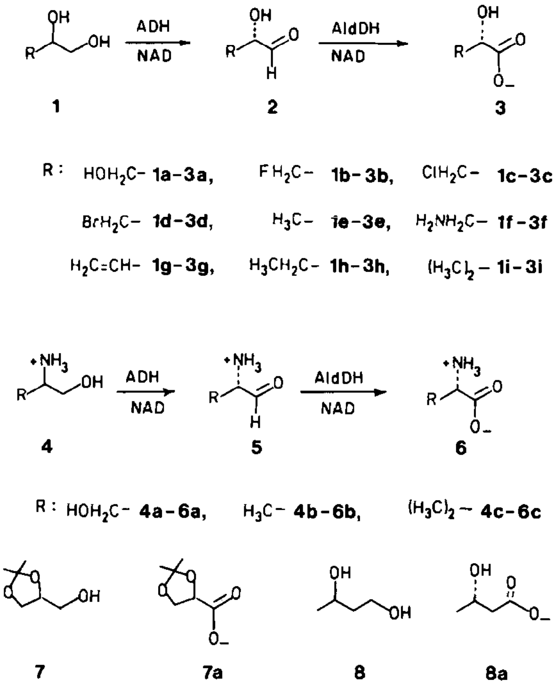


SCHEME 1. Oxidation of alcohols with combined alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (AldDH) as catalysts.

The results of the alcohol dehydrogenase-catalyzed stereospecific oxidation of glycerol to L-glyceraldehyde (*8*) prompted us to test other related compounds such as 1,2-diols and α-aminoalcohols (Scheme 1). Due to the rather low activity of the substrates with yeast alcohol dehydrogenase, we directed ourselves toward horse liver alcohol dehydrogenase for the majority of our synthetic applications. Since most of the products (α-hydroxy- and α-aminoaldehydes) of the enzymatic reactions studied are not stable, they are further oxidized *in situ* by aldehyde dehydrogenase from yeast to α-hydroxy- and α-amino acids accomplishing a threefold purpose; simplifying the isolation of products, providing an easy method for the determination of optical purity, and presenting a new route toward the synthesis of optically active α-hydroxy acids and amino acids from the respective alcohols.

MATERIALS AND METHODS

Enzymes and cofactors were purchased from Sigma without further purification. (*R*)- and (*S*)-butane-1,3-diol, (*S*)-propane-1,2-diol, and (*R*)- and (*S*)-1,2-isopropylidene glycerol were from Aldrich. D- and L-propane-1,2-diol were prepared from L- and D-threonine, respectively, via formation of D- and L-lactaldehyde and subsequent reduction with $NaBH_4$ (*9*). D- and L-aminoalcohols were prepared from the corresponding amino acids by literature methods (*10*). 3-Fluro-1,2-propane diol and 1-butene-3,4-diol were prepared by acid-catalyzed ring opening of the respective epoxides from Aldrich. D- and L-3-methyl-1,2-butanediol were prepared from L- and D-valinol according to literature methods (*11*). Other biochemicals are of analytical grade. Pyruvate and α-ketoglutarate were determined enzymatically with lactic dehydrogenase and glutamate dehydrogenase, respectively (*12*). Aldehydes were determined with horse liver alcohol dehydrogenase and NAD (*12*). Yeast alcohol dehydrogenase and horse liver alcohol dehydrogenase experiments were carried out separately. ^{1}H NMR was determined with an EM390 instrument (90 MHz).

