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Peptide deformylase as biocatalyst for the synthesis of enantiomerically pure amino acid derivatives

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Abstract

Peptide deformylases (PDFs) catalyze the removal of the N-terminal formyl group from nascent polypeptides. In spite of the vast amount of literature on PDF, no information whatsoever is available on its use in organic synthesis. To be able to explore the potential of *E. coli* PDF (*Ec*PDF) in biocatalytic applications, a simple and efficient purification procedure was developed. This method, which was based on one affinity chromatographic step, furnished about 200 mg of pure *Ec*PDF from 1 L of *E. coli* culture. The enzyme combined a good activity (tof $\geq 5\,s^{-1}$) with an almost complete enantioselectivity (*E* ratio >500) in the resolution of *N*-formylated α- and β-amino acids, α-amino acid amides and α-aminonitriles. *N*-Formyl derivatives of non-functionalized amines and β-amino alcohols were hydrolyzed with low to moderate activity and enantioselectivity. *Ec*PDF was also successfully applied in the enantioselective formylation of α-aminonitriles, yielding, e.g. (*S*)-*N*-formyl-phenylalanine nitrile with >99.5% ee. The enzyme also proved very suitable for the mild and selective deprotection of *N*-formyl peptides as was shown for *N*-formyl-Leu-Tle-$NHCH_3$. This deprotection increased the diastereomeric excess of the dipeptide, which was unsatisfactory because of racemization of the N-terminal amino acid in the chemical peptide coupling step.

Keywords: Peptide deformylase; α-Aminonitriles; Amino acids; Resolution reactions; Peptide synthesis

1. Introduction

In eubacteria and eukaryotic organelles, all nascent polypeptides synthesized by the ribosomal pathway initially contain an *N*-formylmethionine residue at their N-terminus [1]. During subsequent elongation of the polypeptide chain, the formyl group is removed by the action of peptide deformylase (PDF, EC 3.5.1.31) [2,3]. This deformylation is required before methionine aminopeptidase (MAP) can remove the N-terminal methionine [4], which in many cases is the second step in the maturation of the polypeptide [5–7]. PDF activity is therefore essential for eubacterial growth, which was also experimentally proven because disruption of the gene encoding this enzyme (*def*) was lethal for amongst others *E. coli* [8]. Hence, PDF is regarded as an attractive novel target in antibacterial drug design [9,10]. The fact that until recently PDF has been regarded an enzyme not present in eukaryotes, further strengthened this interest. Recently, however, searches of genomic databases revealed that eukaryotes including man contain genes that encode proteins of high sequence homology to bacterial PDF. It has now been experimentally proven that these PDF orthologs in the malaria parasite *Plasmodium falciparum* [11], in the plant *Arabidopsis thaliana* [12] and in humans [13] indeed have deformylase activity. Pei and co-workers have shown that human PDF is likely an evolutional remnant without any functional role in protein formylation/deformylation. Therefore, PDF is still regarded as an excellent target in the design of new antibiotics [13].

PDF research has long been hampered by the extreme lability of the protein ($t_{1/2} < 1$ min at ambient temperature) preventing its purification and thorough biochemical

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characterization. This extreme lability of PDF strongly contrasts with its very compact and solid structure [9]. Cloning of the *E. coli def* gene [8,14] and overproduction of the encoded protein enabled the purification of PDF, but still in an inactive form. Nevertheless, *E. coli* PDF (*Ec*PDF) could be characterized as a monomeric enzyme of 168 amino acid residues (19.2 kDa) containing one tightly bound Zn^{2+} ion [14]. Not earlier than in 1995 the reason for the extreme instability of PDF and a satisfactory solution enabling its purification in fully active form were first reported by Wagner and co-workers [15,16]. The active *Ec*PDF contains iron in the ferrous state (Fe^{2+}) as catalytic metal ion. This renders the enzyme very susceptible to oxidation, which leads to rapid and irreversible deactivation of the enzyme. Oxidation of the ferrous enzyme can be prevented by the use of scavengers of reactive oxygen species such as catalase and/or TCEP (tris(2-carboxyethyl)phosphine) [15,16]. Alternatively, the ferrous ion can be replaced by metal cations that are insensitive to oxidation, and almost completely preserve enzyme activity. Examples of such metals are nickel [16,17] and cobalt [18,19], the former of which is preferred because the activity of PDF-Ni is only a little lower than of the native Fe^{2+} containing enzyme.

From the crystal structure of the *Ec*PDF in complex with the tripeptide H-Met-Ala-Ser-OH it was concluded that the S_1' sub-site consists of a deep hydrophobic pocket, which neatly accommodates the methionine side-chain of the natural PDF substrates [20]. Besides this native substrate, also *N*-formyl peptides with other (preferably unbranched) hydrophobic residues at their P_1' position (e.g., *n*-butyl, *n*-pentyl, *n*-hexyl and benzyl) were efficiently deformylated by this enzyme. The presence of hydrophilic side-chains at this position, however, dramatically reduced deformylation efficiencies without exception [21]. This extreme effect of the P_1' side-chain on the catalytic efficiency of the *Ec*PDF contrasts sharply with the much less pronounced influence of the amino acids at the P_2' and P_3' positions [21,22]. This experimental observation is in line with structural information which shows that the S_2' and S_3' sub-sites are large enough to accommodate bulky side-chains. Furthermore, the structure demonstrates that all hydrogen bonds between the *Ec*PDF and H-Met-Ala-Ser-OH involve atoms of the backbone of this tripeptide only [20]. Ragusa et al. even reported the absence of a true S_2' sub-site [21]. Besides the type of amino acid at the different positions in the *N*-formyl substrates, also the length of these peptides has a marked effect on the catalytic efficiency of the deformylase reaction. Groche et al. showed that the catalytic efficiency for *N*-formyl-Met-OH is about three orders of magnitude lower than for *N*-formyl-Met-Ala-Ser-OH and *N*-formyl-Met-Ala-OH [15]. This marked effect of the substrate length was later confirmed in a study of Meinnel et al. [23].

Comparison of the amino acid sequences of seven bacterial PDFs revealed the existence of three PDF signature sequences [24]. Analysis of 150 bacterial genomes for PDF encoding genes using these signature sequences demonstrated that every bacterium contains at least one and at most four PDF gene sequences [25]. Although the level of sequence identity between PDFs is rather low, amino acid sequence alignment and phylogenetic tree analysis revealed that they are closely related. Furthermore, these analyses demonstrated that PDFs can be divided into two sub-families, types I and II. In comparison with type I proteins, type II PDFs contain two sequence insertions located in their N-terminal part, as well as a hydrophobic C-terminus [9,25].

Very recently, X-ray structures of PDFs other than from *E. coli* were elucidated, i.e., from the microorganisms *Pseudomonas aeruginosa* [25,26], *Staphylococcus aureus* [25–28], *Bacillus stearothermophilus* [25], *Haemophilus influenzae* [28], *Streptococcus pneumoniae* [26,28] and *Thermotoga maritima* [26] and from the eukaryote *P. falciparum* [29]. Comparison of these structures shows that all PDFs have an essentially conserved tertiary structure. This also holds for the topology of the active site and the binding mode of the PDF inhibitor actinonin to the various peptide deformylases, which is in line with their identical biological function. On the other hand, also significant differences are observed, mainly at the C-terminus, which are directly related to the deformylase type (i.e., I or II) they belong to [25].

The vast amount of literature on PDFs is clearly driven by the fact that this family of enzymes is regarded as an attractive target for new antibacterial agents. Data on the use of PDF for other applications are extremely limited. Degussa AG disclosed an improved fermentative process for L-lysine using *Corynebacterium glutamicum* strains in which the cellular PDF activity has been reduced by disruption of one of the two *def* genes via recombinant DNA techniques [30]. Bogosian and co-workers showed that coexpression of the PDF gene prevents retention of formylmethionine in bovine somatotropin overproduced in *E. coli* [31]. According to these authors, this approach is generally applicable to increase the efficiency of heterologous protein production. No information whatsoever is available on the use of PDF in organic synthesis.

