

excellent agreement to those of a natural sample (^1H and ^{13}C NMR, IR, TLC, $[\alpha]_{\text{D}}^{20} = +13.0$ ($c = 1.09$, MeOH).

In conclusion, this total synthesis of (+)-discodermolide proceeds in 27 steps and 7.7% overall yield for the longest linear sequence starting from commercial methyl (*S*)-3-hydroxy-2-methylpropionate. The three key subunits were synthesized efficiently using boron-mediated *anti*-selective aldol reactions of chiral ketones (*S*)-**6**, (*S*)-**11**, and (*S*)-**17**. This synthesis has the potential to provide useful quantities of (+)-discodermolide, which will allow detailed biological evaluation, as well as offering a variety of options for analogue chemistry.

Received: September 13, 1999 [Z14006]

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Cofactor-Bound Cross-Linked Enzyme Crystals (CLEC) of Alcohol Dehydrogenase**

Nancy St. Clair, Yi-Fong Wang, and
Alexey L. Margolin*

The use of dehydrogenases in organic synthesis is often limited by the intrinsic instability of enzymes and their nicotinamide cofactors.^[1] The protein part of the molecule can be efficiently stabilized by several techniques such as directed evolution,^[2] immobilization,^[3] and protein crystallization and cross-linking.^[4] The latter approach has turned out to be especially efficient in producing robust and productive biocatalysts for chemical synthesis.^[5] Here, we expand this approach to the stabilization of the cofactor part of the dehydrogenase molecule. Horse liver alcohol dehydrogenase (HLADH) was crystallized in the presence of reduced nicotinamide adenine dinucleotide (NADH), and the resulting crystals were treated with glutaraldehyde to yield the cross-linked enzyme crystals (CLECs). The crystallized and cross-linked HLADH was first introduced by Lee et al., and it demonstrated good activity (26% of that in solution) and an increased stability of the cross-linked crystals in the presence of zinc salts.^[6] In this work, we use this system to address two main questions: 1) Is a cofactor more stable when bound inside the enzyme crystal? 2) Is it possible to regenerate a cofactor using a coupled substrate system, thus making HLADH-NADH-CLEC a useful catalyst for organic synthesis?

The activity of soluble enzyme and various HLADH-CLEC preparations was compared in the reduction of 6-methyl-5-hepten-2-one (**1**) in the presence of isopropanol for cofactor regeneration (Scheme 1). The results, presented in Table 1, afford several conclusions. The HLADH-NADH-CLECs exhibit higher activity when HLADH is cocrystallized with a cofactor and an inhibitor, DMSO. In this case, the resulting complex exhibited 64% of the activity of the soluble enzyme in the absence of an exogenous cofactor. DMSO seems to be

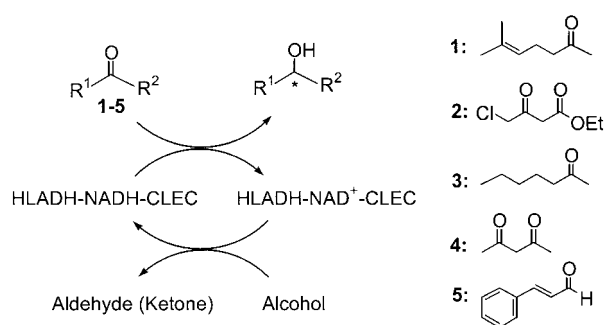
[*] Dr. A. L. Margolin, N. St. Clair, Dr. Y.-F. Wang
Altus Biologics Inc.
625 Putnam Ave., Cambridge, MA 02139-4807 (USA)
Fax: (+1) 617-577-6502
E-mail: margolin@altus.com

[**] This work was funded by the NIH (grant 5R44GM51781).

Table 1. Activity of soluble HLADH and various HLADH-NADH-CLEC in the reduction of 6-methyl-5-heptene-2-one (**1**) with and without addition of an exogenous cofactor.

Enzyme	with added NADH		without added NADH	
	[$\mu\text{mol min}^{-1}\text{mg}$]	[%]	[$\mu\text{mol min}^{-1}\text{mg}$]	[%]
soluble HLADH	14.0	100	0	0
HLADH-NAD(H)-CLEC-I ^[b]	12.9	92	8.9	64
HLADH-NAD(H)-CLEC-II ^[c]	9.6	69	2.5	18
HLADH-CLEC ^[d]	2.1	15	0	0

[a] The activity was based on a 2-h reaction time. [b] Prepared in the presence of both NADH and DMSO. [c] Prepared in the presence of NADH only. [d] Prepared in the absence of both NADH and DMSO.



