

# Preparation of an Amino Acid Intermediate for the Dipeptidyl Peptidase IV Inhibitor, Saxagliptin, using a Modified Phenylalanine Dehydrogenase

Ronald L. Hanson,<sup>a,\*</sup> Steven L. Goldberg,<sup>a</sup> David B. Brzozowski,<sup>a</sup> Thomas P. Tully,<sup>a</sup> Dana Cazzulino,<sup>a</sup> William L. Parker,<sup>a</sup> Olav K. Lyngberg,<sup>a</sup> Truc C. Vu,<sup>a</sup> Michael K. Wong,<sup>a</sup> and Ramesh N. Patel<sup>a</sup>

<sup>a</sup> Process Research and Development, Bristol-Myers Squibb, One Squibb Drive, New Brunswick, NJ 08903, USA  
Fax: (+1)-732-227-3994; e-mail: ronald.hanson@bms.com

Received: January 8, 2007

**Abstract:** The non-proteinogenic amino acid 2-(3-hydroxy-1-adamantyl)-(2*S*)-aminoethanoic acid [**2**, (*S*)-3-hydroxyadamantylglycine], is a key intermediate required for the synthesis of Saxagliptin, a dipeptidyl peptidase IV inhibitor under development for treatment of type 2 diabetes mellitus. Keto acid 2-(3-hydroxy-1-adamantyl)-2-oxoethanoic acid (**1**) was converted to (*S*)-3-hydroxyadamantylglycine by reductive amination using a phenylalanine dehydrogenase from *Thermoactinomyces intermedius* expressed in a modified form in *Pichia pastoris* or *Escherichia coli*. NAD (nicotinamide adenine dinucleotide) produced during the reaction was recycled to NADH (reduced form of nicotinamide adenine dinucleotide) using formate dehydrogenase. *Pichia pastoris* produces an endogenous formate dehydrogenase when grown on methanol, and the corresponding gene was cloned

and expressed in *E. coli*. The modified phenylalanine dehydrogenase contains two amino acid changes at the C-terminus and a 12-amino acid extension of the C-terminus. The modified enzyme is more effective with keto acid **1** than the wild-type enzyme, but less effective with the natural substrate, phenylpyruvate. Production of multi-kg batches was originally carried out with extracts of *Pichia pastoris* expressing the modified phenylalanine dehydrogenase from *Thermoactinomyces intermedius* and endogenous formate dehydrogenase, and further scaled up using a preparation of the two enzymes expressed in *E. coli*.

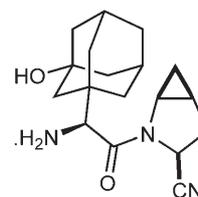
**Keywords:** amino acids; enzyme catalysis; phenylalanine dehydrogenase; reductive amination; Saxagliptin

## Introduction

Glucagon-like peptide-1 (GLP-1) analogues and dipeptidyl peptidase IV (DPP-IV) inhibitors are two promising new approaches currently being explored for treatment of type 2 diabetes.<sup>[1–3]</sup> GLP-1, a peptide secreted by the gut in response to feeding, has the beneficial effects of increasing glucose-stimulated insulin secretion, decreasing glucagon secretion, delaying gastric emptying, and increasing the  $\beta$ -cell mass of pancreatic islets.<sup>[1–2]</sup> GLP-1 (7–36) amide is rapidly inactivated by conversion to GLP-1 (9–36) amide by DPP-IV.<sup>[1–3]</sup> To alleviate the inactivation of GLP-1, more stable analogues of GLP-1 as well as inhibitors of DPP-IV are approaches to provide improved control of blood glucose for diabetics.

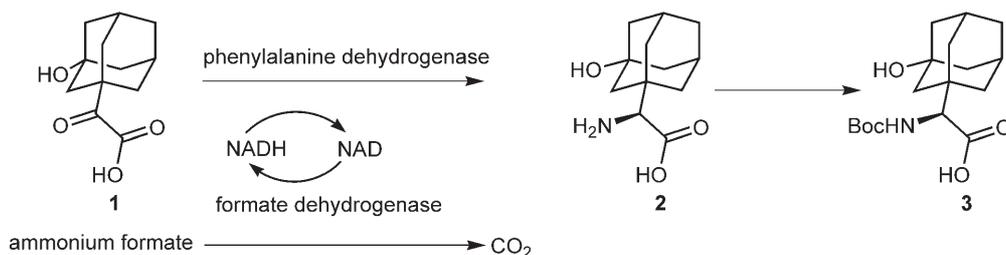
Saxagliptin<sup>[4]</sup> (Figure 1), a DPP-IV inhibitor under development by Bristol-Myers Squibb, requires (*S*)-*N*-BOC-3-hydroxyadamantylglycine (**3**) (Scheme 1) as

an intermediate. This compound was originally prepared using an asymmetric Strecker amino acid synthesis.<sup>[4]</sup> We have previously prepared several unnatural amino acids<sup>[5–7]</sup> as intermediates for synthetic routes by reductive amination of the corresponding keto acids using L-amino acid dehydrogenases and have investigated this approach to afford an improved



Saxagliptin (BMS-477118)

**Figure 1.** Structure of Saxagliptin.



**Scheme 1.** Enzymatic reaction used for preparation of (*S*)-3-hydroxyadamantylglycine.

synthesis of intermediate **3**. This report describes the preparation of (*S*)-3-hydroxyadamantylglycine (**2**) using a modified form of a recombinant phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* or *Escherichia coli*. The amino acid **2** can be directly protected as its BOC derivative without isolation of **2** to afford intermediate **3**. This biocatalysis procedure and subsequent BOC protection has been scaled up to multi-kg scale to support the development of Saxagliptin.

## Results and Discussion

### Screening of Amino Acid Dehydrogenases and Transaminases

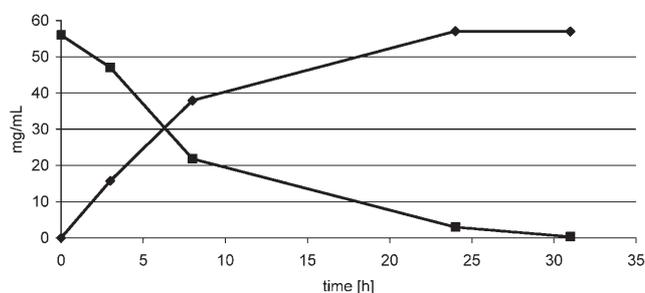
Of six amino acid dehydrogenases screened for reductive amination of **1** to **2**, only two phenylalanine dehydrogenases were found to effect the conversion. Phenylalanine dehydrogenase (PDH) from *Sporosarcina* species gave only a 3% yield and heat-dried cells<sup>[7]</sup> of *Pichia pastoris* containing phenylalanine dehydrogenase cloned from *Thermoactinomyces intermedius* gave a 12% yield. Phenylalanine dehydrogenase from *Rhodococcus*, leucine dehydrogenase from *Bacillus sphaericus*, glutamate dehydrogenase from beef liver, and alanine dehydrogenase from *Bacillus subtilis* were not active. Four transaminases were also screened, and only branched chain transaminase catalyzed the conversion of **1** to **2** (56-mg scale) using glutamate as amino donor with a 49% yield.

