ORGANIC LETTERS

2003
Vol. 5, No. 20
3649−3650

# An Efficient and Selective Enzymatic Oxidation System for the Synthesis of Enantiomerically Pure D-*tert*-Leucine

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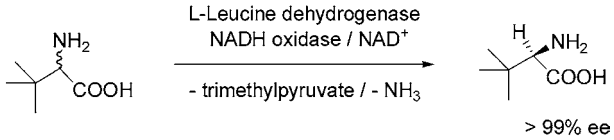
**Received July 16, 2003**

## ABSTRACT




**D-*tert*-Leucine was prepared with an enantiomeric excess of >99% by an enzyme-catalyzed oxidative resolution of the racemic mixture of DL-*tert*-leucine with use of leucine dehydrogenase. The L-amino acid was oxidized completely due to coupling of the primary reaction with a highly efficient irreversible $NAD^+$-regenerating step by NADH oxidase.**

Due to its bulky and hydrophobic *tert*-butyl side chain, *tert*-leucine finds increased use as a building block for the synthesis of chiral auxiliaries and biologically active compounds.[1] Several preparation methods have been reported either by resolution of racemic mixtures or by enantioselective synthesis. Enzymes such as hydantoinases, penicillin acylases, and lipases are extremely useful catalysts for the resolution of racemic mixtures yielding enantiomerically pure L- or D-*tert*-leucine.[2] Enantioselective chemical methods usually include the formation and separation of diastereomers.[3] A direct enzyme-catalyzed route is applied for the production of L-*tert*-leucine at an industrial scale. Degussa developed this synthesis route[4] based on the NADH-dependent reductive amination of the corresponding keto acid catalyzed by leucine dehydrogenase.[5] To reach a complete conversion and to transfer this reaction to a larger scale, it was coupled with an efficient NADH-regeneration step, which is given by the formate/formate dehydrogenase system.[6]

D-*tert*-Leucine, however, cannot be synthesized through such a reductive amination step as a corresponding D-specific leucine dehydrogenase is not known. One route developed by Degussa utilizing D-hydantoinase has the limitation that D-*tert*-leucine must be released from the formed *N*-carbamoyl-D-*tert*-leucine by a treatment with $HNO_2$. Quite recently, a chemoenzymatic approach to synthesize the D-compound has been described by Laumen et al.[7] utilizing the enantioselective cleavage of *N*-acetyl-*tert*-leucine esters by a *Bacillus licheniformis* protease. This route requires a preceding two-step derivatization of the racemic substrate. The synthesis of the preferred substrate, racemic *N*-acetyl-*tert*-leucine chloroethyl ester, and the enzymatic resolution were scaled up to the multi-kilogram level.

We describe herein an enzyme-catalyzed synthesis for D-*tert*-leucine starting with racemic DL-*tert*-leucine. Leucine dehydrogenase, which is the appropriate catalyst for the

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10.1021/ol035314g CCC: $25.00 
**Published on Web 09/04/2003**

reductive amination of the keto acid, was used for the reverse reaction to oxidize L-*tert*-leucine. In general, unnatural derivatives of enzyme substrates are accepted with reduced activity. Table 1 summarizes the kinetic constants of leucine

**Table 1.** Kinetic Data of Leucine Dehydrogenase from *Bacillus cereus* for L-Leucine and L-*tert*-Leucine (0.2 mM $NAD^+$, pH 8.0)

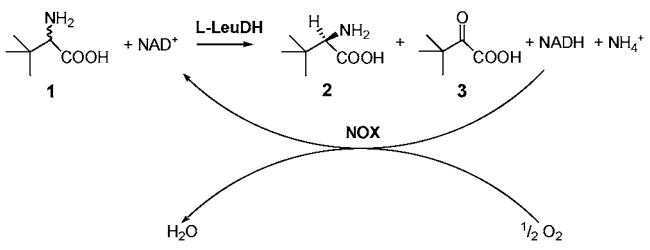
| amino acid | $K_m$ (mM) | $v_{max}$ (U $mg^{-1}$) |
|---|---|---|
| L-leucine | 0.88 | 24 |
| L-*tert*-leucine | 9.0 | 0.33 |

dehydrogenase for L-leucine and L-*tert*-leucine and confirms that the latter is oxidized with decreased activity and affinity. The D-isomer of *tert*-leucine inhibits the oxidation of the L-compound slightly; the kinetic data point to a noncompetitive inhibition type ($K_i$ = 89.4 mM).

