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Cutting Long Syntheses Short: Access to Non-Natural Tyrosine Derivatives Employing an Engineered Tyrosine Phenol Lyase

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Abstract: The chemical synthesis of 3-substituted tyrosine derivatives requires a minimum of four steps to access optically enriched material starting from commercial precursors. Attempting to short-cut the cumbersome chemical synthesis of 3-substituted tyrosine derivatives, a single step biocatalytic approach was identified employing the tyrosine phenol lyase from *Citrobacter freundii*. The enzyme catalyses the hydrolysis of tyrosine to phenol, pyruvate and ammonium as well as the reverse reaction, thus the formation of tyrosine from phenol, pyruvate and ammonium. Since the wild-type enzyme possessed a very narrow substrate spectrum, structure-guided, site-directed mutagenesis was required to change the substrate specificity of this C–C bond forming enzyme. The best variant M379V transformed, for example, *o*-cresol, *o*-methoxyphenol and *o*-chlorophenol efficiently to the corresponding tyrosine derivatives without any detectable side-product. In contrast, all three phenol compounds were non-substrates for the wild-type enzyme. Employing the mutant, various L-tyrosine derivatives (3-Me, 3-OMe, 3-F, 3-Cl) were obtained with complete conversion and excellent enantiomeric excess (>97%) in just a single 'green' step starting from pyruvate and commercially available phenol derivatives.

Keywords: biotransformations; C–C bond formation; non-natural amino acids; tyrosine derivatives; tyrosine phenol lyase

Introduction

Tyrosine derivatives are key intermediates in the synthesis of several anticancer compounds: For example, 3-chlorotyrosine is a building block for Cryptophycin 1 and Cryptophycin 52, which show antimitotic activity.[1] 3-Methoxytyrosine was used as building block in the synthesis of cyclopeptide ligands binding to the Grb2-SH2 domain, an attractive target for anticancer therapeutic interventions.[2] 3-Methyl-DL-tyrosine[3] was used as a precursor for anti-cancer therapeutics as well as 3,5-dimethyltyrosine which is a starting material for a $P2X_7$-receptor antagonist.[4] Other derivatives are part of pharmaceuticals like safracin, ecteinascidin-743, cribrostatin 4 and a tumour necrosis factor-related apoptosis-inducing ligand (TRAIL).[5] 3-Methoxytyrosine is also one of the main biochemical markers for aromatic L-amino acid decarboxylase deficiency in humans[6] and 3-chlorotyrosine is discussed as a marker for atherosclerosis.[7]

The classical synthetic route for these tyrosine derivatives usually comprises several steps including protection and deprotection of the functional amino acid groups making it very laborious and time-consuming.[1,8,9] Our aim was to evaluate chemical and biocatalytic options for the preparation of optically enriched 3-substituted tyrosine derivatives.

Results and Discussion

Chemoenzymatic Approach

3-Methyltyrosine **1a** was chosen as a first target, since the proposed synthesis of optical enriched material would involve only four steps starting from commercial precursors: that is, three chemical steps and one biocatalytic kinetic resolution (Scheme 1).[1,8,9] Due to the low overall yield (<1%) no kinetic resolution attempts were performed.

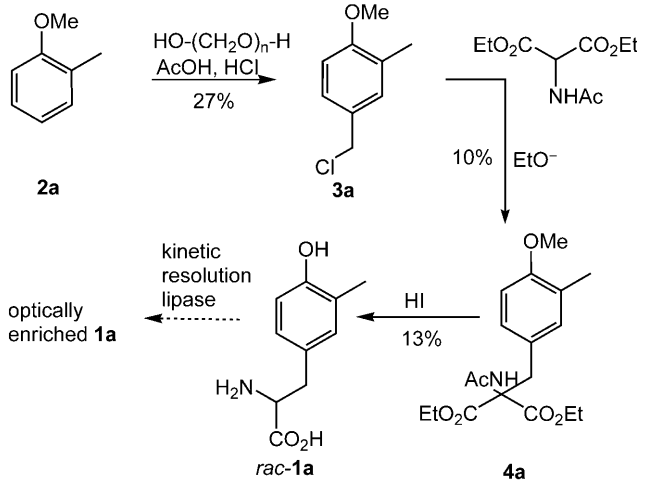
Since this reaction sequence led to unsatisfactory results, we envisaged an alternative route for the synthesis of non-natural tyrosine derivatives.