### Preparation of **2** with Phenylalanine Dehydrogenase from *Thermoactinomyces intermedius* Expressed in *Pichia pastoris*

Using fresh cells, the reaction was evaluated under various conditions with phenylalanine dehydrogenase from *Thermoactinomyces intermedius* expressed in *Pichia pastoris* SMD 1168 (pPDH9K/10)[SC16176].<sup>[7]</sup> The enzymatic reductive amination reaction requires ammonia and NADH (Scheme 1). NAD produced during the reaction was recycled to NADH by the ox-

idation of formate to CO<sub>2</sub> using formate dehydrogenase (FDH) which is produced by *Pichia pastoris* during growth on methanol. Wet cells, heat-dried cells and cell extracts were compared. *Pichia pastoris* extract was more effective than wet cells or heat-dried cells. Wet cells gave 64% yield, heat-dried cells gave 80% yield, whereas an extract gave close to 100% yield. The best conditions using *Pichia* extract were 0.25M keto acid, 0.5M ammonium formate, 1 mM NAD, 1 mM dithiothreitol, pH 8, and 12.5% w/v *Pichia* extract, at 40°C. The total turnover number for the NAD cofactor was 237 with this keto acid input and NAD concentration. With these conditions, a total of 1.6 kg **2** was prepared in lab batches. Yields before isolation were close to 100% with no (*R*)-enantiomer detected. The progress curves for a 30-g reaction are shown in Figure 2.

The product from early batches was isolated as the free amino acid **2**. The procedure for isolating the amino acid consisted of boiling the enzymatic reaction mixture to drive off ammonia, adjusting to pH 3 with formic acid, filtration to remove precipitated protein, adsorption of the amino acid on Dowex 50 (H<sup>+</sup>) resin, elution with 1.5M ammonia and concentration of the rich effluent to give **2** as a crystalline solid. The last run using this isolation procedure (787 g keto acid input) gave 804 g of **2** with a purity of 94.3 weight% and a yield of 96.0% from **1**. All of the batches of **2** isolated by this procedure performed well in subsequent reactions for the synthesis of Saxagliptin. Several additional batches were conducted to prepare the *N*-BOC amino acid **3** without the resin purification



**Figure 2.** Time course of reaction for conversion of 30 g keto acid **1** to amino acid **2** by *Pichia pastoris* extract. ■, keto acid **1**; ◆, amino acid **2**.

step. To facilitate the BOC protection, protein was first removed by microfiltration and ultrafiltration, then ammonia was removed by boiling at pH 9.6. The input keto acid **1** could also be used without isolation. The input keto acid **1** for one of the batches was a solution containing about 1M NaCl and 0.25M NaHCO<sub>3</sub>, prepared without isolation by ethyl acetate extraction. The reaction was satisfactory, but proceeded a little slower than that with the purified keto acid **1**. Pilot plant batches of 4, 7, 10 and 37 kg gave close to 100% conversion and were directly converted to **3** before isolation.

### Modified PDH Found in *Pichia pastoris*

Production of PDH and FDH in *Pichia pastoris* requires growth of the cells on methanol to induce the enzyme activities.<sup>[7]</sup> The PDH enzyme expressed in *Escherichia coli*, inducible by isopropylthio- $\beta$ -D-galactopyranoside (IPTG), was also evaluated. *Escherichia coli* BL21(DE3) (pPDH155K) SC16144<sup>[7]</sup> extract containing PDH cloned from *Thermoactinomyces intermedius* (but not FDH) combined with commercially available FDH from *Candida boidinii* was less effective (45% conversion) than *Pichia* extract despite having higher PDH activity. *E. coli* heat-dried cells or wet cells gave much less conversion than extracts. Another strain of *E. coli*, SC16487, was constructed to express *Thermoactinomyces intermedius* PDH and *Pichia pastoris* FDH, both inducible by IPTG, to obtain expression levels of both enzymes higher than in *Pichia pastoris* and avoid the use of methanol in the pilot plant. Despite high PDH activity with phenylpyruvate, the PDH enzyme expressed in *E. coli* gave a low yield for conversion of keto acid **1** to amino acid **2** and a low activity when measured in a spectrophotometric assay with keto acid **1** in place of phenylpyruvate (Table 1). This surprising result was investigated further.

Use tests of *E. coli* SC16487 PDH/FDH extracts and another *Pichia* construct with constitutive *Thermoactinomyces intermedius* PDH and formate-induced FDH gave only 53% and 8% yields, respectively. The *E. coli* PDH/FDH enzyme activities were higher than the methanol-induced *Pichia* PDH/FDH SC16176 and the formate-induced *Pichia* had PDH/FDH activities equal to the methanol-induced *Pichia*. Dialysis of the extracts to remove possible inhibitors did not improve performance in the use test. After 32 h of use test reaction most of the PDH activity remained, although FDH was decreased. Addition of more FDH, NAD or both after 32 h had little effect on yield. In spectrophotometric enzyme assays, the methanol-grown *Pichia* extract had measurable activity with **1**, but the formate-grown *Pichia* extract and the *E. coli* extract did not. However, the latter two extracts had higher activity with phenylpyruvate (natural substrate for PDH) than the methanol-grown *Pichia* extract. An extract of *Pichia* cells removed from a fermentor just before methanol induction was inactive for reductive amination of **1**, suggesting that the methanol-induced PDH was responsible for the reductive amination reaction, not an endogenous *Pichia* enzyme.

Phenylalanine dehydrogenase from *Thermoactinomyces intermedius* was first cloned and sequenced by Takeda et al.,<sup>[8]</sup> and this sequence information was used for PCR cloning and expression of the gene in *Pichia* and *E. coli*.<sup>[7]</sup> The cloned *pdh* gene was re-isolated from *Pichia* SC16176, and the DNA sequence was compared with the original published sequence of the *pdh* gene. The sequence of the 3'-end of the *pdh* gene was found to be changed in *Pichia* SC16176 from the original *Thermoactinomyces pdh* gene. The modified PDH contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus (Figure 3). This fortuitous alteration in the gene was probably due to lack of a stop codon in the primer used to amplify the *pdh* gene for cloning into the expression vector. The amino acid changes

**Table 1.** Effect of PDHmod on conversion of keto acid **1** to amino acid **2**.

| Strain                         | Enzymes    | Substrate          | U/g wet cells <sup>[a]</sup> | Yield [%] <b>2</b> <sup>[b]</sup> |
|--------------------------------|------------|--------------------|------------------------------|-----------------------------------|
| <i>Pichia pastoris</i> SC16176 | PDHmod/FDH | formate            | 36.4                         | 95                                |
|                                |            | phenylpyruvate     | 21.5                         |                                   |
|                                |            | keto acid <b>1</b> | 5.6                          |                                   |
| <i>E. coli</i> SC16487         | PDH/FDH    | formate            | 85.6                         | 53                                |
|                                |            | phenylpyruvate     | 869.1                        |                                   |
|                                |            | keto acid <b>1</b> | 0.9                          |                                   |
| <i>E. coli</i> SC16496         | PDHmod/FDH | formate            | 132.4                        | 100                               |
|                                |            | phenylpyruvate     | 36.9                         |                                   |
|                                |            | keto acid <b>1</b> | 11.6                         |                                   |

<sup>[a]</sup> Activity measured in 25% w/v extracts as described in Experimental Section.