The oxidation reaction of $NAD^+$-dependent dehydrogenases is severely hampered by their equilibrium, which favors the formation of the reduced products.[8] Therefore, the application of $NAD^+$-dependent enzymes for the oxidative direction is only possible if a highly efficient method for $NAD^+$ regeneration is available. Several methods for the regeneration of $NAD^+$ were demonstrated to be useful, but none was applicable in preparative scale. For example, the reaction rates by coupling with electron-transfer dyes such as methylene blue, methyl viologen, phenazine methosulfate, or FMN are too slow.[9] Other systems such as 2-oxoglutarate/glutamate dehydrogenase, pyruvate/lactate dehydrogenase, oxalacetate/malate dehydrogenase, or acetaldehyde/alcohol dehydrogenase[10] require stoichiometric quantities of unstable keto compounds, and the equilibrium of these reactions prevents a complete oxidation.

Quite recently, we identified, isolated, and characterized a NADH oxidase from *Lactobacillus brevis*,[11] which seems to be well suited for the regeneration of $NAD^+$. The reduced byproduct of this enzyme is $H_2O$. The reaction may be considered as irreversible, which means that it is the driving force in a coupled approach according to Scheme 1 independent of the equilibrium of the primary reaction catalyzed by dehydrogenases.

**Scheme 1.** Leucine Dehydrogenase Catalyzed Preparation of D-*tert*-Leucine **2** by Selective Oxidation of Racemic *tert*-Leucine **1**[a]




[a] $NAD^+$ is regenerated simultaneously by NADH oxidase.

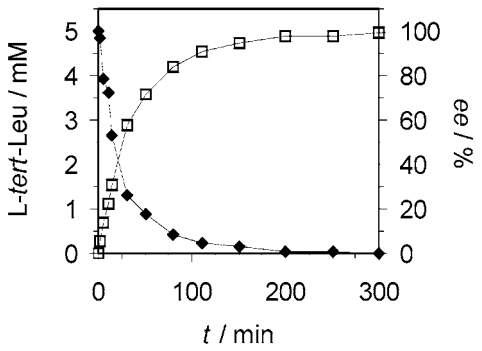



**Figure 1.** Time course of the enzyme-catalyzed oxidation of L-*tert*-leucine out of the racemic mixture (◆) and the ee value for D-*tert*-leucine (□).

NADH oxidase from *L. brevis* is efficiently overproduced in a recombinant *Escherichia coli* strain. Figure 1 shows the time course of the oxidation of L-*tert*-leucine out of the racemic mixture. Starting with 0.25 U $mLl^{-1}$ of leucine dehydrogenase[12] and an excess of NADH oxidase (4.2 U $mL^{-1}$), the reaction was completed after 300 min. The concentration of D-*tert*-leucine remained constant during this process. On the basis of the degradation of L-*tert*-leucine within the first 30 min, a reaction rate of 0.12 $\mu$mol per min was observed, which corresponds to an activity of 0.12 U $mL^{-1}$.

In summary, an oxidative resolution of the racemic mixture of DL-*tert*-leucine with leucine dehydrogenase was applied to obtain D-*tert*-leucine with an excellent enantiomeric excess of >99%. Despite the unfavorable equilibrium of the dehydrogenase reaction, the L-amino acid was oxidized completely due to the coupled irreversible $NAD^+$-regenerating step by NADH oxidase. This kind of $NAD^+$ regeneration will allow the use of other $NAD^+$-dependent enzymes to prepare enantiomerically pure compounds by oxidation.

**Acknowledgment.** This work has been supported by the Degussa AG. We thank Ms. Annika Sterlein for skillful technical support.

**Supporting Information Available:** Experimental procedures for the preparation of NADH oxidase, the reaction conditions for the enzyme-catalyzed oxidation, and the product analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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