Single-Step Biocatalytic Approach

Searching for alternatives we came across tyrosine phenol lyase (TPL, EC 4.1.99.2), a pyridoxal 5-phosphate (PLP)-dependent enzyme which *in vivo* catalyses the reversible β-elimination reaction of L-tyrosine leading to phenol, ammonium ion and pyruvate.[10] Apart from its natural reaction, tyrosine phenol lyase can also catalyse the reversible β-elimination of various β-substituted amino acids and derivatives as well as L-tyrosine structural analogues.[10,11] For instance, L-DOPA was prepared from catechol, pyruvate and ammonium employing the TPL from *Erwinia herbicola*.[12] Furthermore, activity for the formation of various tyrosine derivatives was reported employing the TPL from *Citrobacter intermedius*.[13] However, already the preparation of derivatives bearing a small substituent like a fluoro moiety on the aromatic ring required long reactions times between 3 days up to two weeks.[14] We decided to employ the TPL from *Citrobacter freundii* for our studies (Scheme 2), since it has been characterised in detail concerning structure and reaction mechanism.[15] Additionally, it has been employed in the preparation of 3-azidotyrosine.[16]

The synthetic gene of tyrosine phenol lyase from *Citrobacter freundii* optimised for expression in *E. coli* was purchased from GeneArt. For overexpression the gene was inserted into the commercial plasmid pET21a (+).

In order to verify the activity of the recombinant enzyme, the pH range was tested with phenol as substrate (Figure 1). The pH optimum was found in the range of pH 8–9, which is consistent with the native enzyme.[17] All further experiments were performed in a potassium phosphate buffer (50 mM, pH 8) with ammonium (180 mM) and PLP (0.04 mM).



Scheme 1. Chemoenymatic route for the synthesis of **1a**.

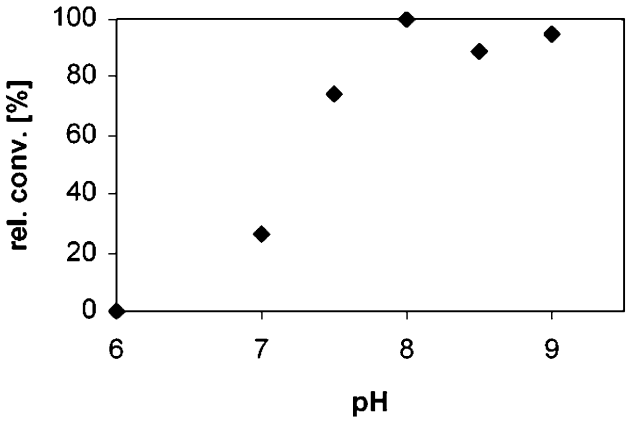
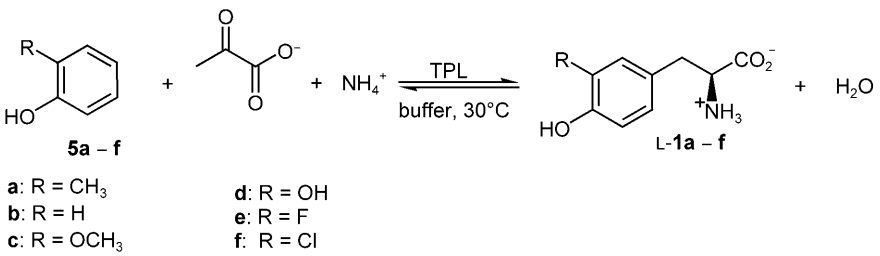


Figure 1. pH range of recombinant tyrosine phenol lyase.



Scheme 2. Single-step biocatalytic synthesis of L-tyrosine derivatives catalysed by tyrosine phenol lyase (TPL).

