A New NAD-dependent Alcohol Dehydrogenase with Opposite Facial Selectivity useful for Asymmetric Reduction and Cofactor Regeneration

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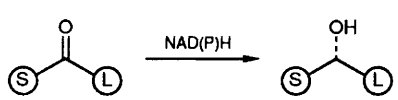
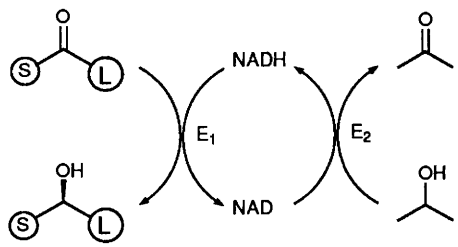
A new NAD-dependent alcohol dehydrogenase isolated from a *Pseudomonas* species catalysed the reduction of many acyclic ketones to optically active alcohols with very high enantioselectivity (90 to >98% enantiomeric excess); the stereochemical course of the reduction was determined to be the transfer of the *pro*-(*R*) hydrogen from NADH to the *Si* face of the carbonyl group, a process different from that for other known alcohol dehydrogenases.

Nicotinamide cofactor-dependent alcohol dehydrogenases have been extensively used in organic synthesis.[1] One common feature of these enzymes is that they normally deliver a hydride from the reduced cofactor to the top face of a carbonyl group with the large (L) and small (S) substituents arranged as shown in Scheme 1, a process known as Prelog's rule.[2] All alcohol dehydrogenases commercially available, *e.g.*, that from yeast,[1a] horse liver,[1a] and *Thermoanaerobium brockii*,[1c] fall into this group. As part of our interest in the development of new enzymes for organic synthesis, we have initiated a new screening strategy using various diol systems as the sole carbon source to search for new alcohol dehydrogenases from micro-organisms. We report here a new NAD-dependent alcohol dehydrogenase (PADH) which is specific for acyclic ketones and possesses an opposite facial selectivity towards the carbonyl group of ketone substrates. This enzyme was isolated from a *Pseudomonas* species, which was identified from a screening medium containing butane-1,4-diol as a sole carbon source.

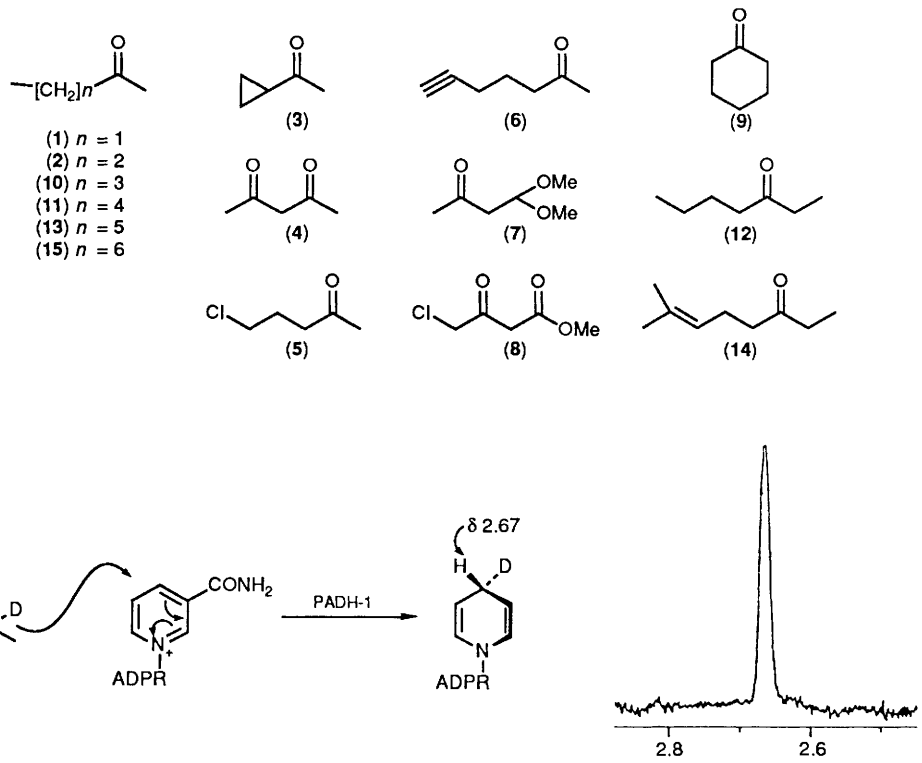


Scheme 1. Alcohol dehydrogenase from yeast, horse liver, and *Thermoanaerobium brockii*. Ⓛ: large group. Ⓢ: small group.

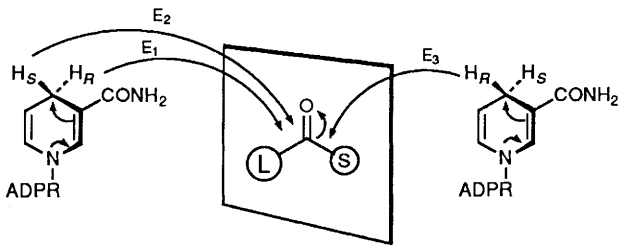
To prepare the enzyme for synthesis, cells obtained (by centrifugation at 15000 g, 4 °C, for 20 min) during the late exponential growth phase were disrupted by a French press (15000 psi) (1 psi ≃ 6.895 × 10^3 Pa) and the resulting



Scheme 2. $E_1 = E_2$ = PADH-1.



Scheme 3



E_1: Alcohol dehydrogenase from *Pseudomonas* species; E_2: from *Mucor javanicus*; E_3: from yeast, horse liver, and *Thermoanaerobium* species.

