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Biocatalytic One-Pot Synthesis of L-Tyrosine Derivatives from Monosubstituted Benzenes, Pyruvate, and Ammonia

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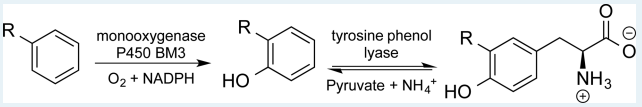
Supporting Information

ABSTRACT: L-Tyrosine derivatives were obtained in >97% ee via a biocatalytic one-pot two-step cascade using substituted benzenes, pyruvate, and NH_3 as starting materials. In the first step, monosubstituted arenes were regioselectively hydroxylated in the *o*-position by monooxygenase P450 BM3 (using O_2 as oxidant with NADPH-recycling) to yield the corresponding phenols, which subsequently underwent C−C coupling and simultaneous asymmetric amination with pyruvate and NH_3 using tyrosine phenol lyase to furnish L-DOPA surrogates in up to 5.2 g L^{-1}. Instead of analytically pure arenes, crude aromatic gasoline blends containing toluene were used to yield 3-methyl-L-tyrosine in excellent yield (2 g L^{-1}) and >97% ee.



KEYWORDS: *tyrosine, lyase, phenol, monooxygenase, hydroxylation*

Proteinogenic (canonical) and nonproteinogenic *α*-amino acids are among the top-five families of natural compounds used for the synthesis of materials, pharmaceuticals, and nutrients.[1] Enantiomerically pure L-tyrosine derivatives represent key precursors for anticancer drugs (e.g., saframycin A), biochemical markers, and are important noncanonical amino acids for synthetic biology.[2] In particular, 3-methoxy-L-tyrosine (L-**4c**) is a pro-drug of L-DOPA, as it is significantly more stable and shows beneficial properties in the treatment of Parkinson's disease.[3] In addition, it constitutes an important biochemical marker for human L-amino-acid decarboxylase deficiency.[2c,4] Chemical *de novo* synthesis of tyrosine derivatives proceeds through several steps and leads to racemic material,[5] which requires late-stage racemate resolution. Direct functionalization of L-Tyr is based on protective group chemistry[2e] or needs harsh reagents, such as F_2 or HNO_3.[6] In order to shortcut chemical synthesis, a biocatalytic C−C coupling was developed employing tyrosine phenol lyase mutant M379V (TPL) from *Citrobacter freundii*.[7] Despite the good yields and excellent stereoselectivity (conversions 27−99%, ee's >97%), this method depends on *o*-substituted phenols (**3a−f**) as indispensable starting materials. The latter are industrially produced by air-oxidation of substituted benzenes through the Hock-process with low efficiency (~5% yield).[8] Due to the harsh reaction conditions (~20 bar, > 100 °C, strong acids),[8,9] isomer purification is necessary to obtain *o*-substituted phenols in high purity. Alternatively, phenol can be synthesized from benzene using a fungal peroxidase and H_2O_2 as oxidant.[10] However, due to overoxidation issues and the use of H_2O_2, this method is unsuitable for a multienzyme cascade. Aiming at a more efficient process for synthesis of L-tyrosine derivatives, we designed a synthetic one-pot biocascade that utilizes the combined activity of a monooxygenase and a C−C lyase (Scheme 1).

In the first step, the regio- and chemoselective *o*-hydroxylation of an arene (**1a−f**) is catalyzed by a mutant of P450 BM3 (variant M2: R47S, Y51W, I401M)[11,12] with high *o*-selectivity (**3a−f** vs **2a−f** > 95/5). The use of atmospheric O_2 as oxidant allowed us to combine it in situ with the TPL-catalyzed C−C coupling of the *o*-phenolic intermediate (**3a−f**) to pyruvate under consumption of NH_3 to afford L-**4a-f**.[7] The feasibility of this cascade was evaluated using **1c** as model substrate.[7a,12a] P450 BM3 and TPL were both employed as cell-free lyophilized lysate providing an "off-the-shelf" catalyst that can be stored over several months. Initial conversions of **1c** using reported reaction conditions for TPL[7a] yielded ~5% of L-**4c**, proving that both enzymatic steps can be performed simultaneously in a one-pot fashion (see Supporting Information (SI); Figure S1). However, the majority of intermediate **3c** (83%) remained unconverted, thus identifying the C−C coupling (step 2) as bottleneck.