<sup>[b]</sup> Percent conversion of 50 mg mL<sup>-1</sup> keto acid **1** to amino acid **2** by 12.5% w/v extract.

3'-end of native PDH gene and corresponding amino acids:

AAC AGC GCA AGG AGG TAA

Asn Ser Ala Arg Arg Stop

3'-end of PDHmod gene and corresponding amino acids

(changed or new amino acids in bold):

AAC AGC GCG **GAG GGG TAC CTC GAG CCG CGG**

Asn Ser Ala **Glu Gly Tyr Leu Glu Pro Arg**

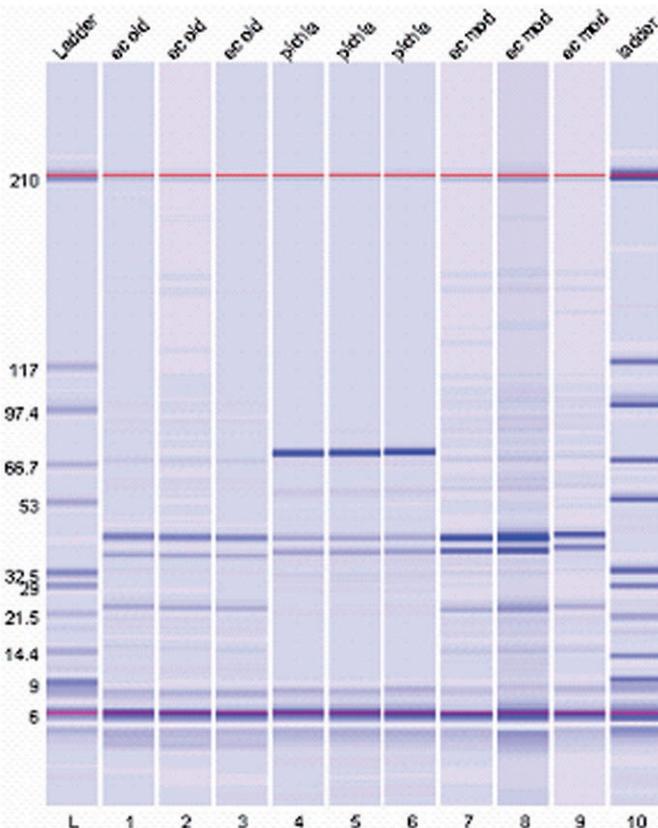
**CGG CCG CGA ATT AAT TCG CCT TAG**

**Arg Pro Arg Ile Asn Ser Pro Stop**

**Figure 3.** Comparison of 3'-ends of native *pdh* gene with modified gene reisolated from *Pichia pastoris* SC16176.

and C-terminal extension should result in a 1353 dalton increase in the subunit molecular weight vs. unmodified PDH. The modified PDH protein (PDHmod) is apparently better able to accept keto acid **1** as substrate than is the unmodified PDH expressed in *E. coli* SC16487. After this finding, the PDHmod gene was subsequently cloned into *E. coli*

along with the FDH gene from *Pichia pastoris* to give strain SC16496. Comparison of the protein subunits in extracts of *E. coli* SC16487, *Pichia pastoris* SC16176, and *E. coli* SC 16496 as shown in Figure 4 is consistent with a slightly increased molecular weight for PDHmod. The PDH subunit molecular weights in *E. coli* SC16487, *Pichia* SC16176, and *E. coli* SC16496 were 37.9 kD, 38.6 kD and 39.3 kD, respectively (mean of 3 lanes for each extract). The FDH subunit in all strains had a molecular weight of about 42.9 kD. The higher level of expression of PDH and FDH in the new *E. coli* strain (SC16496) compared to the old *E. coli* strain (SC16487) and to *Pichia* SC16176 is also apparent in Figure 4. PDH from *E. coli* SC16487 and PDHmod from *Pichia* SC16176 extracts were partially purified by gel filtration chromatography on an FPLC Superdex 200 column to compare molecular weights of the native enzymes. The activity peak for each enzyme was found in the same fraction number indicating a similar native molecular weight.



**Figure 4.** Comparison of PDH/FDH in *E. coli* SC16487 (ec old) with PDHmod/FDH in *Pichia pastoris* SC16176 and in *E. coli* SC 16496 (ec mod). 25% w/v extracts were diluted 10-fold with water and analyzed with an Agilent 2100 Bio-analyzer using a Protein 200 Plus Labchip.

#### Preparation of **2** with the Modified Phenylalanine Dehydrogenase from *Thermoactinomyces intermedius* Expressed in *Escherichia coli*

Reductive amination was conducted using cell extracts from *E. coli* strain SC16496 expressing PDHmod and cloned FDH from *Pichia pastoris*. Cells from a 15-L tank had 133 U/g FDH, 65 U/g PDH (phenylpyruvate assay), and 12.7 U/g PDH (assayed with keto acid **1**). The extract was used for conversion of 30 g **1** to **2** in close to 100% yield, and this material, after filtration for protein removal, was converted to **3** by BOC protection. Further experiments showed that the *E. coli* extract could be used at 2.5% w/v concentration instead of the 12.5% concentration used for batches with *Pichia pastoris* extract. The pH optimum for the reductive amination of **1** by PDHmod was 8.4–9.0. The apparent  $K_m$  for keto acid **1** with the *E. coli* extract at pH 8.75 using assay C

from the Experimental Section with varied keto acid was  $23 \pm 5$  mM (std. error). Because of the lower pH optimum (6.5–7.5<sup>[9]</sup> or 7.5<sup>[10]</sup> with more than 80% of maximal activity at pH 8<sup>[9–10]</sup>) for FDH, reactions were carried out at pH 8.

After some further modifications to the procedure, PDHmod and FDH expressed in *E. coli* have now been used to prepare several hundred kg of BOC-protected amino acid **3** to support the development of Saxagliptin.