Scheme 4

homogenate was centrifuged at 30 000 g for 30 min at 4 °C. The supernatant was freeze-dried to a powder, which was stored in a refrigerator or used directly for chemical synthesis. Typically, the homogenate from 10 g of wet cells contains approximately 800 U (1 U = 1 μmol of propan-2-ol oxidised per min) of enzyme. Further purification of the crude enzyme *via* diethylaminoethyl (DEAE) sepharose and affinity chromatography provided a pure protein with a single band on polyacrylamide gel electrophoresis. The kinetic parameters of the purified enzyme were determined to be: $V_{max.}$ = 25 U mg^{-1} at 25 °C and pH 8.5 based on propan-2-ol and NAD, K_m for NAD = 0.36 mM and K_m for propan-2-ol = 0.19 mM. The enzyme was stable at room temperature in a phosphate buffer (0.01 M; pH 7.0) containing 10% of propan-2-ol. After one week at room temperature, no loss of activity was observed.

The crude enzyme was then used in asymmetric reduction. Reactions were carried out in a phosphate buffer (0.01 M; pH 7.0) containing the enzyme, a ketone substrate, NAD (1 mM), and 20% of propan-2-ol for regeneration of NADH (Scheme 2). In a representative synthesis, to a solution of NAD (20 mg), crude enzyme (0.8 g), and propan-2-ol (12.2 ml) in 60 ml of phosphate buffer (0.01 M; pH 7.0) was added 20 mmol of a ketone. The solution was gently stirred at room temperature until all the substrate had been consumed according to GC analysis.† The mixture was then extracted with dichloromethane (100 ml × 3) and the combined organic phase was dried over sodium sulphate. After the solvent had been

Table 1. Comparison of stereospecificity of NAD-dependent PADH with NADP-dependent TADH.

	TADH[a]		PADH	
Substrate	% E.e.	A.c.[b]	% E.e.[c]	A.c.[b]
(1)	48	*R*	87	*R*
(2)	79	*S*	97	*R*
(5)	98	*S*	>98	*R*
(11)	99	*S*	>98	*R*
(14)	99	*S*	>98	*R*

[a] TADH: the alcohol dehydrogenase from *Thermoanaerobium brokii* (ref. 1e). [b] A.c.: absolute configuration. [c] Determined by ^{1}H NMR analysis of the alcohol products in the presence of chiral shift reagent Eu(hfc)$_3$ (hfc = 3-heptafluoropropylhydroxymethylenecamphorato) or of the corresponding (+)-MTPA (MTPA = methoxytrifluoromethylphenylacetyl) ester derivatives (J. A. Dale, D. L. Dull, and H. S. Mosher, *J. Org. Chem.*, 1969, **34**, 2543).

† GC analyses were performed on a Hewlett-Packard 5890 instrument with a 20 m DB-5 megabore column. Column temperature 32 °C or 32—120 °C gradient; gradient temperature 2—10 °C min^{-1}.

removed by evaporation *in vacuo* at room temperature, the residue was separated by chromatography on a silica gel column using n-hexane–dichloromethane (1:0 to 2:1) or dichloromethane–ether (4:1) to obtain the product. Compounds (**1**) and (**2**) are reduced to (*R*)-butan-2-ol and (*R*)-pentan-2-ol respectively with very high enantiomeric excess (e.e.) (Table 1). Compounds (**4**)—(**6**) and (**10**)—(**15**) are also reduced to (*R*)-alcohols with >98% e.e. The yield for each reaction was in the range 60—95%. Other compounds as indicated are not substrates. It is interesting that the enzyme possesses anti-Prelog enantioselectivity in the reduction of acyclic ketones. Cyclic or aromatic ketones are not substrates. Interestingly, compound (**4**) is reduced to (*R*)-4-hydroxypentan-2-one in 80% yield and >98% e.e. Compared to the other commonly used alcohol dehydrogenase TADH (Table 1), this enzyme showed higher enantioselectivity with opposite facial selectivity.

To determine the stereochemical course of the hydride transfer, NAD (4 mM) and [2-$^2H_1$]propan-2-ol (180 mM) were incubated with 15 U of PADH in a hydrogen carbonate buffer (9 ml; 5 mM; pH 8.0) for 2 days. The reduced cofactor [4-^{2}H]NADH was isolated[3] and the ^{1}H NMR (500 MHz) spectrum was measured to show a chemical shift at δ 2.67 for 4(*S*)-H, indicative of *pro*-(*R*) specificity for the hydride transfer from NADH to the substrate[3] (Scheme 3). A complete stereochemical course for the reduction was thus determined and shown in Scheme 4. It is worth noting that the alcohol dehydrogenases from horse liver, yeast, *Thermoanaerobium brokii*,‡ and the *Pseudomonas* species described here all use the *pro*-(*R*) hydrogen of the reduced cofactor, except that from *Mucor javanicus* which is specific for the *pro*-(*S*) hydrogen.[2] The enzyme PADH, however, reduces the ketone substrate with an opposite facial selectivity as compared to the alcohol dehydrogenase from the first three species, yet with the same facial selectivity as that from *Mucor javanicus*.[2] This new enzyme thus possesses a completely different stereospecificity and appears to be useful as a catalyst for asymmetric reduction and for cofactor regeneration. The single enzyme system used for both cofactor regeneration and synthesis makes the process convenient and practical. Research is in progress to characterize the enzyme, and to determine its structure and the sequence of substrate binding.

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‡ Using deuteriated propan-2-ol as substrate, NADP was reduced by the enzyme as described and the chemical shift of the 4-H was determined to be 2.66 ppm, indicating that the enzyme is specific for the *pro*-(*R*) hydrogen of NADPH.

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