Pergamon

Tetrahedron, Vol. 52, No. 5, pp. 1669-1676, 1996

0040-4020/96 $15.00 + 0.00

0040-4020(95)00995-7

Bacillus stearothermophilus Alcohol Dehydrogenase: a New Catalyst to Obtain Enantiomerically Pure Bicyclic Octen- and Hepten-ols and -ones.

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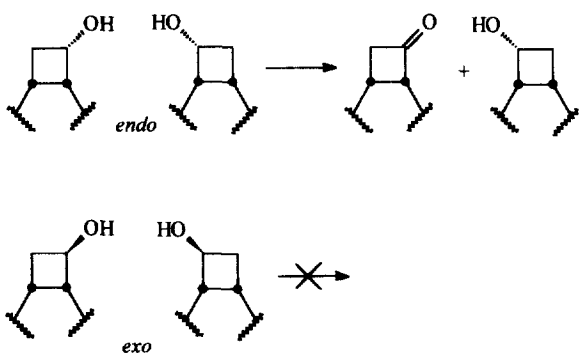
Abstract From the cells of *Bacillus stearothermophilus* a new enzyme has been isolated, which catalyses the stereospecific redox reactions of bicyclic octen- and hepten- ols and -ones.

Bacillus stearothermophilus lactate dehydrogenase (BSLDH) is known to be a very stable, thermophilic enzyme that has been principally used in organic synthesis to obtain the enantioselective reduction of α-keto acids to homochiral α-hydroxy acids[1]. We recently reported the use of *Bacillus stearothermophilus* for the kinetic resolution of 1-aryl ethanols[2] and *endo*-bicyclic octen- and heptenols[3], important starting materials in the synthesis of cyclopentenoid natural products, prostacyclin and prostaglandin analogues[4], both in aqueous and biphasic systems[5]. The action of this microorganism, however, is specific with respect to the *endo* stereochemistry of the substrate, as demonstrated by the absence of oxidation on *exo*-bicyclic octen- and heptenols, in the same experimental conditions[6], Scheme 1.

Scheme 1



The major limitation in the use of whole cells in the transformations mentioned above is the confinement within the oxidative direction and, consequently, the achievement of two of the four possible stereoisomers. *Bacillus*

stearothermophilus, for example, shows no reaction with bicyclic octen- and heptenones. To overcome this drawback and in order to find other enzymes from thermophilic sources, starting from the same cells we have obtained a cell-free solution containing a pyridine dinucleotide dependent alcohol dehydrogenase (BSADH), which catalyses not only the stereospecific oxidation of these compounds, but also the stereospecific reduction of the related ketones. Note that microorganisms other than *Bacillus stearothermophilus* were reported to reduce bicyclic ketones to the related alcohols[7,8]. These reductions, however, displayed good chemical yields but low selectivities, giving with the *endo* alcohols variable amounts of *exo* derivatives. Beside stereoselectivity, BSADH showed an almost unaltered activity of oxidation and reduction also in the presence of organic solvents.

The new alcohol dehydrogenase BSADH was easily released from the cells of *Bacillus stearothermophilus* following treatment with lysozyme in TEA buffer. The purification and characterization of this enzyme is under way. The oxidative and reductive activity of the BSADH, occurring in the presence of NAD^+ and NADH respectively, has been tested on three model bicyclic substrates: *endo*-bicyclo[3.2.0]-hept-2-en-6-ol (±) **1**, *endo*-bicyclo[3.3.0]-oct-7-en-6-ol (±) **3**, *endo*-norborn-5-en-2-ol (±) **5** and the corresponding ketones (±) **2, 4, 6**. As the microorganism[3], the BSADH gave good results in the kinetic resolution *via* oxidation of (±) **1, 3, 5**, Table 1. In this Table reaction times have been optimized in order to achieve the best ketone *enantiomeric excess-yield* balance. On the other hand, it has been proved[3] that longer reaction times increase the kinetic resolution in favor of a better remaining alcohol *enantiomeric excess-yield* balance, as demonstrated by the results obtained on the model (±) **1** at 18 and 24 hours, respectively. Worthy of note is the higher substrate concentration that can be employed in these experiments, approximately 10 fold higher than that used in the microbial oxidation. The BSADH-catalyzed oxidation of (±) **1** has been also used to check the stability of the enzyme. A decrease of ca. 30 % of the enzyme activity was observed after a complete oxidation cycle, extraction of the reaction products with heptane and addition of fresh substrate. Only after three cycles and three extractions of the products, the enzyme becomes completely inactive.