Determination of optical purity. For determination of the optical purity of α-hydroxy acids, a solution containing 100 μl NAD (0.1 M), 2.7 ml glycine/hydrazine buffer (0.5 M/0.4M; pH 9.0) and 100 μl L-lactic dehydrogenase (0.5 mg/ml) was incubated at 25°C until the background absorbance at 340 nm had equilibrated. To this solution was added 100 μl of sample (20 mM), and the change in absorbance after quantitative consumption of the L enantiomer of substrate versus background monitored at 340 nm was recorded. A separate assay was carried out under the same conditions and with the same components as above except that D-lactic dehydrogenase, instead of L-lactic dehydrogenase, was used. The change of absorbance should reflect to the concentration of D-α-hydroxy acid in the sample. On comparison of the results from the separate two runs, optical purity was determined. This procedure was simply used to determine the optical purity of compounds **3a**–**3h**, because it was reported that L-lactic dehydrogenase is specific for each of the L enantiomers and D-lactic dehydrogenase for each of the D enantiomers (*12*). To verify that the enzymatic procedure for determination of the optical purity was reliable, we prepared the methyl esters of compounds **3c**, **3e**, and **3h** and converted them to the corresponding (*R*)-(+)-α-methoxy-α-trifluoromethylphenylacetyl (MTPA) derivatives (*13*) for ^{1}H NMR measurement. The intensities of the resonances due to the methoxy protons of the diastereomers prepared from racemic **3c**, **3e**, and **3h** and the diastereomers produced from the enzymatic reactions were compared [δ($CDCl_3$) 3.68 for D-**3c**, 3.63 for L-**3c**, 3.65 for D-**3e**, 3.60 for L-**3e**, 3.62 for L-**3h**, and 3.56 for D-**3h**]. Neither one of the enzymatic products showed enantiomeric contamination. As reported previously (*13*), the method based on NMR technique would be accurate for determination of enantiomeric excess up to 97%.

The optical purity of L- and D-amino acids was determined with L- and D-amino acid oxidase (EC 1.4.3.2 and EC 1.4.3.3), respectively, as described previously (*12*), except for L-serine, **6a**, which is not a substrate for L-amino acid oxidase (*12*). L-Serine was determined with alanine dehydrogenase, which is specific for L-alanine and L-serine (*12*).

Alcohol dehydrogenase-catalyzed oxidation. To test compounds as substrates for the enzyme, 1 ml of glycine–NaOH buffer (0.1 M, pH 9.0) containing 2 mg of enzyme and 5M NAD was incubated at 25°C until the absorbance at 340 nm was stable. Substrate (10 μl from a 0.5 M solution in water) was then added to the mixture and the increase of absorbance versus time was recorded.

Alcohol dehydrogenase/aldehyde dehydrogenase-catalyzed oxidation. To oxidize alcohols to acids, the reaction was carried out in 3 ml Tris buffer (0.1 M, pH 8.2) containing potassium bicarbonate (50 mM), alcohol substrate (20 mM), NAD (14 mM), and 2 mg each of alcohol dehydrogenase and the yeast aldehyde dehydrogenase (EC 1.2.1.5). The reaction progress was monitored by measuring the concentration of NAD with glucose 6-phosphate and glucose-6-phosphate dehydrogenase (*12*) (the reaction was usually complete in 1 day). The solution was then heated in a boiling-water bath for 10 min and centrifuged to remove denatured enzyme. The acid produced was then determined with lactic dehydrogenase. Since one enantiomer of the racemic diol substrate is oxidized faster than the other, the reaction was controlled so that only 70% of the favorable substrate could be oxidized to the acid, i.e., the maximum yield of α-hydroxy acid would be 35% based on the racemic diol substrate. For reactions containing cofactor regeneration systems, the following conditions were employed. A 10-ml solution of Tris buffer (0.1 M, pH 8.2) containing substrate (100 mM), α-ketoglutarate monoammonium salt (70 mM), NAD (0.5 mM), alcohol dehydrogenase (5 mg), aldehyde dehydrogenase (2 mg), and glutamic dehydrogenase (2 mg) was incubated under argon at room temperature. The reaction was continued over night until the α-ketoglutarate was completely consumed. For the oxidation of aminoalcohols, similar procedures were employed except that pyruvate and L-lactic dehydrogenase were used for cofactor regeneration, and the amino acids produced were determined enzymatically with amino acid oxidase or alanine dehydrogenase as described above. Conditions used for the yeast alcohol dehydrogenase catalysis were the same except that 15 mg of the enzyme was used.

