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Technical Notes

A "Second-Generation Process" for the Synthesis of L-Neopentylglycine: Asymmetric Reductive Amination Using a Recombinant Whole Cell Catalyst†

Harald Gröger,*,‡ Oliver May,*,‡ Helge Werner,‡ Anne Menzel,§ and Josef Altenbuchner*,§

Degussa AG, Service Center Biocatalysis, P.O. Box 1345, 63403 Hanau, Germany, and Universität Stuttgart, Institut für Industrielle Genetik, Allmandring 31, 70569 Stuttgart, Germany

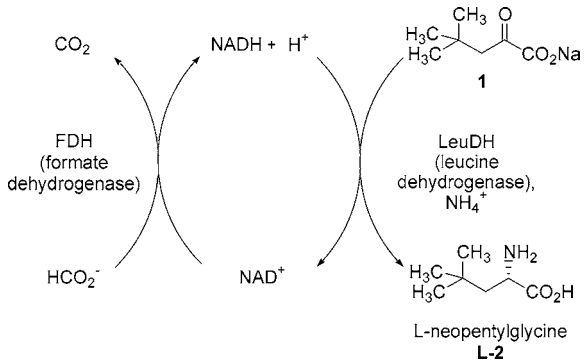
Abstract:

A "second-generation process" for the enantioselective synthesis of L-neopentylglycine has been developed, realizing the first whole cell-catalyzed approach to this pharmaceutically interesting, bulky amino acid. The synthesis of this nonnatural amino acid proceeds highly efficiently via a reductive amination of the corresponding α-keto acid in the presence of a recombinant whole cell catalyst, bearing a leucine dehydrogenase and formate dehydrogenase. The desired L-neopentylglycine was obtained with >95% conversion and a high enantioselectivity of >99% ee at substrate concentrations of up to 88 g/L.

Introduction

The preparation of enantiomerically pure nonnatural L-amino acids is of wide importance due to pharmaceutical applications thereof.[1] Among them, a particular interest is directed towards bulky L-amino acids. For example, L-*tert*-leucine is produced on tons scale.[2] Another bulky L-amino acid of pharmaceutical interest is L-neopentylglycine, L-**2** (L-2-amino-4,4-dimethyl-pentanoic acid).[3] With respect to its synthetic preparation, however, synthetic routes are still rare.[4,5] Among them, the reductive amination of the corresponding α-keto acid or the sodium salt thereof, **1**, in the presence of two isolated enzymes, namely a leucine dehydrogenase and a formate dehydrogenase, is regarded to be one of the most efficient routes.[5c] The reaction concept is shown in Scheme 1. The reductive amination step under consumption of the cofactor NADH is catalyzed by a leucine dehydrogenase, forming the desired L-neopentylglycine, L-**2**, as well as the oxidized cofactor form, NAD^+. This oxidized cofactor form, NAD^+, is subsequently reduced under formation of NADH by means of a formate dehydrogenase-catalyzed oxidation of formate. This continuous recycling of the cofactor NADH, required as the reducing agent, enables its use in catalytic amounts only, which is of importance due to its high price. This enzymatic reaction has already proven its technical feasibility on large scale.

Scheme 1. **General reductive amination concept**



Despite high conversion and enantioselectivity, however, the need for isolated, costly enzymes as well as the requirement for significant amounts of the expensive cofactor NAD^+, albeit needed in catalytic amounts only, are disadvantageous. To improve the efficiency and process economy of the synthetic approach to L-neopentylglycine, L-**2**, we envisaged that the extension of this process towards a whole cell-catalyzed reductive amination represents a potential

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* Authors for correspondence: H.G.: Telephone: +49 (0)6181 596401. Fax: +49 (0)6181 592961. E-mail: harald.groeger@degussa.com. O.M.: Telephone: +49 (0)6181 593035. Fax: +49 (0)6181 592961. E-mail: oliver.may@degussa.com. J.A.: Telephone: +49 (0)711 6857591. Fax: +49 (0)711 6856973. E-mail: Josef.Altenbuchner@po.uni-stuttgart.de.

‡ Degussa AG.

§ Universität Stuttgart.