Scheme 1. Reductions catalyzed by HLADH-NADH-CLEC.

necessary in maintaining the optimal HLADH conformation in the crystal.^[6, 7]

As expected, HLADH-CLEC, which was prepared in the absence of a cofactor, had no catalytic activity in the reaction without added NADH. The addition of exogenous NADH increased the activity of both HLADH-NADH-CLEC and HLADH-CLEC. This effect was especially profound for CLECs crystallized in the presence of NADH, or NADH and DMSO, reaching 69 and 92 % of the activity of the soluble enzyme, respectively.

HLADH-NADH-CLEC-I (Table 1) was then applied to the reduction of several additional ketones **1–4** (Scheme 1). The degree of conversion in these reactions depends on the substrate structure, while the enantioselectivity and stereochemical preference of the soluble HLADH remains unchanged (Table 2).

The ability of the CLECs to maintain the bound cofactor was assayed by incubating the HLADH-NADH-CLEC at a concentration of 30 mg mL⁻¹ (dry weight) in 50 mM 2-[[tris-(hydroxymethyl)methyl]amino]1-ethanesulfonic acid (TES) buffer (pH 7.5) containing 10 % ethanol, at 40 °C for 48 hours in a stirred cell. Following incubation, the CLECs were removed from the solution by filtration and the supernatant

Table 2. Reduction of ketones **1–4** catalyzed by HLADH-NADH-CLEC-I.

Ketones	Conversion [%] ^[a] ee [%]	Stereochemistry of the product	Ref.
1	25.5	96	<i>S</i> [5d]
2	55.8 ^[b]	98	<i>R</i> [7]
3	26.7	98	<i>S</i> [8]
4	5.8 ^[c]	> 98	<i>S</i>

[a] Conversion based on 20 hour reaction. [b] The product was exclusively ethyl 3-hydroxy-4-chlorobutyrate. [c] The product was exclusively 4-hydroxy-2-pentanone.

was assayed spectrophotometrically at 280 nm for protein and at 340 nm ($\epsilon = 6230 \text{ M}^{-1}\text{cm}^{-1}$) for NADH. Only background absorbance was detected indicating that neither protein nor cofactor leach from the CLEC.

The data on both activity and leaching are consistent with structural studies which indicated that in the presence of cofactor, HLADH undergoes a large conformational change from an open form to a closed form.^[7] The catalytic and coenzyme binding domains in each subunit rotate against each other, enclosing the cofactor and thereby preventing its dissociation from the holoenzyme after the crystals are cross-linked. The very low activity of HLADH-CLEC, even in the presence of the exogenous cofactor (Table 1), can be explained by the markedly different conformation of apo- and holoenzymes and the difficulty the cofactor has accessing the binding center of the apoenzyme in the CLEC form. The fact that the addition of exogenous NADH increases activity in all cases indicates that some cofactor binding sites in the crystalline HLADH remain vacant. In addition, it is known that about 4 % of the enzyme surface is covered by the bound cofactor.^[10] It is also possible that a portion of surface-bound NADH was lost during the processing of the crystals. It is worth mentioning that NADH transport in and out of the crystal may proceed differently in different dehydrogenases. For instance, lactate dehydrogenase crystallized in the absence of a cofactor exhibited 30 % of the activity of the soluble enzyme in the presence of the exogenous NADH.^[11]

To understand whether the cofactor is protected from inactivation within a protein crystal, the oxidized form of nicotinamide adenine dinucleotide (NAD⁺) and NADH were incubated in different aqueous buffers (pH 6–8 at 30 °C; Figure 1), and the integrity of the cofactor was measured enzymatically by addition of fresh HLADH and a substrate (see the Experimental Section). Under these conditions the half-life ($t_{1/2}$) of the cofactors ranges from 2.6 d for NADH in phosphate buffer at pH 6 to 10.7 d in TES/2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) buffer at pH 6 for NAD⁺.

Next, we determined the stability of HLADH under the same conditions (Figure 2). The half-life of the soluble HLADH turned out to be close to 14 d regardless of the buffer used. The residual activity of HLADH was assayed in the presence of freshly added NADH and thus the data reflect the stability of the HLADH, not the cofactor. In contrast to the soluble enzyme and cofactor, the HLADH-NADH-CLEC complex retained its full activity after three months of incubation at 30 °C (Figure 2), indicating that not only the