In order to avoid inhibition of TPL, the concentration of reaction components was varied to elucidate their effect on the cascade productivity and (chemo)selectivity (defined as % of L-**4a-f** in the product mixture). A substantial effect on the selectivity for L-**4c** was identified when varying concentrations of $NADP^+$ and P450 BM3, whereas the presence of gluconic acid (<100 mM, derived from nicotinamide-recycling) or intermediate **2c** had no influence on TPL activity (see SI). However, the most critical parameter for the insufficient coupling between steps 1 and 2 turned out to be the drop in pH due to release of gluconic acid during NADPH-regeneration,[13] which shifts the equilibrium of the TPL-reaction unfavorably toward C−C cleavage (pH ≤ 7).[7a,c] In order to maintain a slightly alkaline pH to support C−C coupling, the

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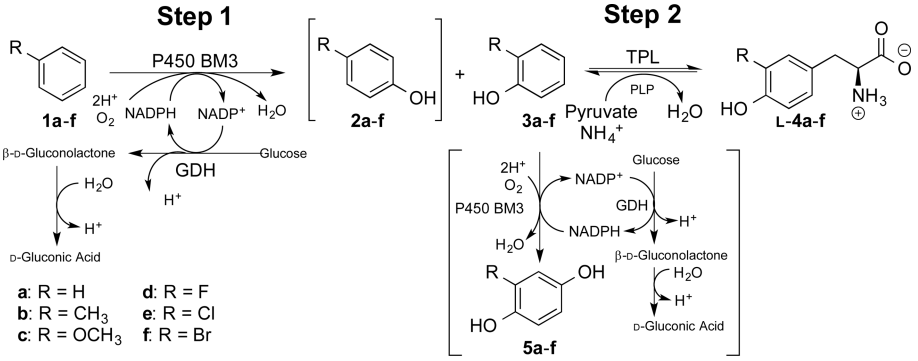
Scheme 1. One-Pot Two-Step Biocascade Employing P450 Monooxygenase BM3 Variant M2 (R47S, Y51W, I401M)[11] and Tyrosine Phenol Lyase Mutant M379V (TPL)[7a] To Form L-Tyrosine Derivatives (L-4a-f) from Substituted Benzenes (1a−f)[a]



[a]Minor side products/reactions are highlighted in brackets. PLP = pyridoxal phosphate; GDH = glucose dehydrogenase.

KPi-buffer (pK_a 7.2; 48% conversion of **1c**; 18% L-**4c**) was exchanged by a Tris-system (pK_a 8.2; 21% conversion of **1c**; 71% L-**4c**), which improved the formation of L-**4c** by a factor of 1.7 (Figure S2, SI). As alternative to the GDH/NADPH/glucose-recycling system, the use of formate dehydrogenase/NADH/formate[14] was less efficient (14% conversion of **1c**, 9.7% L-**4c**, SI; Figure S5), most likely due to the lower coupling efficiency of P450 BM3 with NADH as cofactor. Similarly, disappointing results were obtained with a NADH-dependent P450 BM3-variant[15] in combination with the FDH-system (55% of the GDH system; 24% conversion of **1c**; 19% L-**4c**). The isomeric *p*-phenolic side product (**2c**) was detected in trace amounts (<5%)[12a] together with *p*-hydroquinone **5c** as a consequence of secondary hydroxylation of **3c** by the P450 catalyst.[16] In order to monitor the reaction in more detail, a time study was performed (Figure 1). Substrate **1c** was oxidized rapidly (57 μM min^{-1} of **3c** formed) by the P450 catalyst and L-**4c** was already detectable after 10 min (20 μM min^{-1} of L-**4c** formed), indicating efficient coupling of both steps. **2c** was produced only in traces (<5%) and **5c** started to accumulate at

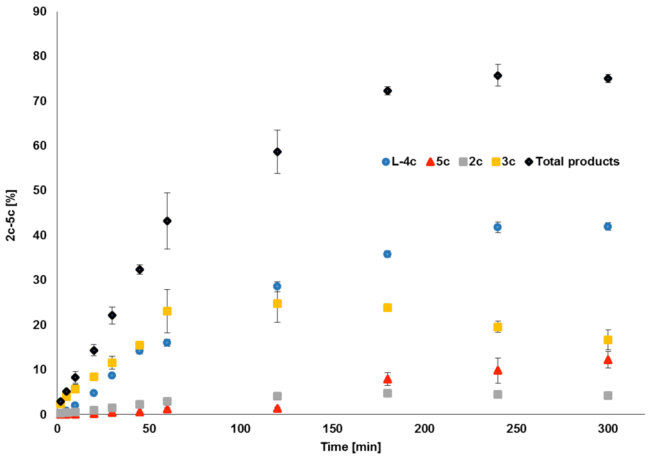


Figure 1. Time-dependent conversion of **1c** (10 mM) by the P450-monooxygenase-TPL-lyase cascade employing 2 μM P450 BM3 variant M2 (R47S, Y51W, I401M), 400 μM NADP$^+$, 3 mg TPL mutant M379V, 40 mM pyruvate, 1200 U catalase (supplemented for removal of traces of H_2O_2 formed by uncoupling of P450 BM3), 40 μM PLP, 12 U GDH, 100 mM D-glucose, 1% (v/v) DMSO and 180 mM NH_4^+ in Tris-HCl buffer (pH 8.0, 0.1 M) in a final reaction volume of 1 mL.