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solution for process improvement. This concept is based on a direct use of a recombinant whole cell catalyst, containing both desired enzymes in overexpressed form, and the use of only the intracellular amount of cofactor. Accordingly, access to the biocatalyst is simplified and economically more attractive since costly enzyme isolation steps are avoided. In addition, there is no need for the addition of external amount of cofactor (due to the use of cell internal cofactor only). We have already developed such types of "designer bugs"—whole cells as efficient biocatalysts—and preliminarily demonstrated their potential very recently for the synthesis of L-*tert*-leucine.[6,7] In this technical note, we report the successful extension of the "designer bugs"—whole cell concept towards a simple, efficient, environmentally friendly, and economically attractive "second-generation process" for the synthesis of the commercial L-amino acid L-neopentylglycine, L-**2**.

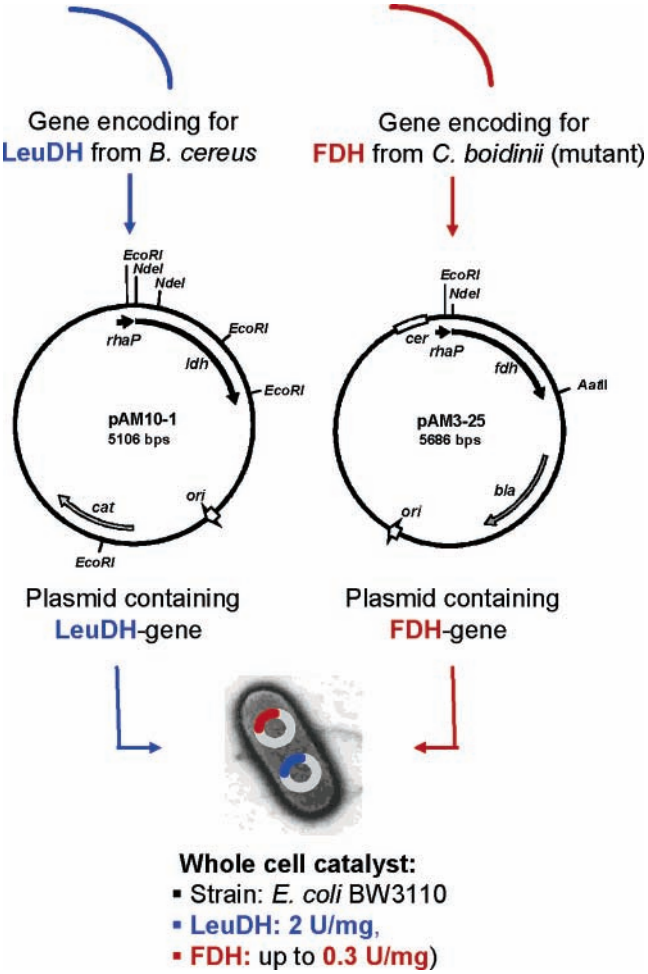
Results and Discussion

To start with an overview about the properties of the developed "designer bugs"—whole cell biocatalyst,[6] which has been now used for the asymmetric reductive amination of **1**, good coexpression of both desired enzymes in the *Escherichia coli* host organism and its feasibility for high cell density fermentation are key features. In more detail, the leucine dehydrogenase from *Bacillus cereus* and a mutant of a formate dehydrogenase from *Candida boidinii* are produced in an *E. coli* organism BW3110 (for the concept of this whole cell biocatalyst, see Scheme 2).[6] A specific challenge was the successful coexpression of both genes, encoding for the LeuDH and FDH, leading to comparable activities for both enzymes due to the large difference in the specific activities of both enzymes by factor >50 (~400 U/mg for the LeuDH, 6 U/mg for the FDH).

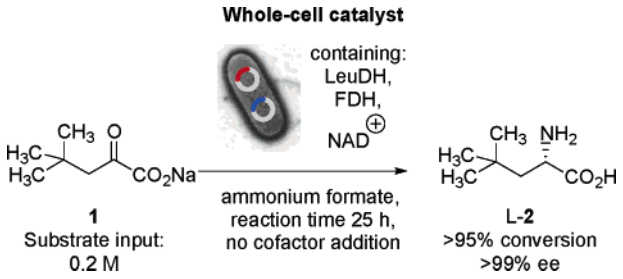
The coexpression was realized by location of the two related genes on two plasmids with different copy numbers, producing LeuDH and FDH at different levels. Due to the low specific activity of the FDH, the related gene was inserted in the plasmid with the higher copy number. For the expression of the gene for the LeuDH, a medium copy number plasmid was used. As a consequence we obtained a "designer bug"—whole cell catalyst, bearing the LeuDH and FDH, with activities in a similar range (Scheme 2).[6]