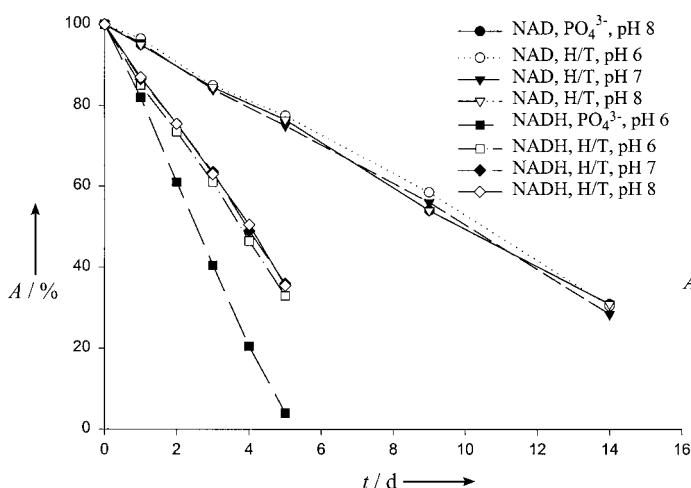


Figure 1. Stability of NAD⁺ and NADH in phosphate buffer and HEPES/TES (H/T) buffer at 30°C. (For details, see the Experimental Section.)

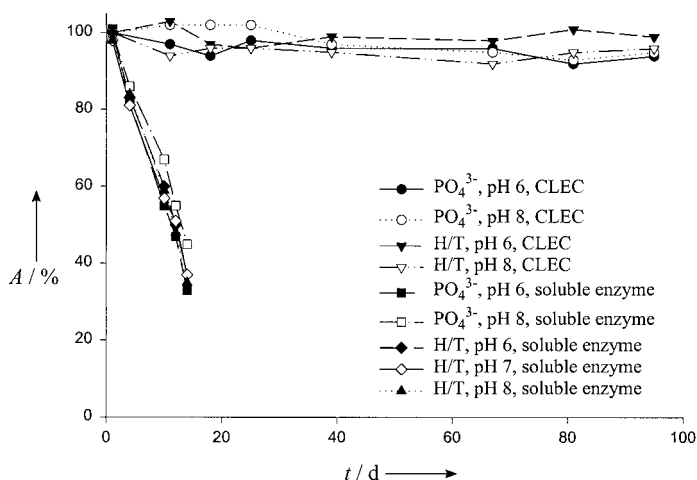


Figure 2. Stability of soluble HLADH and HLADH-NADH-CLEC in buffer at 30°C. (For details, see the Experimental Section.)

enzyme, but also the cofactor was protected by the crystal environment.

Similar to many hydrolases, HLADH in a CLEC form exhibits high stability towards organic solvents and elevated temperature (Figure 3). Soluble enzyme was rapidly inactivated at 40°C in the presence of 25% 2-propanol ($t_{1/2} = 4$ h). At lower temperature (25°C) the half-life of soluble enzyme was extended to 24 h. In contrast, HLADH-NADH-CLEC-I was stable for 96 h in 25% isopropanol at 25°C, maintaining almost full activity for 48 h, and had a half-life of more than 4 d at 40°C.

The stability of HLADH-NAD(H)-CLEC toward organic solvents is important because high concentrations of isopropanol, as well as ethanol or 1,4-butanediol, can be used for cofactor regeneration in a coupled substrate system (Scheme 1).^[12] We found that HLADH-NADH-CLEC-I retained full activity for 2 d in 90% isopropanol at 25°C. Therefore, high concentrations of alcohols can be used for the regeneration of cofactor in a reaction catalyzed by HLADH-NADH-CLEC-I. In contrast, soluble HLADH precipitated and lost activity immediately under the same conditions.

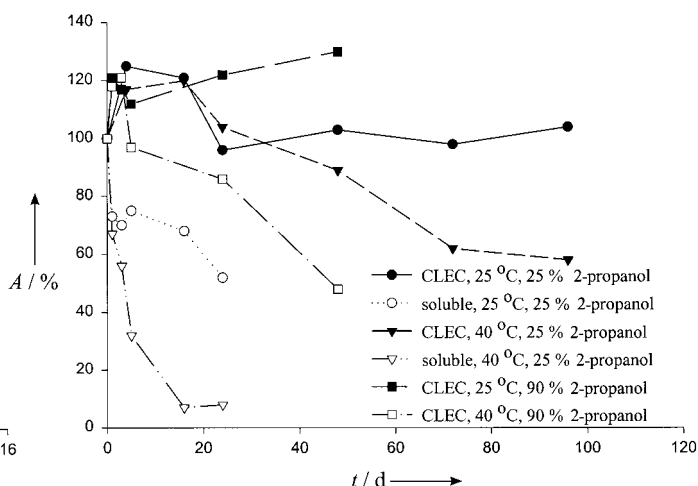


Figure 3. Stability of soluble HLADH and HLADH-NADH-CLEC-I at 25°C and 40°C in 25% and 90% isopropanol. (For details, see the Experimental Section.)