Since the non-natural phenol derivatives to be transformed are poorly soluble in aqueous media, three different approaches were investigated: the phenolic substrate was pre-dissolved (i) in buffer at pH > 11, (ii) in a water miscible organic solvent (acetonitrile) or (iii) in a non-water miscible organic solvent (diethyl ether) leading to a two-phase reaction system. The first approach (i) proved to be not successful. Nevertheless, measuring the activity of the reaction for phenol (23 mM) at varied acetonitrile as well as diethyl ether concentrations showed that the organic solvent did not cause a significant loss of apparent activity of the enzyme when applied in the range of 5–10% (v/v) (Figure 2). As a compromise between high apparent activity and high amount of solvent, 7% (v/v) of organic solvent was applied for the further experiments.

In a second optimisation step the phenol concentration was varied between 23 mM and 237 mM using acetonitrile or diethyl ether as additional solvent as described above. The ammonia concentration was kept constant at 180 mM while two equivalents of pyruvate with respect to the amount of phenol were added. These results were compared to reactions carried out without cosolvent (Figure 3). For acetonitrile and in the absence of cosolvent the highest apparent activity was achieved at 55 mM substrate concentration. At 237 mM no activity was observed for these two approaches. In the case of diethyl ether, however, activity comparable to lower substrate concentration was observed even at 237 mM substrate concentration. It should be added that without cosolvent at 55 mM and 118 mM tyrosine formation could be easily followed due to the precipitation of the product.

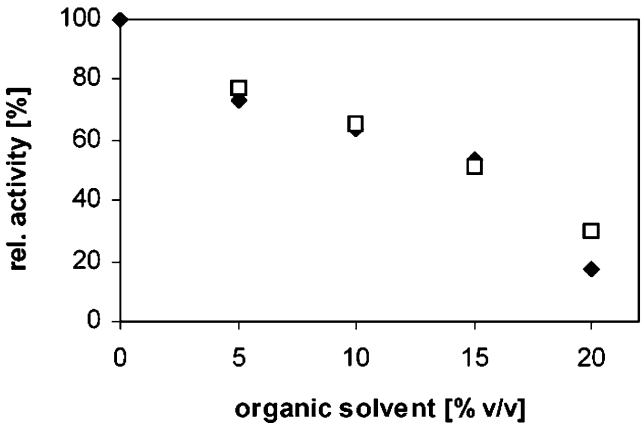


Figure 2. Activity of TPL at varied organic solvent concentrations. □ Diethyl ether (2 phases); ◆ acetonitrile (single phase).

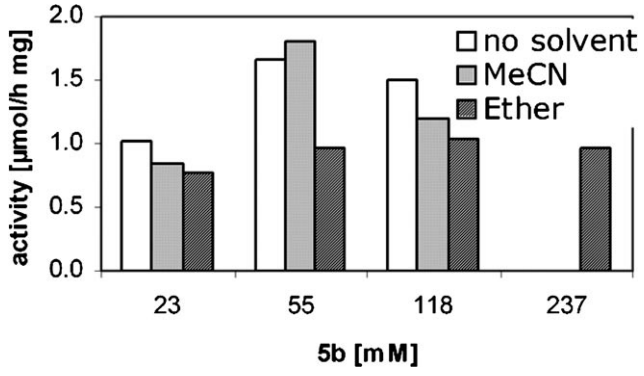


Figure 3. Tyrosine formation at varied phenol concentrations (pyruvate 46 mM, ammonia 180 mM).

Thus, substrate concentrations of 240 mM can be applied by using a two-phase system with diethyl ether.

Recombinant wild-type TPL from *Citrobacter freundii* was able to transform catechol **5d** as well as *o*-fluorophenol **5e**, but failed to catalyse the conversion of phenol derivatives of interest such as *o*-methyl, *o*-methoxy, and *o*-chlorophenol (Table 1). Therefore, the next aim was to engineer the active site of the enzyme by structure-guided, site-directed mutagenesis.[18]

We analysed the active site in the crystal structure of the TPL from *Citrobacter freundii* in complex with 3-(4′-hydroxyphenyl)-propionic acid (PDB-entry 2TPL)[15c] and identified five amino acids – Phe36, Thr125, Met288, Met379 and Phe448 – in the vicinity of the phenolic moiety of the bound ligand, which could (unfavourably) interact with additional substituents on the aromatic ring (Figure 4). Replacement of these residues by smaller amino acids should widen the active site and provide space for substituents in the *o*-position of phenol. Thr125 is located on the same side as the cofactor PLP while the other four amino acids are situated on the opposite side (Figure 4). Other amino acids in the active site, such as Tyr71, Thr124 and Arg381, were not considered, because they have previously been described to be re-