For the use of this biocatalyst in the preparative reductive amination of 4,4-dimethyl-2-oxopentanoic acid sodium salt, **1**, at first the substrate concentration was set at 0.2 M, which is close to the solubility limit of the resulting L-neopentylglycine, L-**2**. As a cosubstrate for cofactor recycling (through transformation of formate into carbon dioxide and reduction of the oxidized cofactor NAD^+ into the required reduced form NADH), 3 equiv of ammonium formate was used, which is in accordance with previous reductive amination reactions.[5c,6] In addition, the ammonium moiety functions as nitrogen donor in the reductive amination reaction

Scheme 2. **Concept of the "designer bug" whole cell biocatalyst (according to ref 6)**



Scheme 3. **Whole cell-based synthesis of L-neopentylglycine**



catalyzed by the leucine dehydrogenase. When carrying out the reaction in the presence of a whole cell biocatalyst loading of 66.4 g/L (wet biomass), a fast reaction was observed leading to the formation of the desired L-amino acid L-neopentylglycine, L-**2**, with a conversion of >95% and an enantioselectivity of >99% ee after a reaction time of 25 h (Scheme 3).

In a subsequent step, we focused on an increase of the substrate concentration up to 0.53 M, thus exceeding the solubility limit of the formed L-amino acid. Such a high substrate concentration is advantageous with respect to volumetric productivity and process economy. When carrying out this process at an overall substrate concentration of 0.53 M, corresponding to a substrate input of 88 g/L, we were pleased to find that the resulting whole cell-catalyzed

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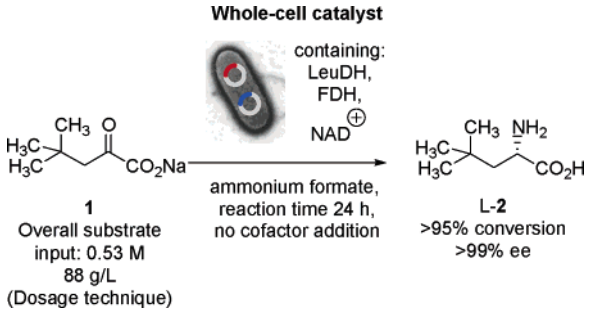
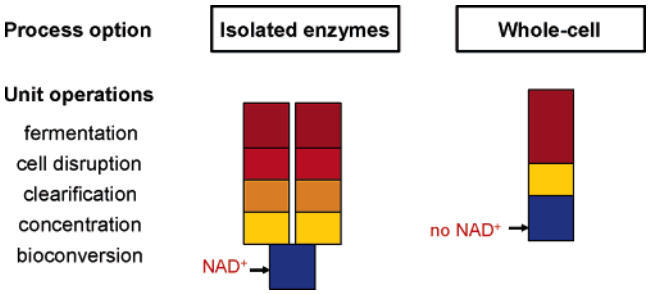
(7) For a previous work on LeuDH and FDH-containing whole cell catalysts reported by Esaki et al., see: Galkin, A.; Kulakova, L.; Yohimura, T.; Soda, K.; Esaki, N. *Appl. Environm. Microbiol.* **1997**, *63*, 4651−4656.

Scheme 4. **Improved process at high substrate concentration**



Scheme 5. **Comparison of the overall economy of both concepts: isolated enzymes vs whole cells**



reductive amination of **1** under formation of L-neopentylglycine, L-**2**, proceeds with a conversion of >95%, and with an enantioselectivity of >99% ee (Scheme 4).

The reaction time for this whole cell-catalyzed asymmetric process, which also does not need addition of external amount of cofactor, is 24 h. Subsequent (nonoptimized) downstream processing comprised solubilization of the precipitated L-amino acid in basic media, followed by separation of the biomass by centrifugation and ultrafiltration, and workup of the (neutralized) filtrate via ion-exchange chromatography (the latter step has been carried out in analogy to the ion-exchange chromatographic procedure described in ref 5c, leading to a yield of 83%). During the initial basification step, the biocatalyst is deactivated, which is acceptable in case of recombinant whole cells due to their economically attractive production costs. This economical impact of using recombinant whole cells rather than isolated enzymes is graphically underlined in the comparison of the process economy of both concepts—isolated enzymes versus whole cells—in Scheme 5.