The ability of the bound cofactor to be recycled inside HLADH-NADH-CLEC was demonstrated in the reduction of cinnamaldehyde (**5**) using 1,4-butanediol to regenerate the cofactor (Scheme 1). A 10 mm × 10 cm column (Pharmacia C) was packed with 50 mg (dry weight) of HLADH-NADH-CLEC-I, and a total amount of 15 mmol of cinnamaldehyde (**5**) was reduced to cinnamyl alcohol. The catalyst had retained nearly full activity when the experiment was terminated. To calculate the cofactor total turnover number (TTN) we assumed that the total protein as measured by dry weight was active enzyme (water content and active-site inactivation were assumed to be negligible). This assumption suggested there was 1.2 μmol of NADH bound to 50 mg of HLADH-NADH-CLEC (given two active sites per enzyme molecule and a holoenzyme molecular weight of 80000). Under these conditions the CLEC yielded a turnover number for cofactor regeneration of greater than 12000. Given the conservative assumptions used in calculating TTN, we were extremely encouraged by the performance of the HLADH-NADH-CLEC since it suggests great potential for a much higher level of catalyst productivity.

Experimental Section

Enzyme crystallization: HLADH was crystallized by a method modified from Lee et al.^[6] Enzyme crystals were recovered by low-speed centrifugation and dissolved in a buffer solution of 0.2 M NaCl/50 mM TES (pH 7.5). Insoluble precipitate was removed by filtration and the soluble enzyme was dialyzed exhaustively against 50 mM TES buffer (pH 7.5). Following equilibration (18 h) the dialysis bags (12000 molecular weight cut off (MWCO) containing 5 mL of enzyme solution (11 mg mL⁻¹) were dialyzed against 50 mM TES buffer (pH 7.5) containing 5% DMSO, 0.5 mg mL⁻¹ NADH, and 8% 2-methyl-2,4-pentanediol (MPD) at 4°C. The MPD concentration was increased by increments of 2% at intervals of 30 min to 1 h until the final MPD concentration was 22%. Crystallization was observed beginning at 16% MPD. Greater than 90% of the enzyme was recovered in crystalline form after 48 h incubation.

Cross-linking of enzyme crystals: HLADH crystals were cross-linked in 1-mL reaction volumes containing 10 mg mL⁻¹ protein crystals, 1.0% glutaraldehyde, 5% DMSO, 0.5 mg mL⁻¹ NADH, and 100 mM TES buffer (pH 7.5). Cross-linking was allowed to proceed for 4 h with stirring. Residual glutaraldehyde and DMSO were removed by exhaustive washing

with TES buffer (pH 7.5) containing 20% ethanol. The HLADH cross-linked crystals were stored in 50 mM TES buffer (pH 7.5).

Stability of NAD⁺ and NADH in buffer: Solutions (20 mM) of NAD⁺ or NADH were incubated at 30 °C in 50 mM TES/HEPES buffer at pH 6, pH 7 and pH 8. Additionally, 20 mM NAD⁺ or NADH was incubated at 30 °C in 25 mM sodium phosphate buffer at pH 6 and pH 8. Incubations were performed in duplicate. Active cofactor was quantified enzymatically. A 100-μL aliquot of 20 mM cofactor solution was removed at intervals and added to 900 μL of TES buffer (50 mM, pH 7.5) at 30 °C containing 10 μmol substrate and 2 mg of HLADH. The substrate for reduced cofactor was cinnamaldehyde and the substrate for oxidized cofactor was cinnamyl alcohol. Moles of product formed were measured by HPLC and assumed to be proportional to moles of active cofactor added to the reaction. (HPLC conditions: 15 cm Rainin C₁₈ microorb 5 μ 300 Å column; mobile phase: acetonitrile/water = 1/1; flow rate: 0.5 mL min⁻¹; monitoring at 260 nm; retention time = 2.38 (cinnamyl alcohol); 2.97 min (cinnamaldehyde). Standard curves were constructed from known quantities of cofactor. Data is presented as a percentage of the initial cofactor quantity (20 μmol).

Stability of HLADH in buffer: HLADH (2 mg mL⁻¹) was incubated at 30 °C in 50 mM HEPES/TES buffer at pH 6, pH 7, and pH 8 and also in 25 mM phosphate buffer at pH 6 and pH 8. Aliquots (50 μL) were removed at intervals and enzyme activity was measured in a cosubstrate assay of 20 mM cinnamaldehyde, 1.5 M isopropanol, and 100 mM NAD⁺ in 50 mM HEPES/TES buffer at 30 °C. The rate of product formation was measured by HPLC. Data is presented as a percentage of the initial activity.