Preparative synthesis. A representative synthesis of L-β-bromolactic acid, **3d**, was carried out. To a solution (100 ml) containing DL-3-bromopropane-1,2-diol (**1d**, 0.2 M), α-ketoglutarate monoammonium salt (0.14 M), NAD (0.5 mM), and dithiothreitol (2 mM) was added horse liver alcohol dehydrogenase (10 U), aldehyde dehydrogenase (30 U), and glutamic dehydrogenase (54 U) coimmobilized in polyacrylamide gels (*14*). The reaction mixture was controlled at pH 8.2 by adding 0.2 M KOH solution and continued for 2 days until no α-ketoglutarate was detected. After removal and washing (with three 10-ml portions of buffer) of the enzyme-containing gels, the washings and reaction mixture were continuously extracted with ether to remove the unreacted substrates. The aqueous solution was then acidified with concentrated H_2SO_4, reduced to 70 ml volume by evaporation, and extracted with ether to give **3d** (6 mmol, 30% yield based on the racemic alcohol substrate). Enzymatic analysis indicates that no D-bromolactic acid was produced; mp 89–90°C (toluene : benzene, 1 : 10; v/v). ^{1}H NMR ($CDCl_3$) 3.60 (d, 2H, methylene), 4.55 (m, 1H, methine), 9.0–11.0 (broad, OH, exchangeable with D_2O). Similar procedures were employed for the preparation of other α-hydroxy acids. The isolated yield for each preparation was in the range 26–32%. The physical constants for each of the products are as follows. Compound **3a**: δ ($CDCl_3$) 4.0 (d, 2H, methylene); 4.5 (m, 1H, methine); 9.0–11.0 (OH, exchangeable with D_2O). Compound **3b**: mp 87–88°C; δ ($CDCl_3$) 4.73 (dd, 2H, J_{CHF} = 48 Hz, J_{CHCH} = 3.6 Hz); 5.3 (m, 1H, methine). Compound **3c**: mp 88–89°C; δ ($CDCl_3$) 3.86 (d, 2H, methylene); 4.55 (m, 1H, methine); 9.0–11.0 (broad, OH, exchangeable with D_2O). Compound **3e**: δ ($CDCl_3$) 1.56 (d, 3H, CH_3); 4.50 (m, 1H, methine): 9.0–11.0 (broad, OH, exchangeable with D_2O). Compound **3f**: δ (D_2O) 4.24 (dd, 1H, J = 7.6, 4.8 Hz, C_2-H); 3.40 (dd, 1H, J = 13.0, 4.8 Hz, C_3-H); 3.08 (dd, 1H, J = 13.0, 7.6 Hz, C_3-H). Other ^{1}H exchangeable with D_2O: mp 199–201°C. Compound **3g**: δ 4.8 (ddd, 1H, J = 7.0, 2.0, 1.5 Hz, C_2-H); 5.21 (m, 1H, C_4-H); 5.26 (m, 1H, C_4-H); 5.96 (ddd, 1H, J = 17.0, 10.0, 7.0 Hz, C_3-H); bp 128–130°C (23 mm Hg). Compound **3h**: δ ($CDCl_3$) 1.01 (t, 3H, CH_3); 1.92 (m, 2H, CH_2); 4.51 (t, 1H, methine).

RESULTS AND DISCUSSION

Enantiomeric Excess and Enantioselectivity of Enzyme Catalysis

Because α-hydroxy- and α-aminoaldehydes are unstable, they were converted *in situ* to the more stable acid, from which enantiomeric purity was determined. Since D- and L-lactic dehydrogenase are specific for a number of α-hydroxy acids, including those prepared here (*12, 14, 16*), these two enzymes were routinely

TABLE 1

THE ENANTIOSELECTIVITY OF THE ALCOHOL DEHYDROGENASE FROM HORSE LIVER (HLADH) AND YEAST (YADH) IN THE OXIDATION OF 1,2-DIOLS AND AMINOALCOHOLS