In this paper we describe for the first time the application of PDF in the stereoselective synthesis of amines and amino acid derivatives and for the selective deprotection of *N*-formyl protected peptides. Enzymatic resolution of racemic amines and amino acid derivatives is well studied and used on industrial scale to produce chiral intermediates for pharmaceuticals and agrochemicals. Of the various options, enantiopure α-amino acids, β-amino alcohols and amines can be obtained by (de)acylation reactions using acylases or lipases [32–36]. Amino acid derivatives in addition offer the opportunity to be resolved on the carboxylate group, for example, by using the highly stereoselective amide route applied at DSM Pharma Chemicals [37]. A special class of compounds is formed by the α-aminonitriles. These compounds are valuable building blocks for chemical synthesis, as they are readily accessible via Strecker

reaction starting from aldehydes and ketones [37], and can be converted into a broad range of interesting synthons. α-Aminonitriles can be resolved by nitrilase or nitrile hydratase catalyzed hydrolysis ending up with enantiomerically enriched amino acids or amino acid amides [38], but in general the remaining (enantiomerically enriched) aminonitriles are not isolated. As α-aminonitriles are not very (optically) stable, only few enzymatic resolution routes are known that furnish enantiopure (*N*-acyl)-α-aminonitriles [39–42]. In this study, we explored the potential of PDF in the enantioselective synthesis of a wide range of amino acid derivatives. Both the PDF catalyzed stereoselective hydrolysis of racemic *N*-formylated precursor molecules as well as the stereoselective formylation of the racemic amino acid derivatives were investigated. In addition, we report the application of the *E. coli* PDF as catalyst for the mild removal of the *N*-formyl protecting group from a dipeptide. Part of this work has been published in patent applications [43,44].

2. Experimental

2.1. Materials

All standard chemicals were of the highest grade obtainable. *N*-Formyl-Met-Ala-OH, H-Met-Ala-OH, and *N*-formyl-Met-Lys-OH were purchased from Bachem (Bubendorf, Switzerland); bovine liver catalase and 4-(2-aminoethyl)-benzenesulfonyl-fluoride (AEBSF, Pefabloc SC) were obtained from Roche Diagnostics (Mannheim, Germany); tris-(2-carboxyethyl)-phosphine (TCEP) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were obtained from Fluka (Buchs, Switzerland).

The Met-Lys-Sepharose affinity matrix was prepared as described by Groche et al. [16]. The binding capacity was calculated from the ligand coupling efficiency and the formyl deprotection efficiency and appeared to be 2–2.5 μmol/mL of affinity matrix. This corresponds to about 45 mg PDF/mL of matrix.

Racemic *N*-formyl amines were prepared by reaction of the corresponding ketones with an excess of formamide or ammonium formate (Leuckart–Wallach reaction) according to Smith and Rajevsky [45] or by formylation of the corresponding amines in a refluxing mixture of formic acid and 1 equiv. of acetic acid anhydride.

Racemic aminonitriles were prepared by Strecker reaction starting from the corresponding aldehydes or ketones as described previously [37]. Racemic amino acid amides were prepared from the corresponding aminonitriles by hydrolysis with benzaldehyde/NaOH as described in the same reference.

All amino acid derivatives were formylated using the mixed anhydride of acetic and formic acid as described above for the *N*-formyl amines.

N-Formyl aminonitriles appeared to be chemically and optically stable for several days in aqueous solution at pH 6–7 and at 20–40 °C, but slowly racemize at higher pH (1% racemization at pH 8.0, 20 °C after 18 h). Aminonitriles appeared to be chemically and optically stable under acidic aqueous conditions and can be isolated as their HCl salt. However, in diluted aqueous solution at pH > 5 they irreversibly decomposed.

2.2. PDF producing microorganisms and cultivation conditions

*Ec*PDF was produced from expression vectors p925C or pBAD*def*. The construction and use of plasmid p925C, which is based on *E. coli* expression vector pKK223-3 (Pharmacia) has been described before [16]. For the construction of plasmid pBAD*def*, the *E. coli def* gene (e.g., nts. 1426–1935 of X77091) was amplified by PCR from the gDNA from *E. coli* W3110 using the primers 5′-gCCTCA*ggTCTC*T**CATgTCAgTTTTgCAAgTgTTAC**-3′ and 5′-CgTggA*ggTCTC*gA**ATTCTgACACgTTAgTTCTTAT-C**-3′ (*Bsa*I recognition site italicized, *def* complementary parts bold). The first primer is complementary to the 5′ end of the *def* gene, whereas the second one is complementary to the nucleotides directly downstream of the *def* gene. The PCR product obtained was digested with the restriction enzyme *Bsa*I, generating *Nco*I and *Eco*RI compatible sticky ends. The fragment was ligated into vector pBAD/Myc-His C (Invitrogen, Breda, The Netherlands), which had been digested with *Nco*I and *Eco*RI, yielding the desired plasmid pBAD*def* (4584 bp). This vector was routinely maintained in *E. coli* TOP10 cells (Invitrogen).

Both *E. coli* JM109 carrying plasmid p925C and TOP10 transformed with plasmid pBAD*def* were cultivated at 28–30 °C in TB medium supplemented with 50–100 mg/L carbenicillin for 14–18 h at a constant pH of 7. The expression of the *def* gene was induced when the OD_{620} reached 0.5–1.0 by the addition of 0.1 mM IPTG (JM109/p925C) or 0.002 wt.% arabinose (TOP10/pBAD*def*). Cells were harvested by centrifugation and stored frozen (−80 °C). Typically, 250 g of cells (wet weight) were obtained from 10 L of fermentation volume.

2.3. Purification of PDF

All purification steps were performed at 4 °C. After thawing, cells were resuspended in 20 mM Hepes–KOH, supplemented with 100 mM KF, 10 mg/L catalase from bovine liver and 1 mM AEBSF having pH 7.7 (1 g of wet weight cells + 2 mL of buffer). Cells were disintegrated by sonication, and cell debris was removed by centrifugation (200,000 × *g* for 1 h). The protein content of the clear supernatant (cell free extract, CFE) was determined, followed by the addition of 1 mL 10% (w/v) polyethylenimine (pH 7.7) per gram of protein. The precipitated nucleic acids were subsequently removed by centrifugation (50,000 × *g* for 30 min).

The clear supernatant was applied to a Met-Lys-Sepharose affinity column that had been equilibrated with start buffer

(20 mM Hepes–KOH, supplemented with 100 mM KF and 0.2 mM TCEP (pH 7.7)). After sufficient washing of the column with start buffer (about 6 column volumes), the bound PDF was eluted from the column with about 6–8 column volumes of elution buffer (20 mM Hepes–KOH, supplemented with 100 mM KCl and 0.2 mM TCEP (pH 7.7)). PDF containing fractions were pooled, and the TCEP concentration was adjusted to 1 mM. Finally, the PDF was concentrated, preferably to a concentration >30 mg/mL, by ultrafiltration using a filter with a cut-off value of 10,000 NMWL (Amicon YM10 or PM10 ultrafiltration discs, Millipore). The resulting PDF stock solution was stored at −80 °C till further use.

After thawing, this PDF stock solution could be used as such in most of the (de-)formylation experiments described below. If, however, applications required a lower PDF concentration, the PDF stock solution was diluted to the desired concentration with a 20 mM Hepes–KOH buffer containing 100 mM KCl, 1 mg/mL of bovine serum albumin, 10 mg/L catalase and 2 mM TCEP (pH 7.7).