Table 1. Conversions (%) for the formation of tyrosine derivatives employing wild-type TPL from *Citrobacter freundii* and engineered variants thereof.[a]

Substrate		Mutants					
	R	wt	F36V	T125S	M288V	M379V	F448V
5a	CH_3	n.c.	35	n.c.	n.c.	99	n.c.
5b	H	40	13	35.3	12	27	39
5c	OMe	n.c.	3.4	17	n.c.	52	n.c.
5d	OH	24	n.c.	n.c.	n.c.	n.c.	n.c.
5e	F	99	6	63	0.5	89	19
5f	Cl	n.c.	n.c.	6	n.c.	58	n.c.

[a] *Reaction conditions:* 23 mM phenol derivative, 180 mM ammonia, 46 mM pyruvate, 2 mg enzyme preparation, 30 °C, 24 h. Conversion measured by HPLC. wt = recombinant wild-type enzyme; n.c. = no conversion.

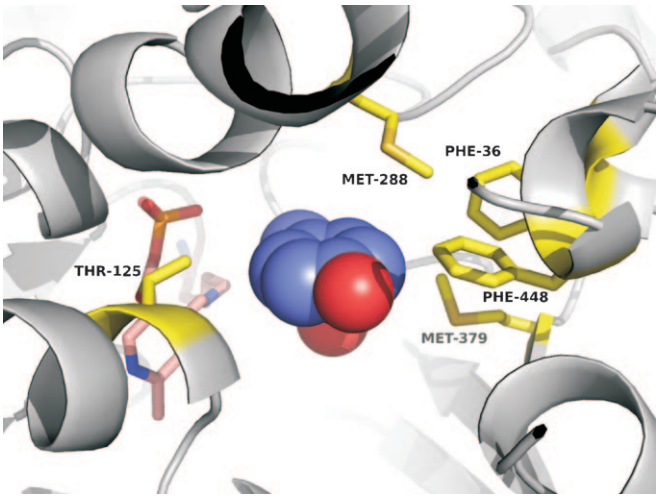


Figure 4. Active site of tyrosine phenol lyase from *Citrobacter freundii* with target amino acids for site-directed mutagenesis (yellow), inhibitor bound (blue), and pyridoxal 5-phosphate (pink).

quired for the catalytic activity.[19] To minimise structural alterations, the non-polar amino acids were replaced by valine, while Thr125 was substituted by serine in order to preserve the hydrogen bonding interaction to Glu103.

The corresponding variants were tested for the transformation of the phenol derivatives **5a–f** (Table 1). Additionally, the variant T125V was prepared but showed low activity for our purposes.

The variants T125S and F448V showed comparable conversions as the wild-type enzyme for phenol. However, only T125S showed a slightly broader substrate spectrum accepting also guaiacol **5c** and *o*-chlorophenol **5f**. Definitely the most suitable variant for the *o*-substituted phenol derivatives **5a**, **5c**, and **5f** turned out to be M379V. All three phenol derivatives as well as *o*-fluorophenol **5e** led to good conversion. Interestingly the conversion for the natural substrate phenol was reduced and the activity for catechol **5d** had vanished.

The preparative applicability of the biocatalytic synthesis of the tyrosine derivatives **1a**, **1c**, **1e** and **1f** was demonstrated using the M379V variant on a 60-mg scale. The reaction was run until all phenol derivative as starting material was consumed (24 h). The products L-**1a**, L-**1c**, L-**1e** and L-**1f** were then purified employing a strong cationic exchanger and characterised by ^{1}H NMR and after derivisation [silylation with (*N,O*-bis(trimethylsilyl)acetamide) BSA and trimethylchlorosilane (TMCS)] by GC-MS. Analysis of the optical purity by HPLC on a chiral stationary phase showed that all products obtained resulted exclusively in one single peak (>97% *ee*).