### Heat Stability of PDHmod

Phenylalanine dehydrogenase from the thermophilic actinomycete *Thermoactinomyces intermedius* is a thermostable enzyme.<sup>[8]</sup> To compare the heat stability of PDHmod with PDH, extracts of *E. coli* SC16496 (FDH/PDHmod) and SC16487 (FDH/PDH) were incubated for 1 h at 0, 40, 50, 60 or 70 °C, then assayed for activity with phenylpyruvate or keto acid **1** as substrate. Samples of the untreated centrifuged extracts were also run on an Agilent 2100 bioanalyzer protein 200 labchip to separate and quantitate the PDH subunit bands. Specific activities are expressed as units per mg PDH or PDHmod protein in the subunit band. Figure 5 shows that the PDHmod enzyme has lower specific activity with phenylpyruvate but higher specific activity with keto acid **1** than the original PDH enzyme. PDHmod also has lower heat stability than PDH, with PDHmod losing all activity after 1 h at

60 °C, whereas PDH retains most of its activity after 1 h at 60 °C.

### Conclusions

*Pichia* extracts containing PDH from *Thermoactinomyces intermedius* and endogenous FDH were used for pilot plant batches (up to a 37-kg scale) giving close to 100% conversion of keto acid **1** to (*S*)-amino acid **2** with no detectable (*R*)-enantiomer. PDH cloned from *Thermoactinomyces intermedius* and expressed in *Pichia pastoris* was a modified version (PDHmod) of the original enzyme. The modified PDH contains two amino acid changes at the C-terminus and a 12 amino acid extension of the C-terminus. PDHmod is more effective with **1** and less effective with phenylpyruvate than the original PDH. PDHmod is less heat-stable than PDH. *E. coli* extracts expressing both PDHmod and *Pichia pastoris* FDH catalyze the conversion effectively and have been used for further scale-up to convert several hundred kg of keto acid **1** to BOC-protected (*S*)-amino acid **3**.

### Experimental Section

#### Preparation of Keto Acid **1**

The preparation of keto acid **1** has been described.<sup>[11]</sup> Detailed procedures for the synthetic route to Saxagliptin are provided in a published patent application.<sup>[12]</sup>

#### Construction and Growth of Recombinant Strain *P. pastoris* SMD1168 (pPDH9K/10) (SC16176, ATCC 74408)

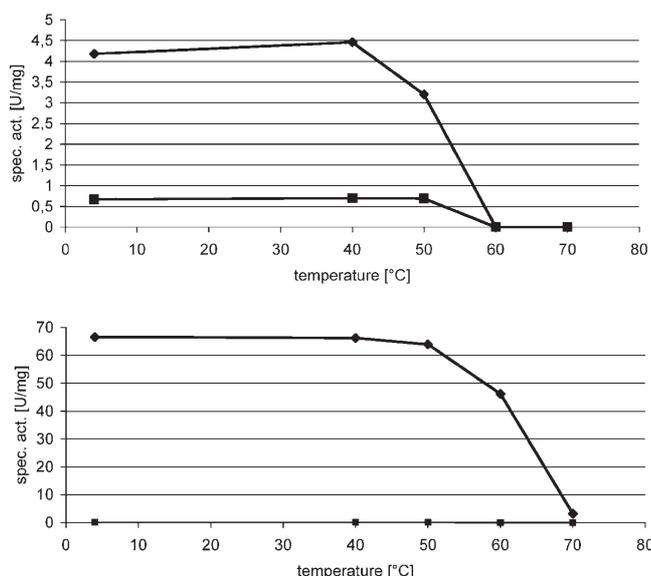
Construction of the final recombinant vector (pPDH9K), transformation into *P. pastoris* strain SMD1168, growth of the cells and expression of the enzyme activities were described previously.<sup>[7]</sup> PDH expression in this system is under the control of the AOX1 promoter with induction of expression by methanol. Expression of the endogenous FDH in this strain is also inducible with methanol.

#### Sequencing of PDH and PDHmod

Plasmids pBMS2000-PDH and pBMS2000-PDHmod were sent to the Bristol-Myers Squibb Core Sequencing Facility, and sequencing was performed using the BigDye terminator kit and a model 377 DNA sequencing unit (Applied Biosystems, Foster City, CA) using a series of external and internal primers.

#### Construction of *E. coli* Strain Containing PDHmod and FDH

A two-step construction of the expression vector pBMS2000-PPFDH-PDHmod was employed. The *P. pastoris*



**Figure 5.** Heat Stability and substrate specificity of PDHmod and PDH expressed in *E. coli*, Upper panel PDHmod, lower panel PDH; ■, specific activity with keto acid **1**; specific activity with ♦ phenylpyruvate.

5'-TCGTCATGAAAATCGTTCTCGTTTTG-3' (5'-end, sense)

*Bsp*HI

5'-TCGGATCCTTATGCGACCTTTTTGTCAT-3' (3'-end, antisense)

*Bam*HI

**Scheme 2.** Oligonucleotides used for amplification of *P. pastoris* FDH gene. Restriction sites used for subsequent ligation to pBMS2000 are indicated.

FDH gene was subcloned into expression vector pBMS2000<sup>[13]</sup> using oligonucleotide primers containing the 5' and 3' end of the *P. pastoris* FDH gene along with compatible restriction endonuclease cleavage sites (see Scheme 2).

High-fidelity PCR amplification of the *P. pastoris* FDH gene was carried out in four 100- $\mu$ L aliquots, each containing 1 X TaqPlus reaction buffer (Stratagene, LaJolla, CA), 0.2 mM each deoxynucleotide triphosphate (dATP, dCTP, dGTP, and dTTP), 0.4 nM each oligonucleotide, 2.5 U Taq-Plus DNA polymerase (Stratagene), and 10 pg plasmid DNA containing the cloned *P. pastoris* FDH gene. The amplification conditions included incubation at 94°C for 4 min, followed by 25 cycles of incubation at 94°C for 1 min; 50°C for 1 min; and 72°C for 1.5 min, using a Perkin-Elmer Model 480 thermocycler with autoextension.

The PCR reaction mixture was extracted with an equal volume of 1:1 phenol:chloroform (GibcoBRL, Gaithersburg, MD), and centrifuged at 13,000 $\times$ g for 5 min. The upper aqueous phase was removed and placed in a new microcentrifuge tube. DNA was precipitated by addition of 0.1 volume 3M sodium acetate and 2 volumes ice-cold ethanol. After centrifugation at 13,000 $\times$ g for 5 min, liquid was aspirated from the tube, and the pellet washed with 0.5 mL ice-cold 70% ethanol. Liquid was aspirated again, and the pellet was allowed to air dry for 30 min at room temperature.