Although with a lower rate, BSADH catalyzes the reduction of (±) **2, 4, 6** to the corresponding alcohols in excellent yield and enantiomeric excess. In addition to kinetic resolution with respect to the ketone, the reductions are stereoselective and afford exclusively the *endo* alcohols. A summary is given in Table 2 and Scheme 3. Of particular interest are the results obtained with (±) **6**, 46 % yield and 93 % e.e., compared to those found with the related alcohol (±) **5**, 16 % yield and 88 % e.e.

The NADH formed during oxidation of (±) **1, 3, 5** and the NAD^+ obtained upon reduction of (±) **2, 4, 6** might be recycled enzymatically. As shown in Scheme 4, NADH is reoxidized to NAD^+ in the presence of pyruvate and lactate dehydrogenase, whereas the NAD^+ formed during the reduction process may be transformed to NADH by adding glucose-6-phosphate and glucose-6-phosphate dehydrogenase to the reaction mixture.

Table 1. Results of BSADH-Catalyzed Oxidation of Bicyclic Octen- and Heptenols (±) 1, 3, 5. Substrates and products are shown in Scheme 2.

substrate	conc. (g/l)	enzyme sol. (mL)	time (h)	medium	products (% yield[c], % e.e.)	
(±) **1**	15.4	3.7	18	H_2O[a]	(-) **2** (43,98)	(-) **1** (52,92)
	15.4	3.7	24	H_2O[a]	(52,90)	(47,97)
	14.0	3.7	48	heptane[b]	(28,100)	(67,60)
(±) **3**	17.4	3.7	35	H_2O[a]	(-) **4** (46,93)	(+) **3** (50,85)
	14.0	3.7	48	heptane[b]	(12,94)	(85,30)
(±) **5**	2.2	3.7	24	H_2O[a]	(-) **6** (16,88)	(+) **5** (81,18)

a. H_2O refers to TEA buffer, total reaction volume 100 mL; b. Two-phase system TEA buffer (90 mL) : heptane (10 mL), total reaction volume 100 mL.; c. After chromatography

Scheme 2

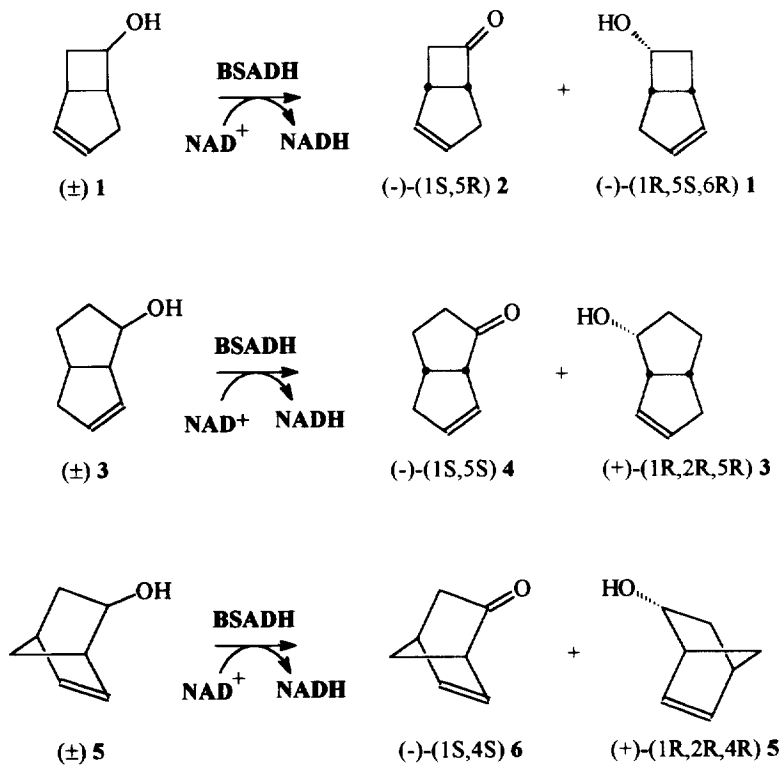
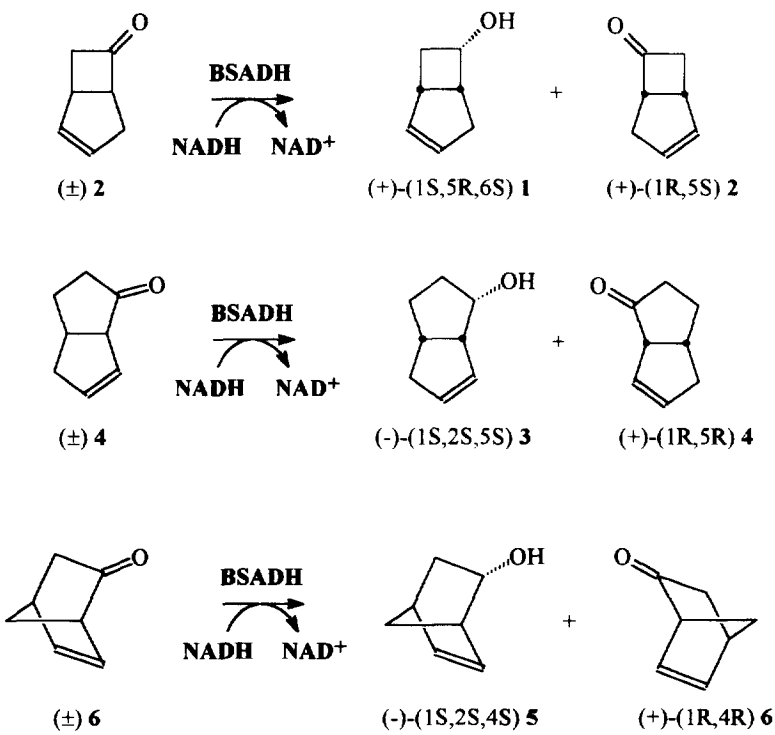