2.4. PDF activity assay and Protein determination

PDF activity was determined at pH 7.2 and 30 °C using *N*-formyl-Met-Ala-OH as substrate in the presence of catalase as described earlier [16], with the exception that the reactions were terminated after 5 min by the addition of 50 μL of 0.1 M TNBS instead of with 4 wt.% aqueous $HClO_4$. Then, 100 μL of the stopped reaction mixtures were mixed with 100 μL of 0.1 M $Na_2B_4O_7$ in 0.1 M aqueous NaOH. After exactly 5 min at ambient temperature, the staining reaction was stopped by the addition of 400 μL of a freshly prepared mixture of 98.5 mL 0.1 M aqueous NaH_2PO_4 and 1.5 mL 0.1 M aqueous Na_2SO_3. After removal of the precipitate by 2 min centrifugation in a minifuge, the absorbance at 420 nm was measured. The amount of the deformylation product H-Met-Ala-OH was calculated from an *N*-formyl-Met-Ala-OH/H-Met-Ala-OH complementary calibration curve, that was treated accordingly throughout the TNBS staining procedure. Furthermore, a blank reaction was included to compensate for the primary amino groups initially present in the PDF solution. In this blank reaction, the order of PDF and TNBS addition was reversed.

The protein concentration in PDF containing samples was determined according to the biuret method using bovine serum albumin (BSA) as reference protein.

2.5. PDF catalyzed resolution of N-formyl amines and amino acid derivatives

The analytical deformylase reactions of the *N*-formyl derivatives were performed in 1.5 mL Eppendorf test tubes. Three different methods were used.

- Method A: deformylation in the presence of Li_2SO_4 at pH 7.2 and 37 °C

  The reaction mixtures with a total volume of 200 μL contained 100 mM MOPS–NaOH, 2 M Li_2SO_4 buffer (pH 7.2) and the concentration of the formylated compound was as indicated in Table 4. Reactions were started by the addition of purified PDF in the concentration as indicated in Table 4. After regular time intervals samples were withdrawn and mixed with aqueous $HClO_4$ to stop the reaction by inactivating the PDF. The exact amounts of sample and aqueous $HClO_4$ are given in the part describing the different HPLC methods used (vide infra).
- Method B: deformylation in the absence of Li_2SO_4 at pH 7.2 and 37 °C

  The reactions were performed as described in method A with the exception that 100 mM MOPS–NaOH, 250 mM NaCl, 0.1 g/L catalase buffer (pH 7.2) was used instead of the 100 mM MOPS–NaOH, 2 M Li_2SO_4 buffer (pH 7.2).
- Method C: deformylation in the absence of Li_2SO_4 at pH 6.2 and 37 °C

  Also in this case the reactions were performed as described in method A with the exception that 100 mM MES–NaOH buffer (pH 6.2) was used instead of the 100 mM MOPS–NaOH, 2 M Li_2SO_4 buffer (pH 7.2).

2.5.1. HPLC analysis

- Method 1: without derivatization

  Samples of 5 μL were withdrawn from the reaction mixtures and immediately mixed with 95 μL of 10 mM aqueous $HClO_4$ to terminate the reaction. Protein precipitate was removed by brief centrifugation and 20 μL of the clear supernatant was subsequently applied to a Crownpak CR(+) column (150 mm × 4 mm) (Daicel) [46]. Further specific chromatographic conditions as well as the compounds analyzed by this method are given in Table 1.
- Method 2: with precolumn derivatization

  Samples of 25 μL were withdrawn from the reaction mixtures and immediately mixed with 25 μL of 100 mM aqueous $HClO_4$ to terminate the reaction. After centrifugation, 40 μL of the clear supernatant was added to 80 μL of 1 M aqueous H_3BO_3–NaOH buffer (pH 9.4). Subsequently, 20 μL OPA reagent (consisting of *o*-phthalaldehyde in H_2O/CH_3OH 1:1 v/v) and 20 μL NAC reagent (consisting of *N*-acetyl-L-cysteine in H_2O/CH_3OH 1:1 v/v) were added. The derivatization reaction was terminated by the addition of 80 μL 0.25 M aqueous H_3PO_4 and subsequently 20 μL of the solution was instantaneously applied to a Nucleosil 120-5 C_{18} column (250 mm × 4 mm) (Machery-Nagel). Flow rate was always 1 mL/min, temperature was always ambient and spectrophotometric detection was performed at a wavelength of 257 nm and/or 340 nm. As mobile phase, a mixture of 0.05 M aqueous H_3PO_4–NaOH buffer (pH 7.0) and acetonitrile was used.

  For the derivatization reaction of valine nitrile, the pH of the borate buffer was adjusted to 11 and the addition of the NAC reagent was done 10 min after the OPA reagent

Table 1
Conditions for HPLC method 1 (without derivatization)

Entry	Compound	Mobile phase	Flow rate (mL/min)	Temperature (°C)	Detection (nm)	Retention time (min)		
						Amine[a]	Amine[a]	Formyl amine
1	*N*-Formyl-phenylglycine	10 mM aq. $HClO_4$	1.0	40	210	2.1 (*R*)	3.8 (*S*)	9.6
2	*N*-Formyl-3-amino-3-phenylpropionic acid	85% 100 mM aq. $HClO_4$/15% CH_3OH	0.7	5	210	23.7	26.7	11.4
3	*N*-Formyl-phenylglycine amide	10 mM aq. $HClO_4$	0.8	22	210	3.2 (*R*)	12.6 (*S*)	6.3
6/7	*N*-Formyl-phenylglycinol	95% 10 mM aq. $HClO_4$/5% CH_3OH	0.8	5	210	4.8 (*S*)	5.7 (*R*)	10.0
9	*N*-Formyl-phenylalanine nitrile	90% 10 mM aq. $HClO_4$/10% CH_3OH	0.8	5	210	11.8	15.1	28.6
10	*N*-Formyl-valine nitrile	100 mM aq. $HClO_4$	0.4	5	200	–[b]	–[b]	11.8
11	*N*-Formyl-*m*-methoxyphenylalanine nitrile	90% 10 mM aq. $HClO_4$/10% CH_3OH	0.8	5	210	23.8	30.7	52.0
12	*N*-Formyl-1-(1-naphthyl)ethylamine	85% 10 mM aq. $HClO_4$/15% CH_3OH	1.0	40	210	26.5 (*S*)	31.2 (*R*)	73.5
13	*N*-Acetyl-phenylglycine amide	10 mM aq. $HClO_4$	0.8	22	210	3.2 (*R*)	12.6 (*S*)	6.9
14	*N*-Formyl-proline	100 mM aq. $HClO_4$	0.4	5	200	3.8[c]	3.8[c]	5.7

[a] In case a stereoprefix is given, this was assigned based on the retention time of enantiopure reference compounds.
[b] No clear peak was observed, because valine nitrile in the protonated form shows only very little UV-absorption at 200 nm.
[c] The two proline enantiomers could not be separated on the Crownpak CR(+) column.

had been added. In this case, the concentration of the aqueous H_3PO_4 to terminate the derivatization reaction was 0.5 M.

Derivatization conditions, the percentage acetonitrile in the mobile phase and the compounds analyzed by this method are given in Table 2.

2.6. PDF catalyzed deformylation of *N*-formyl-β-amino acids

The deformylation of racemic *N*-formyl-3-amino-3-phenylpropionic acid and racemic *N*-formyl-3-amino-3-(4-methoxyphenyl)propionic acid was performed according to the following procedure: to a 10 mM solution of the *N*-formyl-β-amino acid in 100 mM MOPS–NaOH buffer (pH 7.2) containing 0.1 g/L catalase and 250 mM NaCl, was added CFE to a final concentration of ∼20 μM *Ec*PDF. The reactions were stirred at 38 °C and monitored by TLC. The conversion as well as the ee of the two deformylated reaction products was determined by HPLC essentially as was described for method 1. Both the (*R*)-3-amino-3-phenylpropionic acid and the (*R*)-3-amino-3-(4-methoxyphenyl)propionic acid were obtained in an ee of >99.5% at 39 and 34% conversion, respectively.