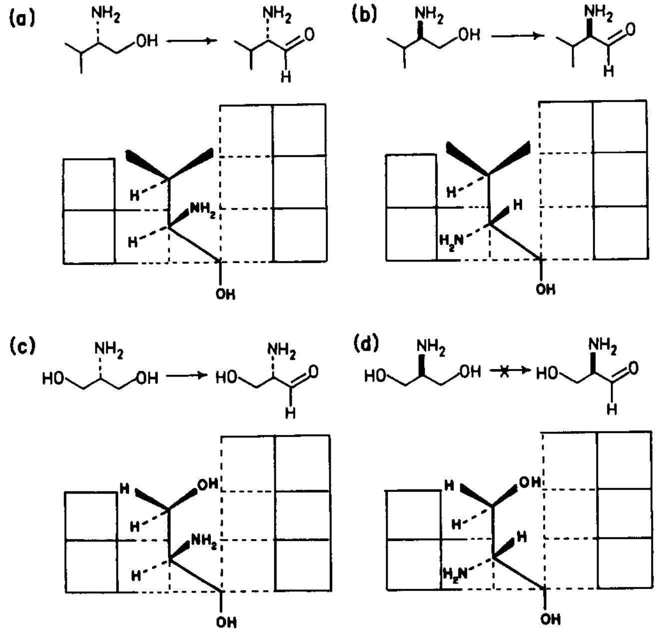
		Relative activity		%ee of Acid	
Substrate	Product	HLADH	YADH	HLADH	YADH
1a	**3a**	1.0[a]	0.0006	>97	>97
1b	**3b**	6.0	0.0001	>97	>97
1c	**3c**	5.5	0.01	>97	>97
1d	**3d**	5.5	0.03	>97	>97
1e	**3e**	29.5	0.01	>97	>97
1f	**3f**	3.0	0.01	>97	>97
1g	**3g**	30.0	0.055	>97	>97
1h	**3h**	33.0	0.073	>97	>97
1i	**3i**	26.0	—[c]	0	—[c]
4a	**6a**	1.0	—[c]	96	—[c]
4b	**6b**	16.0	—[c]	84	—[c]
4c	**6c**	11.0	—[c]	0[b]	—[c]
7	**7a**	7.0	0.013	0[b]	0[b]
8	**8a**	50.0	0.013	0[b]	70[b]

[a] The specific activity was 0.01 U/mg enzyme for glycerol and 1.2 U/mg enzyme for ethanol. Relative activities were determined by comparison of initial rates at 50 mM substrate, 0.2 mg/ml enzyme, and 5 mM NAD concentrations in 0.1 M glycine–NaOH buffer (pH 9.0) (1 U = 1 μmol substrate oxidized/min).

[b] Determined by measuring the initial oxidation rate of each enantiomer at the same condition described above.

[c] Neither enantiomer was detected as a substrate for the enzyme at the condition described above.

used to determine the enantiomeric purity of each of the α-hydroxy acids produced, which should reflect the optical purity of the α-hydroxy aldehydes. The results based on enzymatic analysis are in agreement with those based on the ^{1}H NMR method as described in the Experimental. Of the diols tested (**1a–1i**), each was converted exclusively to the L-α-hydroxy aldehyde (**2a–2i**) in the alcohol dehydrogenase catalysis and to the L-α-hydroxy acid (**3a–3i**) in the aldehyde dehydrogenase catalysis except DL-3-methylbutane-1,2-diol, **1i**, which was oxidized nonselectively to the DL-α-hydroxy aldehyde, **2i**, catalyzed by horse liver alcohol dehydrogenase. No accumulation of any aldehydes (determined by alcohols dehydrogenase) was detected, indicating that all the α-hydroxy aldehydes produced were further converted to α-hydroxy acids. To determine if the aldehyde dehydrogenase accepts D- and L-aldehydes equally well, separate reactions of D- and L-lactaldehyde (**2e**) have been carried out where it has been found that both are oxidized at the same rate; the same results have been found at different concentrations of the substrates. From the results shown in Table 1, it is clear that the hydroxyl group at position 2 is essential for the selectivity in the oxidation of diols. Modification of the OH group via alkylation, as shown in the case of glycerol acetonide, diminishes the selectivity completely, although the derivative is still a substrate. A change of the OH group from position 2 to position 3 reduces the selectivity to some extent. Replacement of the OH group with the NH_2 group cannot be accepted by yeast alcohol dehydrogenase but can still be accepted by the horse liver enzyme as a substrate, and similar enantioselectivity is observed.