Table 2. Results of BSADH-Catalyzed Reduction of Bicyclic Octen- and Heptenones (±) 2, 4, 6. Substrates and products are shown in Scheme 3.

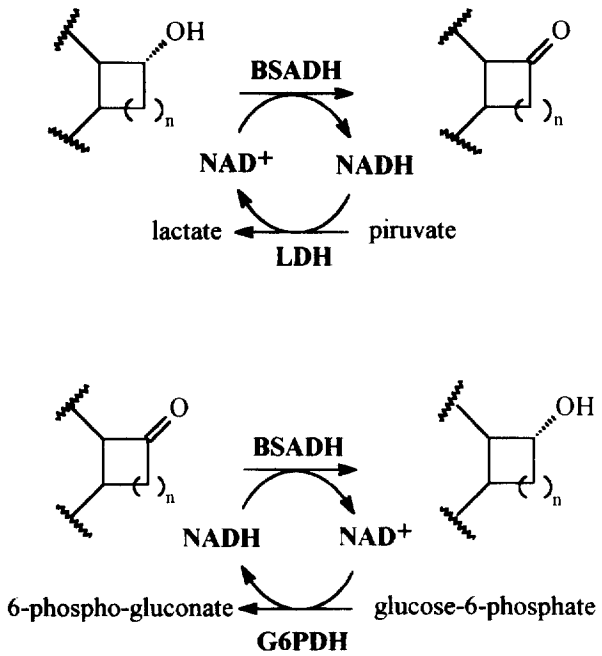
substrate	conc. g/l	enzyme sol.(mL)	time h	solvent	products (% yield[c], % e.e.)	
(±) **2**	4.4	7	36	H_2O[a]	(+) **1** (43,94)	(+) **2** (54,62)
	4.4	7	72	heptane[b]	(24,100)	(72,26)
(±) **4**	4.8	7	36	H_2O[a]	(-) **3** (46,100)	(+) **4** (51,84)
	4.4	7	72	heptane[b]	(41,100)	(56,82)
(±) **6**	2.2	7	36	H_2O[a]	(-) **5** (45,93)	(+) **6** (53,88)
	2.2	7	48	heptane[b]	(37,55)	(50,38)

a. H_2O refers to TEA buffer, total reaction volume 100 mL; b. Two-phase system TEA buffer (90 mL) : heptane (10 mL), total reaction volume 100 mL.; c. After chromatography.