In the concept based on the use of enzymes two separate fermentations are needed, followed by an extensive downstream processing consisting of cell disruption, clarification, and concentration for both fermentation runs. For comparison, the whole cell approach only requires one fermentation run (typically done at a somewhat larger scale) and simple cell separation as a concentration step. Thus, the whole cell approach offers significant advantages not only with respect to the lack of the need for "external" cofactor addition in the biotransformation but also with respect to a cost-attractive biocatalyst access.

Conclusions

In conclusion, the first whole cell-catalyzed reductive amination for the synthesis of L-neopentylglycine, L-**2**, has been successfully developed as a "second-generation process" for this amino acid. This process does not require the addition of "external" amount of cofactor, and gives the desired pharmaceutically important product L-neopentylglycine, L-**2**, with high conversion and an enantioselectivity of >99% ee at substrate concentrations of up to 88 g/L. The extension of this whole cell-based reductive amination technology towards the synthesis of other L-amino acids, as well as further process development, is currently in progress.

Experimental Section

Preparation of the Biocatalyst (biomass *E. coli* BW3110/pAM 3.25/pAM10.1). The preparation of the biocatalyst, comprising the construction of the recombinant strain (*E. coli* BW3110/pAM 3.25/pAM10.1), containing the leucine dehydrogenase from *Bacillus cereus* and a mutant of a formate dehydrogenase from *Candida boidinii*, as well as the preparation of biomass for the biotransformation, is carried out according to the protocols described earlier in ref 6.

Procedure for Whole Cell Reductive Amination at 0.2 M (for the reaction according to Scheme 3). To a mixture of 3.78 g of ammonium formate (3 equiv related to **1**) and 6.64 g of the biocatalyst (biomass *E.* coli BW3110/pAM 3.25/pAM10.1) in a 250 mL-flask was added 100 mL of an aqueous solution of 4,4-dimethylpentanoic acid **1** (0.2 M, pH 7.15). After a reaction temperature of 30 °C was reached, the pH was adjusted to pH 7.0 with a 32% aqueous solution of ammonia. After a reaction time of 25 h a conversion of >98% and an enantioselectivity of >99% ee was obtained.

Procedure for Whole Cell Reductive Amination at 0.53 M (for the reaction according to Scheme 4). To 50 mL of a mixture of 15.0 g of ammonium formate (3 equiv related to **1**) and 26.6 g of the biocatalyst (biomass *E. coli* BW3110/pAM 3.25/pAM10.1) in water in a 250-mL flask was added 12.5 mL of an aqueous solution of 4,4-dimethyl-2-oxopentanoic acid sodium salt **1** (0.8 M, pH 7.15). After a reaction temperature of 30 °C was reached, the pH was adjusted to pH 7.0 with a 32% aqueous solution of ammonia. Further portions of an aqueous solution of 4,4-dimethyl-2-oxopentanoic acid sodium salt **1** (0.8 M, pH 7.15) of 12.5 mL each were added after 1, 2, 3.5, 5, 6.5, 8, and 9.5 h. The final total volume was ~150 mL, and the overall substrate concentration (substrate input) was ~0.53 M, corresponding to ~88 g/L. After a reaction time of 24 h a conversion of >95% and an enantioselectivity of >99% ee was obtained. The subsequent downstream processing of the reaction mixture is carried out by an inital solubilization of the precipitated L-neopentylglycine in basic media at pH 12 (by means of addition of a 5% aqueous solution of ammonia), followed by separation of the biomass by centrifugation and ultrafiltration (molecular weight cut off: 10.000 Da). After neutralization of the resulting filtrate (pH 6.5) isolation of the product L-neopentylglycine was carried out via ion-exchange chromatography. This chromatographic step was done in analogy to the ion-exchange chromatographic procedure described in ref 5c using the acidic ion exchanger, Amberlite 252C, and conducting the final elution of the

L-neopentylglycine with a 5% aqueous ammonia solution and gave a yield of 83%.

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