Amplified DNA was digested with 20 units each of *Bsp*HI and *Bam*HI for 3 h at 37°C in a total volume of 50  $\mu$ L. In parallel, the pBMS2000 vector (2  $\mu$ g) was digested with *Bsp*HI and *Bam*HI. The digested samples were electrophoresed on a 1.0% TAE agarose gel for 2 h at 100 V. The bands corresponding to the FDH gene (1100-base pair fragment) and linearized vector (4700-base pair fragment) were separately excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA). The concentrations of the isolated fragments were estimated by electrophoresis against the low molecular weight mass ladder (Invitrogen Corp., Carlsbad, CA) and ligated with 1 U T4 DNA ligase (Invitrogen) in a 5:1 (insert:vector) molar ratio in a total volume of 10  $\mu$ L at 22°C for 2 h. DNA was precipitated by addition of 15  $\mu$ L H<sub>2</sub>O and 250  $\mu$ L 1-butanol, and pelleted at 13,000 $\times$ g in a microcentrifuge for 5 min. Liquid was removed by aspiration, and the DNA was dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 5 min under low heat. The pellet was resuspended in 5  $\mu$ L H<sub>2</sub>O.

The resuspended DNA was transformed by electroporation into 0.04 mL *E. coli* DH10B competent cells (Invitrogen) at 25  $\mu$ F and 250  $\Omega$ . SOC medium was immediately added (0.96 mL; SOC=0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, and 20 mM glucose per liter), and the cells were incubated

in a shaker for 1 h at 37°C and 225 rpm. Colonies with plasmid DNA were selected on LB agar plates containing 50  $\mu$ g/mL kanamycin sulfate (Sigma Chemicals, St. Louis, MO). Plasmids with the desired insert were identified by colony PCR in capillary tubes using the RapidCycler (Idaho Technology, Salt Lake City, UT). Each reaction mixture contained 50 mM Tris-HCl (pH 8.3), 4 mM MgCl<sub>2</sub>, 0.25 mg/mL bovine serum albumin, 2% sucrose 400, 0.1 mM cresol red, 0.4 nM each primer (Scheme 2), and 2.5 U Taq DNA polymerase (Promega Corp., Madison, WI). The reaction mixture was divided into 10- $\mu$ L aliquots, and pipetted into the wells of a round-bottom microtiter plate. A kanamycin-resistant colony was picked using a disposable plastic inoculation needle, swirled into the reaction mixture, and transferred to LB-kanamycin agar. Each reaction mixture aliquot was drawn into a 30  $\mu$ L capillary tube, and the tube was flame-sealed at both ends. Cells were lysed and DNA denatured by incubation at 94°C for 30 seconds; amplification was performed using 30 cycles of incubation at 94°C for 0 seconds; 40°C for 0 seconds, and 72°C for 60 seconds using a RapidCycler Thermocycler (Idaho Technologies, Salt Lake City, UT). Samples were electrophoresed on a 1.0% TAE agarose gel for 2 h at 100 V. Seven samples out of 17 tested showed a strong band at 1100 base pairs. One colony containing this plasmid (referred to herein as pBMS2000-PPFDH) was chosen for the next step in the plasmid construction.

PDHmod refers to a modified *Thermoactinomyces intermedius* phenylalanine dehydrogenase that differs from the published DNA sequence<sup>[8]</sup> by a change of the last two amino acids and an additional 12 amino acids at the carboxyl terminus. This change was introduced into plasmid pPDH9K/10 which was subsequently transformed into *P. pastoris* SMD1168 (deposited as strain ATCC 74408).<sup>[14]</sup>

Oligonucleotide primers containing the 5'- and 3'-ends of the PDHmod gene along with compatible restriction endonuclease cleavage sites were prepared, as shown in Scheme 3.

Reaction conditions for amplification and purification of the PDHmod by PCR were identical to those used for the *P. pastoris* FDH gene except chromosomal DNA prepared from ATCC 74408 was included as template for the reaction. The resulting fragment was digested with 20 units each of *Nde*I and *Sma*I for 1 h at 25°C, followed by 2 h at 37°C, in a total volume of 50  $\mu$ L. In parallel, a version of the pBMS2000 vector with an *Nde*I site at the initiation codon (2  $\mu$ g) was digested with *Nde*I and *Sma*I using identical conditions. The digested samples were separately electrophoresed on a 1.0% TAE agarose gel for 2 h at 100 V. The bands corresponding to the PDHmod gene (1200-base pair fragment) and linearized vector (4700-base pair fragment) were excised from the gel and purified using the QIAquick Gel Extraction Kit (Qiagen). Ligation of the two fragments,

5'-GATGCTCATATGCGCGACGTGTTTGAATGATG-3' (5'-end, sense)  
*NdeI*

5'-GATCCCGGGCTAAGGCGAATTAATAATTCG-3' (3'-end, anti-sense)  
*SmaI*

**Scheme 3.** Oligonucleotides used for amplification of *T. intermedius* PDHmod gene.

transformation of *E. coli*, and screening for colonies containing inserts with the PDHmod gene (forming pBMS2000-PDHmod) were performed as described above for PPFDH.

For construction of pBMS2000-PPFDH-PDHmod, pBMS2000-PDHmod (2 µg) was cleaved with 10 U each *HindIII* and *SmaI* in a 50-µL reaction for 1 h at 25°C, followed by 1 h at 37°C. Ten units of T4 DNA polymerase (Invitrogen) and 2 µL of a 2.5 mM mixture of all four deoxyribonucleoside triphosphates were added and the sample was incubated at 11°C for 20 min. The reaction was electrophoresed on a 1.0% TAE agarose gel for 2 h at 100 V. The 1800-base pair fragment was excised and isolated using the QIAquick Gel Extraction Kit (Qiagen). This fragment contains, in order, the *tac* promoter, *groES* gene, and the PDHmod gene (as a transcriptional fusion). Next, pBMS2000-PPFDH (2 µg) was digested with 10 units restriction endonuclease *SmaI* in a 50 µL volume for 2 h at 25°C, then treated with 0.4 U shrimp alkaline phosphatase (United States Biochemicals, Cleveland, OH) for 1 h at 37°C. Plasmid DNA was electrophoresed for 2 h at 100 V on a 1.0% TAE agarose gel, isolated, and extracted with the QIAquick kit. The two fragments were ligated with 1 U T4 DNA ligase in a 6.5:1 (insert:vector) molar ratio at 16°C for 4 h in a 10 mL final volume. After 1-butanol extraction and centrifugation, the DNA was transformed into electrocompetent DH10B cells. Kanamycin-resistant colonies were screened for the presence of the PDHmod gene with the two PDHmod-specific primers as previously described for FDH. A second round of PCR screening was conducted by using a DNA primer homologous to the 5' end of the PPFDH gene and an internal primer (antisense) homologous to the PDHmod gene. Only those constructs able to support amplification of a 1400-base pair fragment possessed the two genes in the same orientation. One such plasmid was found and the orientation confirmed by diagnostic restriction digestion with *KpnI*, which gave the expected fragments of 5422 and 1826 base pairs. This plasmid was designated "pBMS2000-PPFDH-PDHmod."