Scheme 3



Scheme 4



The absolute activity of BSADH was investigated in oxidation on (±) **1** and reduction on (±) **2**. In the former reaction 1 μL of the enzyme solution catalyzes the reduction of 1,9 nmoles/min of NAD^+, whereas in the latter case the amount of NADH that has been oxidized was 0.92 nmols/min. Each of (±) **1-6** was a reasonable substrate for BSADS, however norbornenic-type structure reacts more slowly. The rates of oxidation and reduction relative to hepten-ol and one are reordered in Table 3.

Table 3. Relative rates[a] of BSADH-Catalyzed Oxidation and Reduction of (±) 1-6.

substrate	oxidation relative rate	substrate	reduction relative rate
(±) **1**	100[b]	(±) **2**	100[b]
(±) **3**	96,5	(±) **4**	95,3
(±) **5**	13,7	(±) **6**	22,2

[a] Oxidation and reduction rates were measured spectrophotometrically at 20 °C in TEA buffer containing 0.2 mM of NAD^+ or NADH and the mixture of the (±) substrates (15 mM); [b] The rates of oxidation of **1** and reduction of **2** have been arbitrarily setted to 100.

In conclusion, NAD-dependent alcohol dehydrogenase has shown to be an efficient catalyst for the specific oxidation and reduction of bicyclic substrates. Starting from an equimolar mixture of an enantiomeric pair of bicyclic alcohols, only one is enzymatically oxidized. Likewise, starting from an equimolar amount of the related ketones, only one is enzymatically and stereospecifically reduced giving a sterically pure alcohol as well as the unreacted ketone. Note that microorganisms and isolated enzymes are often utilized only in reductive biotransformations[7,9-10]. To our knowledge this is the first example of a more complete use of alcohol dehydrogenase in both directions in order to obtain enantiomerically pure bicyclic ketones as well as *endo* alcohols.

EXPERIMENTAL

Materials and Methods. *Bacillus stearothermophilus* is available from American Type Culture Collection (ATCC2027). Porcine Heart Lactic Dehydrogenases (PHLDH), glucose-6-phosphate (G6P), pyruvate, NAD and lysozyme are from Sigma. *Leuconostoc mesenteroides* glucose-6-phosphate dehydrogenase (LMG6PDH) is from Boehringer Mannheim. Bicyclo[3.2.0]-hept-2-en-6-one is from Merck, norborn-5-en-2-ol is from Aldrich. Bicyclo[3.3.0]-oct-7-en-6-one has been synthesized from 1,3-cyclooctadiene according to Crandall[11]. Yields and enantiomeric excess were determined by GC analysis in the presence of an internal standard, following a previously described procedure[3]. TEA buffer is composed of 50 mM triethanolamine-HCl buffer (pH 7.5) containing 0.1 mM EDTA.

Preparation of the Enzyme. *Bacillus stearothermophilus* ATCC2027 was cultivated in 250 mL of a medium composed of sucrose (10 g), peptone (5 g), yeast extract (2.5 g), $NaH_2PO_4 \cdot 6H_2O$ (1.7 g) and K_2SO_4 (0.65 g). Cultivation is carried out at 39 °C for 48 h with reciprocal shaking. Wet cells (20 g), obtained from four portions of 250 mL cultures, were harvested by centrifugation (8000 rpm/15 min), washed with 200 mL of NaCl 0.15 M, suspended in 100 mL of TEA buffer and treated with 40 mg of lysozyme for 60 min. at 22 °C. Supernatant (enzyme solution) was used for the enzymatic reactions without further treatment.

Relative Rates of BSADH-Catalyzed Reductions and Oxidations of (±) 1-6. Enzymatic activity was measured spectrophotometrically at room temperature (20-22 °C) adding few μL of the enzyme solution to 1 mL of the reaction mixture. Oxidation reactions were performed in TEA buffer containing 0.2 mM of NAD^+ and the mixture of the (±)-alcohols at a concentration of 15 mM. Reduction reactions were carried out in similar conditions, employing TEA buffer containing 0.2 mM of NADH and the ketones (15 mM). The absorbance changes at 340 nm were monitored, using intervals of 10 sec during the first minute, and of 1 min

for the following readings. One unit of enzyme activity is the quantity of enzyme which catalyses the formation of one μmole of product in one minute. Using as substrate the compound **1**, one μl of the enzyme solution catalyses the reduction of 1,9 nmoles/min of NAD^+. Using as substrate the compound **2** the same quantity of enzyme solution catalyze the oxidation of 0,92 nmoles/min of NADH. The relative rates were determined setting the rate of oxidation of **1**, and the rate of reduction of **2** to be 100.