2.7. PDF catalyzed formylation of α-aminonitriles

2.7.1. Determination of pH optimum of PDF catalyzed formylation reaction

Stock solutions of 1 M aqueous H_3PO_4 (A), 1 M aqueous KH_2PO_4 (B) and 1 M aqueous K_2HPO_4 (C) each containing 6 M sodium formate were used for the preparation of the various buffer solutions with the desired pH.

To 10 mL of ∼55 mM racemic 2-amino-3-phenylpropionitrile·HCl (phenylalanine nitrile) in aqueous phosphate buffer of pH 4.0 (A), pH 5.0 (mixture of 15 mL A + 13 mL C), pH 6.0 (mixture of 20 mL B + 5 mL C), and pH 8.1 (C) was added 5 mg purified *Ec*PDF (∼25 μM). The reaction mixtures were stirred at ambient temperature. After 20 and 50 min, samples were withdrawn and the conversion

Table 2
Conditions for HPLC method 2 (with derivatization)

Entry	Compound	[OPA] (mg/ml)	[NAC] (mg/ml)	Time (min)	CH_3CN (vol.%)	Retention time (min)	
						Amine[a]	Amine[a]
2	*N*-Formyl-3-amino-3-phenyl propionic acid	4	4	30	15	19.7	23.3
4	*N*-Formyl-*tert*-leucine amide	8	8	10	22.5	14.9 (*R*)	17.4 (*S*)
5	*N*-Formyl-α-methyl-phenylglycine amide	16	16	30	20	24.4	26.3
8	*N*-Formyl-alaninol	4	4	5	15	16.9 (*S*)	18.8 (*R*)
10	*N*-Formyl-valine nitrile	16	4	5	20	8.6 (*S*)	10.2 (*R*)

[a] The stereoprefixes were assigned based on the retention time of the derivatization products of enantiopure reference compounds.

was determined using HPLC method 1 described above. No *N*-formyl-2-amino-3-phenylpropionitrile formation was observed in the blank reactions.

2.8. PDF catalyzed formylation of rac-2-amino-3-phenylpropionitrile

To 25 mL of a 100 mM aqueous MOPS–NaOH buffer solution containing 0.1 g/L catalase, 6 M sodium formate and 250 mM NaCl (pH 7.4) was added 25 mg purified *Ec*PDF (∼50 μM) and 1.0 g (5.5 mmol) racemic 2-amino-3-phenylpropionitrile·HCl salt. This solution was adjusted to pH 6.7. After 20 h of shaking at ambient temperature the reaction was terminated at 38% conversion (as determined by HPLC method 1). A small amount of the heterogeneous reaction mixture was acidified to pH 2 and extracted with CH_2Cl_2. (*S*)-*N*-Formyl-2-amino-3-phenylpropionitrile with ee >99% was isolated from the organic layer. The precipitate in the remaining part of the reaction mixture was filtered yielding (*S*)-*N*-formyl-2-amino-3-phenylpropionitrile (ee >99.5%) as a white solid. Determination of the ee of the formylated α-aminonitrile was performed by chiral HPLC using a Chiralpak OJ column (Daicel) in combination with an *n*-hexane/2-butanol (60/40 v/v) mobile phase. Flow was 1 mL/min. The compounds were spectrophotometrically detected at 254 nm.

2.9. PDF catalyzed formylation of rac-2-amino-4-methylvaleronitrile

To a solution of 116 mg (0.8 mmol) of 2-amino-4-methylvaleronitrile·HCl (leucine nitrile) in 10 mL of a 100 mM MOPS–NaOH buffer containing 0.1 g/L catalase, 6 M sodium formate and 250 mM NaCl (pH 7.4) was added 25 mg of purified *Ec*PDF (∼130 μM). After 2.5 h at 34 °C a conversion of 39% was reached. The ee of the remaining (*R*)-aminonitrile was 63% and the ee of the (*S*)-*N*-formyl-2-amino-4-methylvaleronitrile was >98%. Conversion of the reaction and ee of the remaining 2-amino-4-methylvaleronitrile was determined by HPLC method 1. In this case the *N*-formyl-2-amino-4-methylvaleronitrile was spectrophotometrically detected at 200 nm, whereas both non-formylated enantiomers were detected by fluorescence measurement ($\lambda_{ex} = 365$ nm; $\lambda_{em} > 420$ nm) after post-column derivatization using fluorescamine. Determination of the ee of the *N*-formyl-2-amino-4-methylvaleronitrile product was performed after acidic extraction of the reaction mixture with $CHCl_3$ by chiral HPLC using a Chiralpak OJ column (Daicel) in combination with an *n*-hexane/2-butanol (90/10 v/v) mobile phase. Flow was 1.5 mL/min. The compounds were spectrophotometrically detected at 210 nm.

2.10. PDF catalyzed deprotection of N-formyl-dipeptides

To a solution of 35 mM *N*-formyl-Leu-(*S*)-Tle-$NHCH_3$ with 88% de (diastereomeric excess), prepared from *N*-formyl-(*S*)-Leu-OH and H-(*S*)-Tle-$NHCH_3$ by the mixed anhydride method [47], in 100 mM aqueous MOPS–NaOH buffer containing 250 mM NaCl and 0.1 g/L catalase (pH 7.2) was added purified *Ec*PDF to a final concentration of 2 μM. The clear reaction mixture was incubated at 37 °C. The progress of the reaction was monitored by quenching samples with an equal volume of methanol followed by reversed-phase HPLC analysis using an Inertsil ODS-3 column (150 mm × 4.6 mm) (Varian) and phosphate buffer pH 2.5/acetonitrile as mobile phase. After completion of the hydrolysis reaction (2–3 h at 96% conversion), the product H-(*S*)-Leu-(*S*)-Tle-$NHCH_3$ was isolated from the remaining part of the reaction mixture via acidification to pH 1.3 and extraction into *i*-propyl-acetate. To find out if the *Ec*PDF catalyzed deprotection reaction was enantioselective, the isolated deformylated dipeptide was completely hydrolyzed by treatment with 6 N HCl at 80 °C for 16 h. Subsequently, the ee of the leucine moiety was determined by chiral HPLC according to the method of Shinbo et al. [46].

3. Results and discussion

3.1. Purification of E. coli PDF via affinity chromatography

The *E. coli def* gene was cloned in two different *E. coli* expression vectors, i.e., pKK223-3 and pBAD/Myc-His C via standard molecular biology procedures. Both expression vectors resulted in a 1000–2000-fold PDF overexpression in *E. coli*. Combined with the high specificity of the Met-Lys-Sepharose affinity column, this enabled purification of PDF by the very simple two-step procedure given in Section 2. The results of a typical purification experiment starting from 20 g (wet weight) *E. coli* TOP10 cells containing pBAD*def* are given in Table 3.

The specific activity of the purified PDF towards the substrate formyl-Met-Ala-OH was 1175 U/mg protein at pH 7.2 and 30 °C. This is almost equal to the 1200 U/mg protein earlier reported for a homogeneous preparation of PDF(Fe) [16], indicating that this simple procedure furnishes pure PDF. A single band on SDS-PAGE supports this conclusion (data not shown). This implies that the purification procedure yields ∼200 mg of pure and highly active PDF from 1 L *E. coli* culture.