### Expression of FDH and PDHmod

pBMS2000-PPFDH-PDHmod was transformed into *Escherichia coli* JM110. In shake-flasks studies, JM110(pBMS2000-PPFDH-PDHmod) was grown for 18 h at 28°C, 250 rpm in MT5 medium (2.0% Yeastamin, 4.0% glycerol, 0.6% sodium phosphate [dibasic], 0.3% potassium phosphate [monobasic], 0.125% ammonium sulfate, 0.0256% magnesium sulfate [heptahydrate; added post-autoclaving from a sterile 1 M solution], and 50 µg/mL kanamycin sulfate [added post-autoclaving from a filter-sterilized 50 mg/mL solution]). The optical density at 600 nm (OD<sub>600</sub>) was recorded and cells sufficient to give a starting OD<sub>600</sub> of 0.35 AU/cm were added to fresh MT5/kanamycin medium. Flasks were shaken at 250 rpm, 28°C until the OD<sub>600</sub> was 0.8–1.0 AU/cm.

Expression of both genes was induced by addition of filter-sterilized 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 35 µM and the fermentation was continued for 24–48 h. Cells were pelleted by centrifugation at 6,500×g for 5 min, washed once with an equal volume of 50 mM ammonium formate pH 7.0, and repelleted. Cells were stored frozen at –20°C or used immediately. The pellet was resuspended in 50 mM ammonium phosphate, pH 7.0 at 10 mL/g wet cell weight and sonicated 3×15 seconds using a Fisher Scientific Model 50 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA), power setting 15 with a microtip. Debris was pelleted by centrifugation at 13,000×g for 5 min at room temperature.

Expression was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). One µL of the cell extract was mixed with 5 µL of 4X NuPAGE™ LDS buffer (Invitrogen) and brought up to 19 µL with distilled water. Samples were heated at 70°C for 10 min. One µL of a 1 M dithiothreitol solution was added to the mixture and 10 µL applied to a 10% NuPAGE™ Bis-Tris polyacrylamide mini-gel. Electrophoresis was carried out at 200 V for 50–60 min and the gel stained in a solution consisting of 0.1% (w/v) Coomassie Blue (Sigma), 40% (v/v) ethanol, and 10% (v/v) acetic acid. The gel, immersed in the stain, was heated in a microwave oven until boiling was evident, then shaken at 40 rpm on an orbital shaker for 15 min. The gel was washed thoroughly with deionized water and covered with destaining solution (GelClear™; Invitrogen). The solution was again heated just to the point of boiling and shaken gently for at least 2 h. Two prominent bands at M<sub>r</sub> 43,000 and 40,000 were seen upon induction, corresponding to the expected molecular weight of the subunits of FDH and PDHmod. Samples were also found to possess both FDH and PDH activities when tested for reductive amination as described below. This recombinant *E. coli* strain was given the internal designation of SC 16496.

### Production of PDHmod/FDH by *E. coli* SC16496 in a 15-L Fermentation

One frozen vial of SC16496 was thawed and the entire contents (1.5–1.8 mL) were transferred to a 4-L flask containing 1 L of MT5 medium. The inoculum flask was incubated at 30°C for ca. 24 h and 250 rpm. The optical density of the resulting inoculum (OD<sub>600</sub>) at this time was ca. 5.5–5.7 AU/cm. Based on a desired starting OD<sub>600</sub> of 0.35 AU/cm for the fermentor stage, the appropriate quantity of broth was then used to inoculate a 23-L tank containing 15 L of the production medium, MT5M1. The modified MT5M1 medium was composed of 4% glycerol, 1.85% Tastone 154 (Sensient Bi-nutrients), 2% Hy-Pea (Quest International), 0.125% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6% Na<sub>2</sub>HPO<sub>4</sub>, and 0.04% UCON LB625 anti-foam (Dow Chemical). The pH of the medium was adjusted to 7.4 with NaOH. Solutions of MgSO<sub>4</sub> heptahydrate and

kanamycin, prepared separately and filter-sterilized, were added to the tank medium prior to inoculation to yield final concentrations of 0.0246% and 0.005%, respectively. The fermentation process conditions for the tank containing 15 L of medium were as follows: temperature 30°C; pH control at or above pH 7.0 with ammonium hydroxide only; agitation 500 rpm (tip speed 130 m/min); aeration 15 Lpm; back pressure 690 mbar; foam controlled by the addition of UCON LB625 on demand.

At the target OD<sub>600</sub> of ca. 0.8 AU/cm, (1.5–3.0 h into the run), a filter-sterilized IPTG solution was added to yield a final concentration of 35 µM. The run was considered completed after glycerol was exhausted and growth ceased, and an OD<sub>600</sub> of 50–53 AU/cm was usually achieved by 35 h. The whole broth was cooled to 4–10°C and cells were recovered by centrifugation followed by a wash with 50 mM ammonium formate pH 7.0 buffer. A total of 560–600 g of cell paste was then recovered and stored at –75°C.

### Screening of Amino Acid Dehydrogenases

Reaction mixtures contained 0.15 M ammonium formate, 0.05 M keto acid **1**, 1 mM NAD, 1 mM dithiothreitol, 1U FDH (Boehringer Mannheim), and an amino acid dehydrogenase in a final volume of 1 mL. The pH was adjusted to 8.5 with NH<sub>4</sub>OH. Beef liver glutamate dehydrogenase, *Bacillus subtilis* alanine dehydrogenase, and *S. ureae* phenylalanine dehydrogenase were from Sigma. *Rhodococcus* phenylalanine dehydrogenase was from Jülich. Leucine dehydrogenase was partially purified from *Bacillus sphaericus* ATCC 4525. Phenylalanine dehydrogenase from *Thermoactinomyces intermedius* expressed in *Pichia pastoris* was prepared previously as dried cells.<sup>[7]</sup> Solutions were incubated at 30°C for 20 h, then analyzed by HPLC.

### Transamination using Branched Chain Transaminase

The solution contained 0.10 M sodium glutamate, 0.05 M **1** (neutralized with 0.05 M NaOH), 0.1 mM pyridoxal phosphate, and 1 mg branched chain transaminase (Biocatalytics) in a final volume of 1.0 mL in 50 mM potassium phosphate buffer pH 8.0. The solution was incubated in a microfuge tube at 37°C for 68 h, then analyzed by HPLC. The solution contained 5.53 mg/mL (49.2% yield) **2** and 7.05 mg/mL remaining **1**.