detectable amounts (>5%) after 2 h. After 6 h, 75% of **1c** was converted by P450 BM3 and L-**4c** peaked at 42% (0.88 g L^{-1}). To explore the substrate scope of the cascade, six arenes (**1a−f**) were converted at three different substrate concentrations (Table 1; see SI for 10 mM values). All substrates were converted to the respective L-amino acids with large variation in conversion of **1a−f** (7−87%) and selectivity (26−82% of L-**4a-f**) (Table 1). The best substrate with regard to productivity was **1c** (20 mM), generating 5.5 mM of L-**4c** (1.16 g L^{-1}; 72% of **1c** converted), whereas significant amounts (5.47 mM, 27%) of intermediate **3c** remained unconverted by TPL. The highest selectivity in amino acid formation (82%, Table 1) was obtained for **1a**, albeit at low conversion (7−18%). The successful transformation of **1a** was unexpected, because P450 BM3 was reported to depend on a perfluorinated fatty acid (PFA) as a decoy molecule to catalyze phenol formation.[17] *p*-Hydroquinone formation was particularly high for halogenated substrates, because **3e** is a good substrate for second oxidation by P450 BM3[16a] due to the lower pK_a of **3e** (pK_a 8.5) compared to **3a** (pK_a 10),[18] which enhances the solubility and accessibility in the aqueous phase. To demonstrate the practical utility of the cascade, all substrates were converted at 30 mL scale and 40 mM **1a−f** (Table 2, entries 1−6). As Tris base impedes the purification of L-**4a-f**, we switched to a KPi-system for prep-scale reactions and pH-stat control to maintain the pH at 8.0. With this reaction setup, the product concentration for L-**4a-f** was improved significantly (3.9 to 22.7 mM L-**4a-f**; conversion of **1a-f** ranged between 10 to 68%). In addition to the enhanced productivity, the cascade displayed also a better selectivity for L-**4b-f** (53−99%; L-**4a** was slightly decreased) due to an equilibrium shift toward carboligation by TPL at slightly alkaline pH. The enhanced performance of step 2 efficiently removes phenols **3a−f** and thereby decreases the formation of *p*-hydroquinones via overoxidation, which was very successul in particular for conversion of **1b** and **1c**. Best conversions in scale-up reactions were obtained for **1b** (53%) and **1c** (68%), yielding 3.51 g L^{-1} (18 mM) of L-**4b** and 5.2 g L^{-1} (22.7 mM) of L-**4c**. The product titer for L-**4c** (Table 2, entry 3) corresponds to a space time yield (STY) of 0.11 g L^{-1} h^{-1}, which is exceptionally high for a P450-supported reaction (0.01% catalyst loading).[19] Although **1a** and **1d−f** displayed improved product formation for the target amino acids (3.9 to 5.8 mM); Table 2), product titers did not exceed 1.3 g L^{-1} (max. 28 % conversion of arene to amino acid). Overoxidation prevailed for **1e** and **1f** yielding 43 and 28% of **5e** and **5f**, respectively (Table 2, entries 5 and 6). In contrast, this could be

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Table 1. Substrate Scope of the P450-TPL Cascade[a]

entry	substrate	concn [mM]	**3a−f** [mM]	L-**4a−f** [mM]	**5a−f** [mM]	conv. [%][b]	selectivity L-**4a−f** [%][c]
1	1a	20	0.05	1.09	0.23	7	80
2	1a	5	0.06	0.74	0.1	18	82
3	1b	20	1.89	2.85	4.97	49	29
4	1b	5	0.24	1.45	2.65	87	33
5	1c	20	5.47	5.49	1.52	72	41
6	1c	5	0.38	3.01	0.25	82	74
7	1d	20	1.44	0.98	n.d.	12	40
8	1d	5	1.01	0.7	n.d.	34	41
9	1e	20	0.41	1.67	3.33	27	31
10	1e	5	0.16	1.19	2.25	72	33
11	1f	20	0.56	1.02	2.24	19	27
12	1f	5	0.28	0.77	1.94	60	26

[a]Reaction conditions: Tris buffer (0.1 M; pH 8.0), 12 U mL^{-1} GDH, 400 μM $NADP^+$, 2 μM P450 BM3 variant M2 (5.43 mg mL^{-1} freeze-dried lysate), 40 μM PLP, 180 mM NH_4^+, substrate (**1a−f**), 1% (v/v) DMSO (cosolvent), 100 mM D-glucose, 40 mM pyruvate, 1200 U ml^{-1} catalase, and 3 mg of TPL. Experiments were done in triplicate in a final volume of 1 mL with 170 rpm shaking at RT and 6 h reaction time. [b]Calculated from the sum of products formed from **1a−f**. [c]Selectivity = % L-**4a−f** in the final product mixture. n.d. = not determined.