In the Met-Lys-Sepharose affinity matrix the ligand is bound via the ε-NH_2 group of the Lys side-chain to the NHS-activated Sepharose. This prevents hydrolysis of the Met-Lys bond on the affinity matrix by, e.g., methionine aminopeptidases. The PDF binds to the Met-Lys-Sepharose affinity matrix in the presence of fluoride ions because these significantly increase *Ec*PDFs affinity for small peptides, most likely because fluoride mimics formate. A switch to, e.g., chlorine ions drastically decreases *Ec*PDFs affinity for the ligand leading to elution of the desired enzyme.

Use of this affinity matrix for the purification of *Ec*PDF has been earlier described [16], but in this case it served as

Table 3
Purification of *Ec*PDF from *E. coli* TOP10/pBAD*def*[a]

Purification step	Total activity ($\times 10^3$ U)	Total protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell free extract	2840	29550	96	100	1.0
PEI precipitation (supernatant)	2338	16840	139	82	1.4
Met-Lys-Sepharose column	2056	1750	1175	72	12.2

[a] Results were obtained starting from 220 g (wet weight) cells.

a fourth and last column step to polish the *Ec*PDF preparation. Also other published methods to purify the *Ec*PDF made use of multiple column steps [17,48]. The results obtained in this study clearly show that the native *Ec*PDF can be purified with this single column step only. Another great advantage of this purification method is its universal applicability and the fact that the native proteins are isolated, because an affinity tag is not required. We successfully applied it to the purification of the native PDF from *Bacillus subtilis*, *Helicobacter pylori*, *H. influenzae*, *P. aeruginosa* and *Synechocystis* sp. (data not shown).

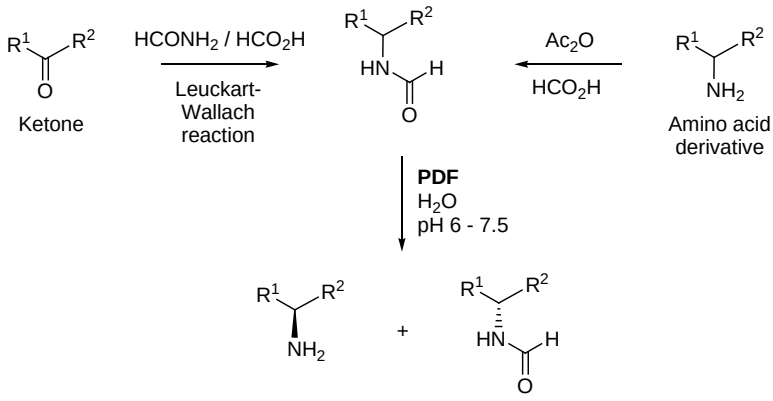
3.2. PDF catalyzed resolution of amines and amino acid derivatives

3.2.1. Hydrolytic (deformylation) concept

In this paper we describe for the first time the application of PDF in the resolution of *N*-formyl amines and *N*-formyl amino acid derivatives. The advantage of resolution of *N*-formyl derivatives is their readily availability. For example, racemic *N*-formyl amines can be prepared in one step from the corresponding prochiral ketones by the Leuckart–Wallach reaction or by reaction of amines and amino acid derivatives with the mixed anhydride of acetic acid and formic acid (see Scheme 1).

To explore the scope and limitations of PDF catalyzed resolution reactions, we incubated a range of racemic *N*-formylated amines and amino acid derivatives with purified *Ec*PDF at 37 °C. Most of these reactions were performed in a buffer at pH 7.2 containing 0.1 g/L catalase to prevent inactivation. In case the activity of the PDF was too low under these conditions, the reaction was repeated in the presence of 2 M Li_2SO_4. For the resolution of *N*-formyl aminonitriles either a pH of 6.2 or 7.2 was chosen. It was expected that the aminonitriles formed were more stable at lower pH values. At both pH values used, *Ec*PDF displays maximal activity because of its broad pH-activity profile, which is nearly constant between pH 6.1 and 11.2 [15]. The results of these experiments are given in Table 4. The resolution of a few typical substrates is described in more detail in Fig. 1.

The results in Table 4 demonstrate high L-stereoselectivity for all *N*-formyl amino acid derivatives tested (entries 1–5), with *E* ratios ranging from 90 to >1500. The activity for the faster reacting enantiomer (i.e., the L-isomer), on the other hand, strongly varied for the different substrates investigated. Replacement of the phenyl group by the very bulky *tert*-butyl side-chain, for example, let to a >1000-fold lower activity (entry 4 versus entry 3), even in the presence of 2 M Li_2SO_4. Comparing the hydrolysis rates of the *N*-formyl derivative of phenylglycine (entry 1) and phenylglycine amide (entry 3) it appears that PDF is more active for formylated amino acid amides than for the corresponding amino acids. This effect was earlier observed for *N*-formyl-methionine and *N*-formyl-methione amide [15] and was explained by the fact that only the amide substrate can interact with *Ec*PDF's Gly 89 residue via a hydrogen bond [49]. It has been postulated that this hydrogen bond is communicated to the metal ligand Cys 90 thereby reducing the energy of the transition state. This is in line with the observation that the higher catalytic efficiency for *N*-formyl-methionine amide is mainly due to a higher k_{cat} and less to a reduced K_m [15]. This additional



Scheme 1. Synthesis and PDF catalyzed resolution of *N*-formyl amines and *N*-formyl amino acid derivatives. For definition of R^1 and R^2, see Table 4.

Table 4
Compilation of kinetic and enantioselectivity data for *Ec*PDF catalyzed deformylation of *N*-formyl model compounds

Entry	Compound	Type	[Compound][a] (mM)	[PDF] (μM)	k_{fast}[b] ($M^{-1}\,s^{-1}$)	k_{slow}[b] ($M^{-1}\,s^{-1}$)	*E* ratio	ee_{max} (%)[c]
1	*N*-Formyl-phenylglycine	α-H-Amino acid	10 (B)	200	10.6	0.0047	>1500	99.6 (*S*)
2	*N*-Formyl-3-amino-3-phenylpropionic acid	β-H-Amino acid	10 (B)	10	7.1	<0.004	>1500	100 (*R*)
3	*N*-Formyl-phenylglycine amide	α-H-Amino amide	10 (B)	5.2	227	0.09	>1500	99.7 (*S*)
4	*N*-Formyl-*tert*-leucine amide	α-H-Amino amide	4.8 (A)	200	0.15	0.0005	300	100 (*S*)
5	*N*-Formyl-α-methyl-phenylglycine amide	α-CH_3-Amino amide	10 (A)	200	0.045	0.0005	90	100 (*S*)
6	*N*-Formyl-phenylglycinol	β-Amino alcohol	10 (B)	200	0.69	0.029	24	90.5 (*S*)
7	*N*-Formyl-phenylglycinol	β-Amino alcohol	10 (A)	200	6.3	0.34	19	93.3 (*S*)
8	*N*-Formyl-alaninol	β-Amino alcohol	10 (A)	200	0.22	0.018	12	85.6 (*S*)
9	*N*-Formyl-phenylalanine nitrile	α-H-Aminonitrile	7.5 (C)	20	880	1	880	98.8 (*S*)
10	*N*-Formyl-valine nitrile	α-H-Aminonitrile	10 (A)	50	29.7	0.62	48	95.5 (*S*)
11	*N*-Formyl-*m*-methoxyphenylalanine nitrile	α-H-Aminonitrile	7.2 (B)	2.5	1370	2	685	99 (*S*)
12	*N*-Formyl-1-(1-naphthyl)ethylamine	Amine	0.42 (A)	200	0.45	0.03	15	90 (*R*)
13	*N*-Acetyl-phenylglycine amide	α-H-Amino amide	10 (A)	10	<0.001	<0.001	–	–
14	*N*-Formyl-proline	α-H-Amino acid	10 (A)	200	<0.004	<0.004	–	–
15	*N*-Formyl-L-methionine	α-H-Amino acid	(B)		92	n.a.[d]	–	–

[a] A, B and C refer to the respective methods given in Section 2.
[b] Initial rate constants for the faster and slower converted enantiomer.
[c] Highest ee value of the product as observed during the course of the deformylation.
[d] Not available.

hydrogen bond with the amide substrate has no effect on the enantioselectivity of *Ec*PDF, which is extremely high for both *N*-formyl-phenylglycine and *N*-formyl-phenylglycine amide: the ee of both deformylated products is >99.5% throughout both reactions, which stop at exactly 50% conversion (Fig. 1A and B). *N*-Acetyl-phenylglycine amide (entry 13) was not hydrolyzed at all by PDF, confirming earlier observations that activity is limited to *N*-formyl derivatives [50].