Stability of HLADH-NADH-CLEC in buffer: HLADH-NADH-CLEC (10 mg mL⁻¹) was incubated at 30 °C in 50 mM HEPES/TES buffer at pH 6 and pH 8 and in 25 mM phosphate buffer at pH 6 and pH 8. Aliquots (50 μL) were removed at intervals and enzyme activity was measured in a cosubstrate assay of 20 mM cinnamaldehyde and 1.5 M isopropanol in 50 mM HEPES/TES buffer at 30 °C. The rate of product formation was measured by HPLC. Data is presented as a percentage of the initial activity.

General procedure for reductions: A suspension of substrate (20 μL) in a mixture of isopropanol (0.2 mL) and phosphate buffer (2 mL, pH 7.0) was incubated with 200 μL of CLEC or soluble HLADH at room temperature with and without adding 0.3 mg of NADH. The conversion was determined by GC analysis. Conditions for GC analysis: Cyclodex B capillary GC 25 m × 0.25 mm column, thickness: 0.25 μm (J & W Scientific, Folsom, CA), flow rate: He, 1 mL min⁻¹; temperature program: initial: 90 °C for 10 min, gradient rate: 2–5 °C min⁻¹, final: 160 °C for 0 min).

HLADH-NADH-CLEC-I catalyzed multi-cycle reaction of cinnamaldehyde: A slurry of CLEC (10 mL, 50 mg dry weight) and 0.5 g of silica gel in a solution containing 100 mM Tris HCl and 0.1 mM ZnCl₂ (pH 9.0) was packed in a 10 mm × 10 cm column (Pharmacia C). A solution of 200 mM 1,4-butanediol and 10 mM cinnamaldehyde in 100 mM Tris HCl and 0.1 mM ZnCl₂ buffer (pH 9.0) was pumped through the column at a flow rate of approximately 15 mL h⁻¹ at ambient temperature. Conversion of aldehyde was measured by HPLC analysis using a 15-cm Rainin C₁₈ microorb 5 μ 300 Å column (mobile phase: acetonitrile/water = 1/1; flow rate: 0.5 mL min⁻¹; monitoring at 260 nm; retention time: 2.38 min (product); 2.97 min (substrate)). A total substrate volume of 3.22 L passed through the column before the experiment was stopped.

Received: August 17, 1999 [Z13893]

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In₈(C₆H₃-2,6-Mes₂)₄ (Mes = C₆H₂-2,4,6-Me₃): A Metal-Rich Main-Group Cluster with a Distorted Cubane Structure**

Barrett E. Eichler, Ned J. Hardman, and Philip P. Power*

The reaction between indium monochloride and the bulky lithium terphenyl (Et₂O)LiC₆H₃-2,6-Trip₂^[1] (Trip = C₆H₂-2,4,6-*i*Pr₃) was recently reported to yield the monomeric compound InC₆H₃-2,6-Trip₂.^[2a] This compound and its thallium analogue^[2b] are unique examples of one-coordinate metal atoms in the solid state. Reactions of InCl or TlCl with less crowded lithium terphenyls are expected to lead to more highly aggregated structures, which may involve interesting metal–metal interactions. This class of compounds is of considerable current interest in Group 13 metal chemistry.^[3] We therefore investigated the reactions of InCl with lithium terphenyls of various sizes.^[4] Initial work focused on the less crowded ligand C₆H₃-2,6-Mes₂^[5] (Mes = C₆H₂-2,4,6-Me₃). Here we report that the reaction of LiC₆H₃-2,6-Mes₂ with InCl leads to the unusual cluster species In₈(C₆H₃-2,6-Mes₂)₄ (**1**) rather than the projected stoichiometric aggregates of formula (InC₆H₃-2,6-Mes₂)_n (*n* ≥ 2).

Compound **1** was synthesized by treating a suspension of InCl in THF with LiC₆H₃-2,6-Mes₂ at about –78 °C. Red crystals of **1** were isolated in approximately 25% yield after recrystallization from hexane. The X-ray crystal structure^[6] of **1** (Figure 1) shows that in the solid state the compound has a

[*] Prof. P. P. Power, Dr. B. E. Eichler, N. J. Hardman
Department of Chemistry
University of California
One Shields Avenue, Davis, CA 95616 (USA)
Fax: (+1) 530-752-8995
E-mail: pppower@ucdavis.edu

[**] This work was supported by the National Science Foundation.