### Reductive Amination using an Extract from Recombinant *Pichia pastoris*

Recombinant *Pichia pastoris* frozen cells (2.25 kg) were added to deionized water (6.75 L) containing ammonium formate (28.65 g, 0.454 mol). After thawing, the cells were suspended using a Janke and Kunkel Ultra-turrax T25 homogenizer, then adjusted to pH 7 with concentrated NH<sub>4</sub>OH and cooled with crushed ice to give a 25% w/v cell suspension in 50 mM ammonium formate. Cells were disrupted by two passages through a microfluidizer at 12000 psi, and cell debris was removed by centrifugation at 20,000×g and 4°C. Supernatant (7024 mL) containing 230998 U (assay A) or 124641 U (assay B) phenylalanine dehydrogenase and 80080 U formate dehydrogenase was added to a 16-L vessel of a New Brunswick Scientific Bio-flow IV bioreactor.

A solution (7024 mL) containing ammonium formate (442.5 g, 7.017 mol) and keto acid **1** (786.7 g, 3.510 mol) was brought to pH 8.0 with 276 mL concentrated ammonium hydroxide. NAD (9.834 g, 14.82 mmol) and dithiothreitol (2.163 g, 14.027 mmol) were added, then the solution was added to the bioreactor containing the *Pichia pastoris* extract. The solution was maintained at 40°C and stirred at 150 rpm. Portions of concentrated ammonium hydroxide were added at 0 (44 mL), 3 (25 mL) and 18 (27 mL) h after the start of the reaction to adjust the pH to 8.0. After 25 h, the solution contained 818.9 g (3.637 mol, 100% yield) of amino acid **2** as measured by HPLC analysis and no detectable keto acid **1** or (*R*)-enantiomer of the amino acid.

To reduce the level of ammonia, the reaction mixture, 14 L, was heated to boiling and sparged with steam, with *n*-butanol (1.3 L) added to control foaming. When foaming subsided, the mixture was cooled to 37°C and adjusted to pH 3.0 with 88% formic acid (579 g). Precipitated protein was removed by filtration. The filtrate was diluted to 80 L with water, applied to a 25 i.d. × 30 hcm column of Dowex 50W-X8 (H<sup>+</sup>), 50–100 mesh, and the column was washed with water. No amino acid **2** was detected in the effluent (TLC, silica gel, butanol-acetic acid-water, 4:1:1, ninhydrin, R<sub>f</sub>=0.33). The product was eluted from the resin with 1.5 M ammonium hydroxide, collecting 4 L fractions, and was detected in fractions 13–20 (TLC). These were combined and concentrated to a solid on a rotary evaporator to give 804 g of **2** with a potency of 94.3% by HPLC. The isolated yield from the keto acid was thus 96%.

### Pilot Plant Scale Reductive Amination

Recombinant *Pichia pastoris* frozen cells (114.7 kg) were thawed and added to deionized water (340.4 kg) containing ammonium formate (1.45 kg) in an 800-L vessel. After thawing, the cells were suspended by stirring, then adjusted to pH 7 with concentrated NH<sub>4</sub>OH. Cells were disrupted by passages through a microfluidizer at 14000 psig, and the cell extract was clarified by centrifugation with a Sharples centrifuge.

Deionized water (196.1 kg), keto acid **1** (40 kg, 178.4 mol), and ammonium formate (22.3 kg, 353.6 mol) were charged into an 800-L vessel. The pH was adjusted to 8 with concentrated NH<sub>4</sub>OH, then NAD (499.5 g, 0.753 mol), dithiothreitol (111 g, 0.720 mol), 30.4 kg deionized water, and 407 kg clarified cell extract containing 3467 kU formate dehydrogenase and 646 kU phenylalanine dehydrogenase (assay C) were added. With slow stirring, the solution was maintained at 40°C, and pH 8.0. After 49 h, the solution contained 0.44 kg keto acid **1** and 36.95 kg of amino acid **2** as measured by HPLC analysis (99% conversion). Insoluble material was removed from the reaction mixture by microfiltration (0.2 micron ceramic Membrane, 88 square feet), followed by three diafiltrations (133.3 kg water each) for a total filtrate of 978 kg. Protein was then removed by ultrafiltration (5000 MW cut-off Millipore Prostack Membrane, 50 square feet), followed by diafiltration (twice with 20 kg deionized water). The filtrate (1013 kg) was adjusted to pH 9.6 with 25% sodium hydroxide solution for concentration and removal of ammonia. Water and ammonia were removed by distillation (jacket temperature 110–130°C) to

give 407 kg solution containing amino acid **2** (~100 g/L) which was converted to the BOC derivative **3**.

### Reductive Amination using an Extract from Recombinant *Escherichia coli* SC16496 JM110[pBMS2000-PPFDH-PDHmod]

Frozen cells of recombinant *Escherichia coli* 25% w/v in 50 mM ammonium formate pH 7 were suspended using a Janke and Kunkel Ultra-turrax T8 homogenizer, then cooled with crushed ice and disrupted by two passages through a microfluidizer at 12000 psi. Cell debris was removed by centrifugation at 20,000×g and 4°C. Supernatant (266 mL) containing 2456 U (assay A) or 768 U (assay C) phenylalanine dehydrogenase and 8801 U formate dehydrogenase was added to a 1-L bottle.

A solution (266 mL) containing ammonium formate (16.74 g, 0.2654 mol) and keto acid **1** (29.76 g, 0.1327 mol) was brought to pH 8.0 with 12.7 mL concentrated ammonium hydroxide. NAD (372 mg, 0.561 mmol) and dithiothreitol (81.8 mg, 0.530 mmol) were added, then the solution was added to the bottle containing the *Escherichia coli* extract. The bottle was maintained at 40°C on a shaker at 40 rpm. Concentrated ammonium hydroxide was added periodically to maintain pH 8.0. After 38 h, the solution contained 31.5 g (0.140 mol, 100% yield) of amino acid **2** as measured by HPLC analysis and no (*R*)-enantiomer of the amino acid.

### Preparation of (*S*)-*N*-BOC-3-Hydroxyadamantylglycine (**3**)

The reaction mixture was filtered through a 0.1 micron filter for clarification and subsequently through a 5 kD ultrafilter to remove soluble proteins. The filtrate was charged to a 1-L reaction flask equipped with a distillation apparatus and a receiving flask. The pH was adjusted to 9.6 with 25 wt% NaOH, then was heated to distill off excess water and remove ammonia. The solution, containing 25 g of **2**, was concentrated to 200 mL and K<sub>3</sub>PO<sub>4</sub> (70 g, 0.33 mol) was added. The reaction mixture was cooled to room temperature then treated with BOC<sub>2</sub>O (26 g, 0.12 mol) in THF (200 mL). The reaction was stirred at room temperature until deemed complete by HPLC (2 h). The bottom layer was discarded, and water (150 mL) was added to the top layer, followed by isopropyl acetate (200 mL). This mixture was adjusted to pH 2 with conc. HCl (15 mL). After mixing, the aqueous layer was separated and the organic layer was concentrated to 150 mL by distillation at 1 atmosphere. The organic layer was stirred, cooled to room temperature and heptane (150 mL) was added over 30 min. The suspension was stirred for another h, then the product was filtered out and dried at 40°C under vacuum to obtain **3** (32 g, 88% yield).