A very low activity was observed for the hydrolysis of the α,α-disubstituted amino acid amide *N*-formyl-(α-methyl) phenylglycine amide (entry 5), most likely due to steric hindrance by the second α-substituent. Also *N*-formyl-proline was not hydrolyzed by PDF (entry 14), probably due to the inability of hydrogen bond formation between the substrate and the Gly 45 carbonyl and/or Glu 133 carboxylate group [20] because of the lacking *N*-formyl–N–H group, although steric hindrance of the cyclic structure cannot be excluded.

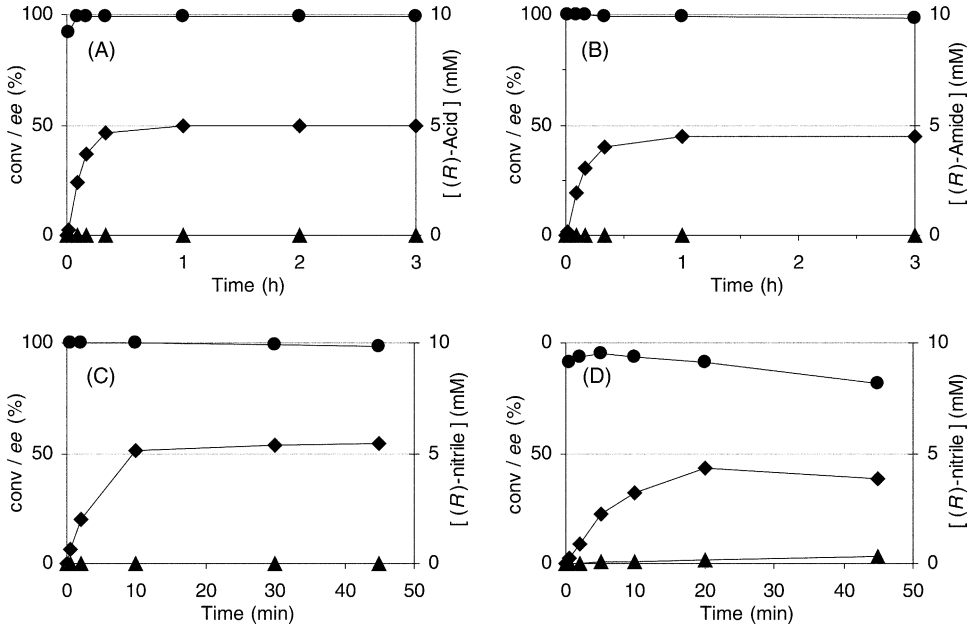


Fig. 1. Progress curves of the *Ec*PDF catalyzed resolution reaction of: (A) *N*-formyl-(*RS*)-phenylglycine, (B) *N*-formyl-(*RS*)-phenylglycine amide, (C) *N*-formyl-(*RS*)-*m*-methoxy-phenylalanine nitrile and (D) *N*-formyl-(*RS*)-valine nitrile. (●) ee of the major deformylated product formed, (◆) conversion and (▲) concentration of the minor deformylation product formed.

Hydrolysis of *N*-formyl-β-amino acids (entry 2) occurred with approximately the same activity as for the related α-amino acid derivative. For two *N*-formyl-β-amino acids a PDF resolution was performed on a semi-preparative scale. Thus, (*R*)-3-amino-3-phenylpropionic acid was obtained in >99.5% ee at nearly 39% conversion (*E* ratio >770) and (*R*)-3-amino-3-(4-methoxyphenyl)propionic acid in >99.5% ee at 34% conversion (*E* ratio >680).

The *N*-formyl derivatives of non-functionalized amines (entry 12, but also of other aliphatic amines not shown in Table 4) and β-amino alcohols (entries 6–8) were hydrolyzed by PDF only with low to moderate activity and stereoselectivity. These molecules lack the carboxyl or carboxamide group which prevents their interaction via hydrogen bonding with the backbone amino groups of Gly 45 and Ile 44 in *Ec*PDF.

To our surprise the activity and enantioselectivity of PDF in the hydrolysis of *N*-formyl α-aminonitriles (entries 9–11) was remarkably high. Although the extreme instability of phenylglycine nitrile prevented a comparison with analogous *N*-formyl substrates of other types, it is clear that *N*-formyl α-aminonitriles are amongst the most active monomeric PDF substrates tested (Scheme 2).

From the initial deformylation rate constants given in Table 4, turnover frequencies (tof) of 3.3 and 4.9 s^{-1} were calculated for the PDF catalyzed hydrolysis of *N*-formyl-phenylalanine nitrile and *N*-formyl-*m*-methoxy-phenylalanine nitrile, respectively. This is significantly >1 s^{-1}, regarded as the minimum value for a successful biocatalyst for industrial applications [51]. The activity and enantioselectivity of PDF for *N*-formyl-valine nitrile appeared to be much lower, although it cannot be excluded that the ee decrease of L-valine nitrile in time is (at least in part) caused by chemical racemization. As known from other aminonitriles the α-proton is slightly acidic and might be deprotonated under (mildly) alkaline conditions [39]. Furthermore, it appeared that the valine nitrile decomposed during the reaction (Fig. 1D), most likely due to a *retro*-Strecker reaction. Because both the racemization and the *retro*-Strecker reactions will be suppressed at acidic pH, execution of this PDF catalyzed resolution reaction at lower pH will probably give significant improvement.

3.2.2. Formylation concept

In addition to the deformylation reaction, the PDF catalyzed formylation reaction also proceeded with high selectivity. Initially, various formyl donors were tested without any success in the formylation of (*RS*)-phenylalanine nitrile under kinetic reaction conditions. With poorly activated formyl donors such as formamide, *N*-methylformamide, *N*-formylethanolamine and *N*-formylglycine no reaction occurred while ethyl formate gave chemical hydrolysis. Only with the very active formyl donor *N*-formyl-Met-Ala-OH a small amount of *N*-formyl-phenylalanine nitrile was formed (data not shown). However, since the hydrolysis of the *N*-formyl donor was much faster than the formylation of phenylalanine nitrile, this formylation is probably the result of reaction with the formate formed during the hydrolysis (vide infra).

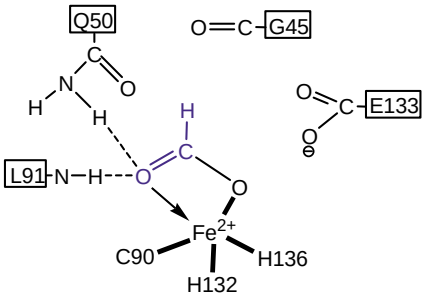
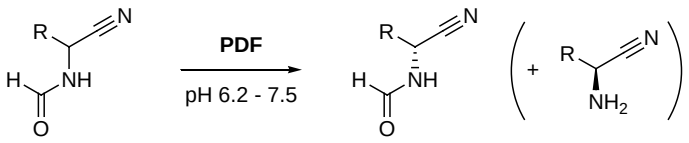


Fig. 2. Main interactions in the activated *Ec*PDF-formate complex as proposed by Becker et al. [20].