SCHEME 2. Cubic-space active-site section model of horse liver alcohol dehydrogenase depicting the favorable (a, b, c) and unfavorable (d) interactions of the substrate with the enzyme. In the oxidation of D-valinol as shown in b, the C_2–NH_2 group enters the forbidden region of the model.

In each oxidation of the α-amino alcohols, the amount of pyruvate consumed was equivalent to twice the amount of the sum of D- and L-amino acids produced, indicating that the aldehyde dehydrogenase catalyzed the oxidation of both D- and L-amino aldehydes to amino acids. As can be seen upon going from hydrophilic to hydrophobic 1,2-diols and amino alcohols (serinol to valinol), the enantioselectivity of the horse liver alcohol dehydrogenase decreases.

The Active Site Model

The cubic active site section model of horse liver alcohol dehydrogenase developed by Jones *et al.* (*2, 5*), has been a valuable contribution to the rationalization of a number of oxidoreductions catalyzed by the enzyme and has been used to predict potential substrates for use in enzymatic synthesis. The top-view of the model associated with some particular substrates is indicated in Scheme 2. Based on the results we obtained regarding the enantioslectivity of horse liver alcohol dehydrogenase in the oxidation reactions, the presence of hydrophobic and other hydrophilic substituents on position 3 can be seen to affect the relative activities determined by comparison of initial rates, as compared to glycerol. Upon removal of the hydrophilic influence, the relative activity for both alcohol systems increases dramatically accompanied by a remarkable loss of enantioselectivity. Analysis of the relative initial rates (Table 2) for some oxidations of enantiomers catalyzed by horse liver alcohol dehydrogenase shows that for a comparable 1,2-diol and α-amino alcohol the relative initial rates are 2:1 in favor of the diol substrate. On comparison of the initial rates of valinol (**4c**) and alaninol (**4b**), an obvious preference for the small hydrophobic substituent is found; the same is the case with 1,2-propanediol (**1e**) and 3-methyl-1,2-butanediol (**1i**). In each case the L enantiomer is oxidized about 20 times as fast as the D enantiomer, except that compounds **1i**, **4c**, **7**, and **8** with hydrophobic substituents at position 3 are surprisingly oxidized with no enantiomeric preference, indicating the limitation of the model.

The Origin of Enantioselectivity

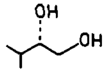
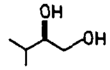
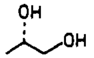
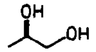
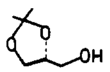
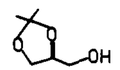
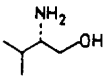
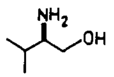
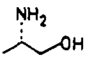
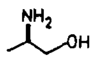
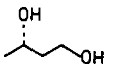
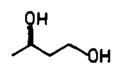
In addition to the relative reactivity for the oxidation of each enantiomer catalyzed by horse liver alcohol dehydrogenase, another factor which affects the enantioselectivity is the affinity of each enantiomer for the enzyme. The K_m value of (R)-**1e** (K_m = 48 mM) for the enzyme, for example, is about 25 times as high as that of (S)-**1e** (K_m = 1.8 mM). Determination of the enantiomeric ratio (E), an intrinsic property of the biochemical reaction formulated as $V_A K_B / V_B K_A$, where V_A, K_A and V_B, K_B represent the maximal velocities and Michaelis–Menten constants for the competing fast (A) and slow (B) enantiomers in a reaction where both compete for the same active site (*17*), yielded a value of approximately 270 based on initial rates as $V_{\max}$.