Conclusion and Outlook

By employing an engineered tyrosine phenol lyase (TPL), it was possible to synthesise various monosubstituted tyrosine derivatives in one single step instead of performing multi-step reactions. The biocatalytic one-step approach represents a 'green' methodology in contrast to the published chemical synthetic sequences. Precursors for anticancer compounds could be prepared after investigating the crystal structure of the TPL from *Citrobacter freundii* and using a rational approach identifying suitable amino acids for site-directed mutagenesis. With the exchange of only one amino acid in the active site of the enzyme the substrate spectrum could be significantly modified. The best identified variant M379V allowed the synthesis of non-natural tyrosine derivatives possessing a chloro-, methoxy or methyl substituent in position 3′ of tyrosine.

Experimental Section

For detailed information on the chemical organic synthesis of *rac*-3-methyltyosine including NMR and GC-MS data see Supporting Information.

General Remarks

Phenol, *o*-cresol, guajacol, 3-fluorophenol, 3-chlorophenol, catechol, sodium pyruvate, NH_4Cl, $(NH_4)_2SO_4$, pyridoxal 5-phosphate and tryptamine were purchased from Sigma Aldrich. All primers for site directed mutagenesis, vector pET21a (+), chemical competent *E. coli* TOP10 cells and chemical competent *E. coli* BL21(DE3) cells were purchased from Invitrogen. dNTP mix (2 mM each), Pfu polymerase, Pfu buffer + $MgSO_4$ 10×, DpnI and H_2O of molecular biology grade were purchased from Fermentas. The gene of tyrosine phenol lyase from *Citrobacter freundii* was purchased from GeneArt and cloned into pET21a (+) for overexpression. All Eppendorf vials and pipette tips for the work with DNA were autoclaved prior to use and water was of molecular biology grade.

Preparation of the Cell-Free Extract

An overnight culture (ONC) of *E. coli* was prepared and 330 mL of LB media (ampicillin 100 μg mL^{-1}) were inoculated with 10 mL of ONC. The cells were grown at 30 °C and 120 rpm until the OD_{600} reached 0.4. Then IPTG (0.5 mM) was added and the cells were incubated for 2 h at 20 °C and 120 rpm. The cells were harvested at 4 °C and 8000 rpm for 20 min. The supernatant was discarded and the cells resuspended in potassium phosphate buffer [10 mL, 50 mM, pH 8, 100 mM NH_4Cl, 40 mM $(NH_4)_2SO_4$, 0.04 mM PLP]. Ultrasonication was performed with 40% amplitude, 1 sec pulse and 2 sec pause for 5 min with ice-bath cooling using a Branson Digital Sonifier 250. The cell debris was removed

by centrifugation at 4 °C and 15000 rpm for 20 min and the supernatant was lyophilised to give the cell-free extract.

General Procedure for Activity Testing

Lyophilised cell-free extract (2 mg) was rehydrated in a potassium phosphate buffer [640 μL, 50 mM, pH 8, 100 mM NH_4Cl, 40 mM $(NH_4)_2SO_4$, 0.04 mM PLP] for 30 min at 30 °C and 120 rpm. Then phenol (derivative) stock (16 μmol in acetonitrile, final concentration 23 mM) and pyruvate (32 μmol, final concentration 46 mM) were added and the sample was incubated at 30 °C and 120 rpm for 24 h and worked up for cation exchange HPLC measurements.

Variation of Substrate Concentration

Lyophilised cell-free extract (2 mg) was rehydrated in potassium phosphate buffer [50 mM, pH 8, 100 mM NH_4Cl, 40 mM $(NH_4)_2SO_4$, 0.04 mM PLP] for 30 min at 30 °C and 120 rpm. Then phenol derivative (1 equiv.) and pyruvate (2 equiv.) were added. The concentration of phenol derivative was varied between 23 mM and 237 mM, the concentration of cosolvent (acetonitrile, diethyl ether) was kept at 10% (v/v).The samples were incubated at 30 °C and 120 rpm for 2 h using the recombinant wild-type enzyme and 4 h for the variant M379V. Then the samples were prepared for cation exchange HPLC measurements. For detailed information see the Supporting Information.