From earlier work, it is known that *Ec*PDF in principle can transfer the formyl group from *N*-formyl-Met-Ala-OH to H-Met-Ala-Ser-OH by a "ping-pong" mechanism [15]. This transformylation reaction proceeds via the activated enzyme-formate complex shown in Fig. 2; the same intermediate is also formed in deformylation reactions. In the latter reactions, this activated intermediate is attacked by water liberating formate and the free enzyme. There is strong evidence that this formate release proceeds by attack of a water molecule on the Fe(II) atom rather than on the formate carbonyl carbon, so by a "ligand exchange" reaction [15]. In transformylation reactions, on the other hand, the carbonyl carbon of the metal ion-formate complex is attacked by the amino group of the second substrate molecule. Our results with *N*-formyl-Met-Ala-OH as formyl donor show that phenylalanine nitrile is a poor formyl acceptor as compared to the tripeptide H-Met-Ala-Ser-OH, probably due to a much lower affinity of *Ec*PDF for this substrate. Therefore, the ligand exchange reaction mentioned above will have occurred long before phenylalanine nitrile to enter the active site, resulting in deformylation instead of transformylation. The transformylation reaction might be



Scheme 2. PDF catalyzed deformylation of *N*-formyl-α-aminonitriles.

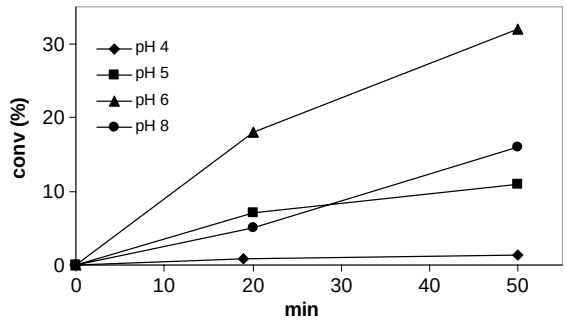


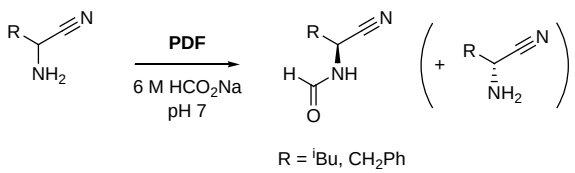
Fig. 3. Determination of the optimum pH of the PDF catalyzed formylation of racemic 2-amino-3-phenylpropionitrile.

promoted by reducing the ligand exchange. It is known that ligand exchange of water molecules on metal ions like Fe^{2+}, Co^{2+} and Zn^{2+} is very fast, i.e., in the order of 10^6–$10^7\ s^{-1}$ [52]. Replacing the active site metal ion by, e.g., Cr^{3+} will dramatically decrease the ligand exchange rate, in which case transformylation might be possible. Further research should reveal if these metal ions can be incorporated in the *Ec*PDF and if the resulting enzyme is still active.

Formylation of aminonitriles under thermodynamic conditions with sodium or ammonium formate gave better results. Using the method of Halling et al. [53], the equilibrium constant for the formylation reaction is estimated to have a maximum value of $K_{eq} = 2 \times 10^2\ M^{-1}$ at pH 4.0 (the pH with the highest concentration of both free formic acid (pK_a 3.5) and free aminonitrile (approximate pK_a 4.6)). Clearly the low pK_a value of the aminonitrile favors product formation, especially at high concentrations.

To optimize the PDF catalyzed formylation reaction, racemic phenylalanine nitrile was incubated with 6 M aqueous sodium formate at various pH values between 4 and 8. From the results, as shown in Fig. 3, it can be concluded that the reaction kinetics are optimal at a pH value between 6 and 7, being the lower value of the pH optimum of PDF. At this pH, the equilibrium constant K_{eq} is estimated to be $10\ M^{-1}$, inferring significantly lower product formation than at pH 4.

The PDF catalyzed formylation reactions were repeated on preparative scale for two racemic aminonitriles, i.e., derived from phenylalanine and from leucine (Scheme 3). At a conversion of 38–39% the two (*S*)-*N*-formyl aminonitriles were obtained in >99 and >98% ee, respectively, by extraction from the acidified reaction mixture, corresponding to an *E* ratio of >200 for both formylation reactions. The ee (and conversion) of the formylation reaction of phenylalanine nitrile was even further improved by crystallization of the product from the reaction mixture, giving (*S*)-*N*-formyl-phenylalanine nitrile with >99.5% ee.



Scheme 3. PDF catalyzed formylation of α-aminonitriles.

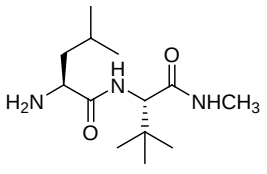
The PDF catalyzed formylation of aminonitriles has also been successfully performed in organic solvents, such as toluene and *n*-heptane. For example, racemic phenylalanine nitrile was formylated with ammonium formate in *n*-heptane using the freeze-dried CFE of PDF ([substrate] 10.3 mM; [E] 175 μM; [HCO_2NH_4] 0.9 M (only partially soluble), 40 °C), albeit with a somewhat lower enantioselectivity than in aqueous solution. After 6 h, (*S*)-*N*-formyl-phenylalanine nitrile was formed in 79% ee. Due to the low solubility in *n*-heptane (0.34 mM for the racemic *N*-formyl-phenylalanine nitrile), the product crystallized in an enriched form (90% ee, no further details given).

Because of the problems with spontaneous racemization and degradation of free α-aminonitriles (vide supra), both PDF catalyzed reaction concepts are complementary. In general, the hydrolysis reactions will only furnish the remaining (*R*)-*N*-formyl-α-aminonitriles, whereas under formylation conditions only the (*S*)-*N*-formyl-α-aminonitrile products can be obtained.

3.3. PDF catalyzed deprotection of N-formyl-dipeptides

Besides the use of PDF in resolution reactions for the synthesis of enantiopure amines and amino acid derivatives, we also investigated its application in peptide synthesis. As the deformylation of (small) peptides resembles the natural function of PDF, we expected a much higher activity in these reactions.

Chemical peptide synthesis, either in solution or by solid phase, generally proceeds by elongation at the N-terminal side. In academic practice N-protecting groups like *tert*-butoxycarbonyl (Boc), benzyloxycarbonyl (Cbz) and



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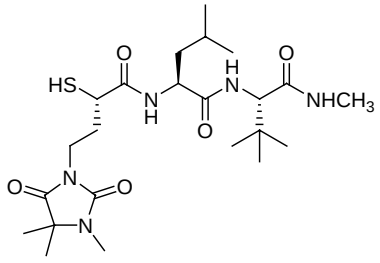