Semipreparative-Scale BSADH-Catalyzed Oxidations of (±) 1, 3, 5. The following general procedure was employed. 100 mL of the reaction mixture contained 50 mM TEA buffer, pH 7.5, 20 mg of NAD^+, 3.7 mL of the enzyme solution, 2 mg of PHLDH, 14 mmols of alcohols **1** or **3**, or 2 mmols of alcohol **5**, and pyruvate at a concentration equimolar to that of the alcohols. The medium for the reactions in biphasic system was prepared adding heptane (10 mL) to the solution described above (90 mL). Depending on the reaction medium *i.e.* aqueous or two-phase system (H_2O-heptane), the substrate was added either as 2 M DMSO solution (7 mL for racemic **1, 3** and 1 mL for **5**) or neat (14 mmol for **1, 3** and 2 mmol for **5**), respectively. After the proper time the reaction mixture was extracted with diethyl ether (100 mL) with a continuous liquid-liquid extractor, dried over anidrous sodium sulfate and concentrated under reduced pressure. The products **2, 4, 6,** were separated by column chromatography (silica gel, using petroleum ether/diethyl ether 7/3 as eluent). Enantiomerically pure (-)-(1S,5R) **2** $[\alpha]_D$=-63 (*c*=1.2 $CHCl_3$)[3]; ^{1}H NMR δ (CCl_4) 2.4-2.9 (m, 3H), 3.0-3.6 (m, 2H), 3.6-4.0 (m,1H), 5.85 (br s, 2H)[12]. Enantiomerically pure (-)-(1S,5S) **4** $[\alpha]_D$=-502 (*c*=1.3 $CHCl_3$)[3], ^{1}H NMR δ 1.0-3.3 (m, 8H), 5.55 (m, 2H)[11]. (-)-(1S,4S) **6** $[\alpha]_D$=-930 (*c*=1.1 $CHCl_3$, optical purity 82%) ^{1}H NMR δ 1.7-2.4 (m, 4H), 2.8-3.3 (m, 2H), 6.0 (dd, *J*=3.6 Hz, 1H), 6.4 (dd, *J*=3.6 Hz, 1H)[13].

Semipreparative-Scale BSADH-Catalyzed Reductions of (±) 2, 4, 6. The following general procedure was employed. 100 mL of the reaction mixture contained 50 mM TEA buffer, pH 7.5, 20 mg of NAD^+, 7 mL of the enzyme solution, 2 mg of LMG6PDH, 4 mmols of ketones **2** or **4**, or 2 mmols of ketone **6**, and glucose 6-phosphate (G6P) at a concentration equimolar to that of ketones. After the proper time the reaction mixtures were extracted and treated as described in the previous section. Enantiomerically pure (-)-(1R,5S,6R) **1** $[\alpha]_D$=-68 (*c*=1.1 $CHCl_3$)[3], ^{1}H NMR δ 1.2-3.3 (m, 7H), 4.4 (m, 1H), 5.8 (br s, 2H)[12]. Enantiomerically pure (+)-(1R,2R,5R) **3** $[\alpha]_D$=151 (*c*=1.5 $CHCl_3$)[3], ^{1}H NMR δ 1.21-2.20 (m, 6H), 2.57-2.8 (m, 2H), 3.25 (m, 1H), 4.22 (m, 1H), 5.62 (m,1H), 5.85 (m, 1H)[14]. (+)-(1R,2R,4R) **5** $[\alpha]_D$=160 (*c*=0.5 $CHCl_3$, optical purity 97%)[3], the racemic mixture is commercially available.

Recycle of the Cofactors. NADH formed during oxidation of the alcohol and NAD^+ obtained on reduction of the ketone may be recycled by means of a multiple-enzyme system. The recycling of NADH is obtained by adding to the reaction mixture 0.05 mg of Porcine Heart Lactic Dehydrogenase (PHLDH), in a total reference volume of 1 mL, and an equimolar amount of pyruvate, with respect to the alcohol. Addition of 0.02 mg of LMG6PDH per 1 mL reference volume, and an equimolar amount of G6P, with respect to ketone, is required in order to recycle NAD^+.

ACKNOWLEDGEMENTS

This work was supported by grants from the Italian MURST (fondi 40% and 60%)

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(*Received in UK* 13 *October* 1995; *revised* 7 *November* 1995; *accepted* 9 *November* 1995)