Synthesis

Application of the enantioselectivity in the horse liver alcohol dehydrogenase-

TABLE 2

RELATIVE INITIAL RATES FOR THE OXIDATION OF SEVERAL ENANTIOMERS CATALYZED BY HORSE LIVER ALCOHOL DEHYDROGENASE[a]

Enantiomers	Relative rate[b]
 : 	26:26
 : 	40:4
 : 	7:7
 : 	11:11
 : 	20:6
 : 	50:50

[a] To a solution of 0.1 M glycine–NaOH buffer (1 ml, pH 9.0) containing NAD (2 mM) and enzyme (0.1 mg) was added 10 μl of 0.5 M substrate, and the initial increase of absorbance at 340 m was recorded.

[b] Determined by comparing the initial rate with that of glycerol under the same conditions described above.

catalyzed oxidation of racemic 1,2-diols to organic synthesis has been illustrated by the synthesis of α-bromo-L-lactic acid (**3d**) from racemic bromoglycerol, **1d**, in 6-mmol scale. The reaction has used coimmobilized horse liver alcohol dehydrogenase and aldehyde dehydrogenase as catalysts and α-ketoglutarate/NH_3 as a regeneration system for NAD cofactor. We should mention that in the synthesis of α-hydroxy acids or esters, consideration must be given to current methods available. The reduction of α-keto esters via β-(3-pinanyl)-9-borabicyclo-[3.3.1]nonane (*18*) has achieved enantiomeric excesses in the range of 100%; however, this method was not tested with β-halogenated acids. Other methods involving reduction of keto esters via hydrosilylation (*19*), or homogeneous hydrogenation (*20*) have achieved optical purities of only 35–80%. Asymmetric halolactonization (*21*), reversed polarity prelog reactions (*22*), and asymmetric addition of allyltrimethylsilane (*23*) have problems of either nonapplicability to halogenated hydroxy acids or enantiomeric excesses in the range of 50%. Fermentative reactions (*24*, *25*) have yielded optically pure products but only for a very narrow range of substrates. Furthermore, in order to generate the desired optically active α-hydroxy aldehyde from the respective acid or ester, a subsequent reduction must be made, decreasing the overall chemical yield. We do not wish to infer that this catalytic method should replace the various chemical procedures available, but should a certain synthetic pathway necessitate extremely mild conditions with enantiomeric excesses in the range of 100% (given a ±3% error in determination of optical purity), this method might be considered.

CONCLUSION

In summary, the work has demonstrated the specificity of alcohol dehydrogenase using aminoalcohols, acyclic 1,2-diols, and derivatives as substrates. The enantioselective oxidation of 1,2-diols and hydrophilic aminoalcohols can be rationalized by the cubic-space section model, i.e., the C-2 group must have the L configuration so that the C-2-OH points toward the allowed region. The nonselective oxidation of **1i** and **4c**, however, is not consistent with what is expected. Apparently, the NH_2 and C-2-OH groups can enter freely into the forbidden or limited region (B1) (Scheme 2). Since horse liver alcohol dehydrogenase has a hydrophobic active site (*4, 2*), it is suggested although not certain that the hydrophobic substituent at position 3 interacts with the hydrophobic residues in the active site and causes some distortion so that both enantiomers can be accepted and oxidized by the enzyme. Further investigation of the model may be necessary. The enantioselectivity of horse liver alcohol dehydrogenase in the oxidation of the compounds studied here has two synthetic applications. First, organic synthesis based on the enzymatic oxidation of 1,2-diols to L-α-hydroxy acids overcomes the difficulties encountered in the preparation of these acids. L-β-Halo lactic acids, for example, can be easily prepared from haloglycerols, which are readily available and inexpensive. These compounds currently are prepared from β-halo pyruvates which are either expensive or not readily available and would inactivate the enzyme via alkylation (*13*). Second, the horse liver alcohol dehydrogenase-catalyzed oxidation of 1,2-diols and α-amino alcohols to aldehydes can be coupled with the aldolase reaction to prepare a series of unusual L-sugars and amino sugars (*26, 28*).

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