Preparative Biocatalytic Synthesis of Tyrosine Derivatives

Pyruvate (0.85 mmol, final concentration 120 mM), and lyophilised cell-free extract (20 mg, recombinant wild-type or variant) were mixed with potassium phosphate buffer [6.8 mL, 50 mM, pH 8, 100 mM NH_4Cl, 40 mM $(NH_4)_2SO_4$, 0.04 mM PLP] in a Sarstedt tube (15 mL). Then phenol (derivative) (0.3 mmol in diethyl ether, final concentration 43 mM) was added in two portions during 2 h to the reaction solution The concentration of the cosolvent was kept at 10%
(v/v). The reaction was incubated at 30 °C and 120 rpm for 24 h until no starting material could be detected anymore. Phosphoric acid was added (2 mL, 85%) to the reaction mixture and mixed well. Then the solution was filtered and the amino acid was purified *via* flash chromatography using the cationic exchanger Lewatit MonoPlus SP-112 (column size: Ø 3 cm, height: 10 cm; ordering number: Fluka 62102). The column was washed prior to use with HCl (6 M, 2 volumes), NaOH (6 M, 2 volumes) and water (2 column volumes). The reaction solution was acidified (pH <3) using phosphoric acid (85%) and applied onto the column. After washing with water the amino acid was eluted with aqueous NH_4OH (8%). The fractions showing a positive ninhydrin reaction were combined and lyophilised yielding the tyrosine derivative with remaining salts. The absolute amount of amino acid was determined using the standard ninhydrin assay.[20] The resulting amino acid was analysed by ^1H NMR and after derivatisation by BSA and TMCS by GC-MS. Optical purity was determined by HPLC on a chiral stationary phase (see Supporting Information).

L-3-Methyltyrosine (1a): yield: 40 mg (68%); ^1H NMR (300 MHz, D_2O, TMS): $\delta = 6.98$ (s, 1H, Ar), 6.89–6.90 (m, 1H, Ar), 6.72–6.79 (m, 1H, Ar), 3.80–3.84 (m, 1H, Ar-CH_2-C*H*), 3.02–3.08 (m, $1H_A$, Ar-CH_AH_B), 2.90–2.95 (m, $1H_B$, Ar-CH_AH_B), 2.09 (s, 3H, CH_3); MS (EI): $m/z = 57$, 73, 100, 117, 147, 193, 218 (100%), 294, 324, 368, 396 (trimethylsilyl derivative); *ee* >97% (HPLC).

L-3-Methoxytyrosine (1c): yield: 38 mg (60%), ^1H NMR (300 MHz, D_2O, TMS): $\delta = 6.76$–6.79 (m, 2H, Ar), 6.63–6.65 m, 1H, Ar), 3.78–3.82 (m, 1H, Ar-CH_2-C*H*), 3.70 (s, 3H, OCH_3), 2.99–3.06 (m, $1H_A$, Ar-CH_AH_B), 2.88–2.95 (m, $1H_B$, Ar-CH_AH_B); MS (EI): $m/z = 73$, 100, 133, 146, 179, 209, 218 (100%) (trimethylsilyl derivative); *ee* >97% (HPLC).

L-3-Fluorotyrosine (1e): yield: 50 mg (83%), ^1H NMR (300 MHz, D_2O, TMS): $\delta = 6.84$–6.96 (m, 3H, Ar), 3.79–3.83 (m, 1H, Ar-CH_2-C*H*), 3.00–3.06 (m, $1H_A$, Ar-CH_AH_B), 2.94–2.96 (m, $1H_B$, Ar-CH_AH_B); MS (EI): $m/z = 73$, 100, 130, 147, 197, 218 (100%), 298 (trimethylsilyl derivative); *ee* >97% (HPLC).

L-3-Chlorotyrosine (1f): yield: 17 mg (26%), ^1H NMR (300 MHz, D_2O, TMS): $\delta = 7.14$ (s, 1H, Ar), 6.86–6.93 (m, 2H, Ar), 3.76–3.79 (m, 1H, Ar-CH_2-C*H*), 2.97–3.03 (m, $1H_A$, Ar-CH_AH_B), 2.86–2.93 (m, $1H_B$, Ar-CH_AH_B); MS (EI): $m/z = 73$, 147, 207, 218 (100%) (trimethylsilyl derivative); *ee* >97% (HPLC).

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