### HPLC Analysis of Amino Acid **2**

Samples of the reductive amination reaction mixture were diluted with water to about 2 mg/mL and placed in a boiling water bath for 1 min to stop the reaction and precipitate proteins. After cooling, the samples were filtered through 0.2 micron nylon filters into HPLC vials. Samples were analyzed with a Regis Davankov Ligand Exchange 15×0.46 cm column. The mobile phase was 25% methanol/75% (6 mM

CuSO<sub>4</sub> in water), flow rate was 1 mL min<sup>-1</sup>, detection was at 240 nm, temperature was 40°C, and injection volume was 10 µL. Retention times were (*S*)-enantiomer 3.2 min, (*R*)-enantiomer 11.2 min, keto acid **1** 5.2 min.

### Enzyme Assays

Phenylalanine dehydrogenase assay A contained in 1.0 mL at 40°C: 0.4 mM NADH, 5 mM sodium phenylpyruvate, 0.75 M NH<sub>4</sub>OH adjusted to pH 8.75 with HCl. Absorbance decrease was monitored at 340 nm. Phenylalanine dehydrogenase assay B contained in 1.0 mL at 40°C: 1 mM NAD, 10 mM *L*-phenylalanine, 0.1 M K<sub>2</sub>HPO<sub>4</sub> adjusted to pH 10.0 with 1 N NaOH. Absorbance increase was monitored at 340 nm. Phenylalanine dehydrogenase assay C contained in 1.0 mL at 40°C: 0.4 mM NADH, 50 mM keto acid **1** (dissolved in 1 equivalent NaOH solution), 0.75 M NH<sub>4</sub>OH adjusted to pH 8.75 with HCl. Absorbance decrease was monitored at 340 nm. The formate dehydrogenase assay contained in 1.0 mL at 40°C: 1 mM NAD, 100 mM ammonium formate, 100 mM potassium phosphate buffer, pH 8.0. Absorbance increase was monitored at 340 nm. Enzyme activity units were calculated as µmol/min based on the rates of absorbance change.

### Heat Stability of PDHmod

The heat stability and substrate specificity of PDH and PDHmod are compared in Figure 5. For this experiment, 25% w/v extracts of *E. coli* SC16496 (FDH/PDHmod) and SC16487 (FDH/PDH) in 50 mM ammonium formate pH 7 were microfluidized and centrifuged at 20000×g. One mL samples of the centrifuged extract were incubated for 1 h at 0, 40, 50, 60 or 70°C, then assayed for activity with phenylpyruvate or keto acid **1** as substrate. Samples of the untreated centrifuged extracts were also diluted 1:10 with water and run on an Agilent 2100 bioanalyzer protein 200 labchip to separate and quantitate the PDH subunit bands. The PDH concentration was 2.99 mg mL<sup>-1</sup> and the PDHmod concentration was 5.17 mg mL<sup>-1</sup> in the centrifuged extracts (mean of 4 samples each). Specific activities are expressed as units per mg PDH or PDHmod protein in the subunit band.

### References

- [1] B. Gallwitz, *Treat. Endocrinol.* **2005**, *4*, 361–370.
- [2] L. L. Nielsen, *Drug Discovery Today* **2005**, *10*, 703–710.
- [3] E. M. Sinclair, D. J. Drucker, *Curr. Opin. Endocrinol. Diabet.* **2005**, *12*, 146–151.
- [4] D. J. Augeri, J. A. Robl, D. A. Betebenner, D. R. Magnin, A. Khanna, J. G. Robertson, A. Wang, L. M. Simpkins, P. Taunk, Q. Huang, S.-P. Han, B. Abbo-Offei, M. Cap, L. Xin, L. Tao, E. Tozzo, G. E. Welzel, D. M. Egan, J. Marcinkeviciene, S. Y. Chang, S. A. Biller, M. S. Kirby, R. A. Parker, L. G. Hamann, *J. Med. Chem.* **2005**, *48*, 5025–5037.
- [5] R. L. Hanson, J. Singh, T. P. Kissick, R. N. Patel, L. J. Szarka, R. H. Mueller, *Bioorg. Chemistry* **1990**, *18*, 116–130.

- [6] R. L. Hanson, M. D. Schwinden, A. Banerjee, D. B. Brzozowski, B.-C. Chen, B. P. Patel, C. G. McNamee, G. A. Kodersha, D. R. Kronenthal, R. N. Patel, L. J. Szarka, *Bioorg. Med. Chem.* **1999**, *7*, 2247–2252.
- [7] R. L. Hanson, J. M. Howell, T. L. LaPorte, M. J. Donovan, D. L. Cazzulino, V. Zannella, M. A. Montana, V. B. Nanduri, S. R. Schwarz, R. F. Eiring, S. C. Durand, J. M. Wasyluk, W. L. Parker, M. S. Liu, F. J. Okuniewicz, B.-C. Chen, J. C. Harris, K. J. Natalie Jr., K. Ramig, S. Swaminathan, V. W. Rosso, S. K. Pack, B. T. Lotz, P. J. Bernot, A. Rusowicz, D. A. Lust, K. S. Tse, J. J. Venit, L. J. Szarka, R. N. Patel, *Enzyme Microb. Technol.* **2000**, *26*, 348–358.
- [8] H. Takada, T. Yoshimura, T. Ohshima, E. Nobuyoshi, K. Soda, *J. Biochem.* **1991**, *109*, 371–376.
- [9] C. T. Hou, R. N. Patel, A. I. Laskin, N. Barnabe, *Arch. Biochem. Biophys.* 1982, **216**, 296–305.
- [10] J. J. Allais, A. Louktibi, J. Baratti, *Agric. Biol. Chem.* 1983, **47**, 2547–2554.
- [11] J. D. Godfrey, R. T. Fox, F. G. Buono, J. Z. Gougoutas, M. F. Malley, *J. Org. Chem.* **2006**, *71*, 8647–8650.
- [12] T. C. Vu, D. B. Brzozowski, R. Fox, J. D. Godfrey, R. L. Hanson, S. V. Kolotuchin, J. A. Mazzullo Jr., R. N. Patel, J. Wang, K. Wong, J. Yu, J. Zhu, D. R. Magnin, D. J. Augeri, L. G. Hamann, (Bristol-Myers Squibb Company), *PCT Int. Appl.* WO 2004/052850 A2, **2004**.
- [13] S. W. Liu, T. J. Franceschini, (Bristol-Myers Squibb Company), *US Patent* 6,068,991, **2000**.
- [14] R. Hanson, M. J. Donovan, S. Goldberg, P. A. Jass, W.-S. Li, R. Patel, K. Ramig, L. J. Szarka, J. J. Venit, (Bristol-Myers Squibb Company), *US Patent* 6,140,088, **2000**.