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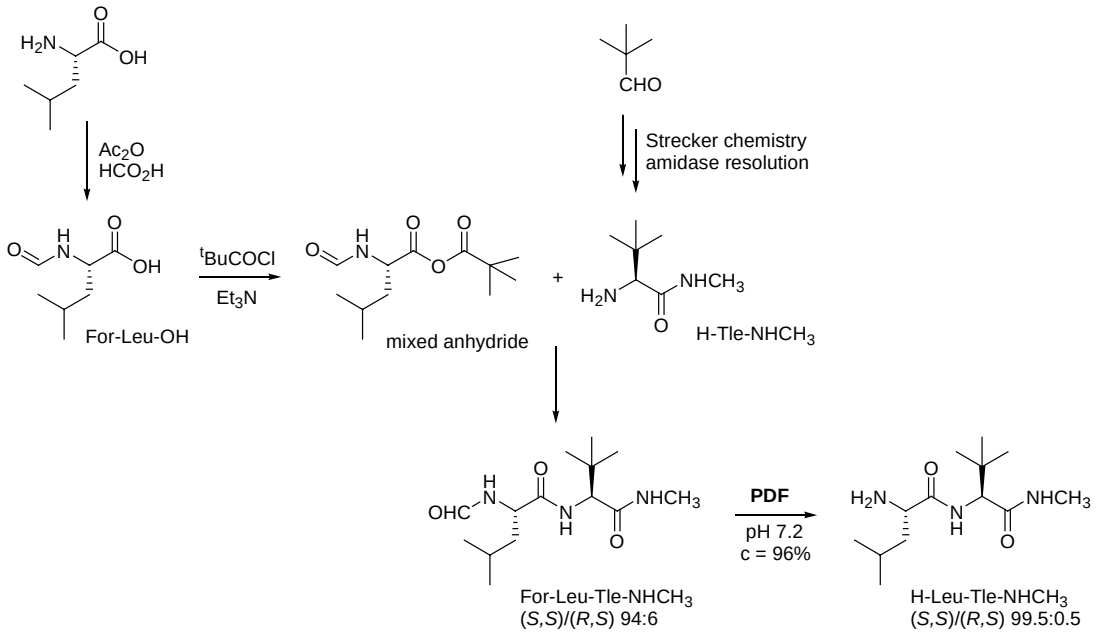
Fig. 4. Structures of H-(*S*)-Leu-(*S*)-Tle-$NHCH_3$ (**1**) and matrix metalloproteinase inhibitor BMS275291 (**2**).



Scheme 4. Synthesis and PDF catalyzed deformylation process for H-(*S*)-Leu-(*S*)-Tle-$NHCH_3$ dipeptide.

especially fluorenylmethoxycarbonyl (Fmoc) are used in combination with advanced coupling reagents like carbodiimides, phosphonium salts or uronium/guanidinium salts, like EDC/HOSu, EDC/HOBt or PyBOP [54].

Although these coupling methods work well on laboratory scale and proceed without substantial racemization, for commercial application more cheap protecting groups and coupling reagents are required. A particularly economically interesting amino protective group is formyl, which can be readily and cheaply introduced using formic acid and acetic acid anhydride. An example of an industrial peptide coupling is described in Scheme 4 for the preparation of *N*-formyl-Leu-Tle-$NHCH_3$. The dipeptide H-(*S*)-Leu-(*S*)-Tle-$NHCH_3$ is an interesting intermediate for a novel class of inhibitors of matrix metalloproteinases (see Fig. 4) [55–57].

The coupling of *N*-formyl-(*S*)-Leu-OH with H-(*S*)-Tle-$NHCH_3$, which was synthesized from 2,2-dimethylpropionaldehyde (pivaldehyde) via DSM Pharma Chemicals' amidase process [37], was performed using the mixed anhydride method, which is also easy to perform on large scale [47]. Disadvantage of this method is that to some extent racemization of the *N*-formyl-(*S*)-Leu mixed anhydride occurs [47]. Because of this racemization, a 94:6 mixture of the (*SS*) and (*RS*) diastereomers was obtained by this procedure. Thus, chemical hydrolysis of the *N*-formyl protecting group resulted in a diastereomeric dipeptide mixture. Additionally, chemical deformylation was accompanied by some peptide bond hydrolysis.

As an alternative for the chemical deprotection we tested the enzymatic deformylation using *Ec*PDF under standard reaction conditions (pH 7.2, 37 °C, [PDF] 2 μM).

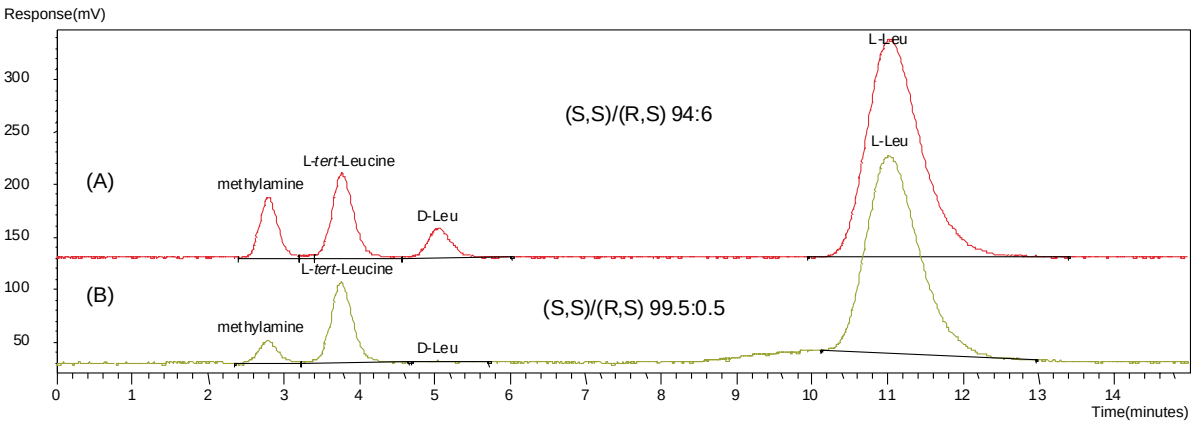


Fig. 5. ee determination of Leu moiety in H-Leu-(*S*)-Tle-$NHCH_3$ obtained from *N*-formyl-Leu-(*S*)-Tle-$NHCH_3$ with 88% de by (A) chemical deformylation and (B) PDF catalyzed deformylation.

This resulted in a completely selective hydrolysis of the formyl group (so without any detectable peptide hydrolysis). Additionally, since PDF is highly L-enantioselective, only the (*SS*) diastereomer was hydrolyzed, leaving the (*RS*) diastereomer unaffected. Thus, from the 94:6 mixture almost diastereomerically pure H-(*S*)-Leu-(*S*)-Tle-$NHCH_3$ was obtained after 96% conversion by deformylation with purified *Ec*PDF. The optical purity of the product obtained was determined by chiral HPLC analysis after hydrolysis of the dipeptide into the separate amino acids compounds, the chromatogram of which is shown in Fig. 5.

It has to be noted that for this application the use of purified *Ec*PDF is essential, because deformylation with cell free extract resulted in simultaneous extensive peptide bond hydrolysis, most likely as a result of leucine aminopeptidase activity originating from the *E. coli* strain used for the overexpression of the PDF (data not shown).

4. Conclusions

Purified *E. coli* PDF as well as cell free extract of an *E. coli* PDF overproducing strain can be successfully applied to prepare various enantiomerically enriched amines and amino acid derivatives by enantioselective hydrolysis of the corresponding *N*-formyl derivatives. Although the *N*-formyl derivatives of non-functionalized amines and β-amino alcohols are only resolved with low to moderate activity and enantioselectivity, the resolution of α- and β-amino acids, α-amino acid amides and α-aminonitriles all show almost complete enantioselectivity with *E* ratios >500. Of these substrates, *N*-formyl-α-aminonitriles are hydrolyzed with the highest activity. In addition to the resolution by deformylation, α-aminonitriles can also be resolved by a PDF catalyzed formylation reaction. In this way both enantiomers of the more stable *N*-formyl-α-aminonitriles can be obtained in enantiopure form.

PDF also appeared to be suitable for the mild and selective deprotection of *N*-formyl peptides as was shown for *N*-formyl-(*S*)-Leu-(*S*)-Tle-$NHCH_3$. Using this PDF catalyzed deprotection, the *N*-formyl protecting group can now find wide application in peptide synthesis. An additional advantage of this enzymatic deprotection is that the N-terminal *R*-isomers are not deformylated, so that the diastereomeric purity of the peptide is significantly improved.

Acknowledgements

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