Table 2. Preparative-Scale Reactions with the P450-TPL Cascade under Continuous pH Control

entry	substrate	**3a−f** [mM]	L-**4a−f** [mM]	5a−f [mM]	conv.[b] [%]	selectivity[c] [% L-**4a−f**]	isol. yield [%]	ee L-**4a−f** [%]
1	1a	0.3	3.9	1.2	14	72	7.6	>97
2	1b	1.4	18.0	1.9	53	85	34	>97
3	1c	2.6	22.7	<0.5*	68	84	49	>97
4	1d	<0.25*	4.0	n.d.	10	>99	6.2	>97
5	1e	0.4	5.8	4.8	28	53	7.8	>97
6	1f	0.6	5.3	2.3	21	65	6.0	>97
7	1c (no TPL)	7.2	0	4.5	31	0	---	---
8	1c (90 mL)[d]	0.8	18.4	<0.5*	52	89	43	>97
9	$TN100_{50\%}$[e]	<0.25*	6.4	0.3	17	95[f]	n.d.	n.d.
10	$TN100_{65\%}$[e]	0.5	15.5	0.1	40	96[f]	26	>97

[a]All experiments were done in duplicate or triplicate, and reaction conditions are described in the experimental section (see SI). [b]Sum of products obtained from **1a−f**. [c]Selectivity is defined as % of L-**4a−f** in the final product mixture. [d]20 h of reaction time with external O_2 supply. [e]Substrate addition led to a final concentration of 40 mM of **1b**; TN100 is a crude technical grade fraction of a commercial gasoline blending (see SI) containing 50−65% **1b**. [f]Selectivity calculation based on conversion of **1b** in TN100 blend (28−39% HPLC area (280 nm) derived from unquantified side products; for details, see Figures S26−S29 in SI); n.d. = not determined. *Limit of detectability.

efficiently suppressed for **5b** and was completely abolished in the case of **5c**. Lower conversions for **1a** and **1d** (14 and 10%) can be attributed to the high volatility substrates and a low coupling efficiency of P450 BM3.[12a,17,20] In the absence of TPL, the oxidation of **1c** decreased by 54%, and formation of the overoxidation product **5c** increased by 3.8-fold (Table 2, entry 7). From this, we conclude that TPL enhances the productivity of P450 BM3 by avoiding the accumulation of inhibiting phenolic intermediates (**3a−f**) and overoxidation products (**5a−f**). FMN-/FAD-containing multicomponent-enzymes, like P450 BM3,[20] are easily inhibited by electron-rich phenols/*p*-hydroquinones through formation of charge-transfer complexes with electron-deficient flavin species,[21] which impede the binding of NAD(P)H.[22] Products L-**4a-f** produced at 30 mL scale were purified yielding 14.9 to 124 mg (6 to 49% isolated yield) all in >97% ee. Reduced KOH consumption (pH stat) was observed upon scale-up of **1c** (40 mM, 90 mL, Table 2, entry 8), indicating limitation in the P450 monooxygenase activity (H^+ release from cofactor recycling; Scheme 1). We assumed limited availability of O_2,[19b] which was circumvented by external O_2 supply resulting in a ∼3-fold increased KOH consumption rate.[13a] Under these reaction conditions (90 mL scale, pH stat, O_2 supply), 3.88 g L^{-1} of L-**4c** (330 mg, 18.4 mM, 89% selectivity) were produced within 20 h (30 mL scale reaction done for 48 h)which corresponds to a STY of 0.19 g L^{-1} h^{-1} and a conversion of 52% of **1c** (Table 2, entry 8). To challenge the system further, a technical-grade aromatic gasoline blend TN100 obtained from petroleum steam-cracking (containing 50 to 65% of **1b** and other benzenes[23]) was used as starting material. Although all ingredients of the latter are potential substrates of P450 BM3 oxidation,[11,12b,20,24] against all expectations, the conversion of gasoline blend TN100 gave L-**4b** in 95−96% selectivity (based on conversion of **1b**) and in good quantity (6.4−15.5 mM; 1.3 to 3 g L^{-1}, 17 to 40% conversion of **1b**, Table 2, entries 9 and 10, see SI). Minor products and residual intermediates **2a** or **2b** (28−39% HPLC area, see SI) were simply removed by extraction. After purification, 60 mg of isolated L-**4b** (a precursor for antitumor agents, such as saframycin A or renieramycin H[2c,f]) were obtained in >97% purity and ee > 97%.

In summary, a one-pot artificial biocascade employing a P450 monooxygenase and a C−C lyase allowed the preparative scale synthesis of L-tyrosine derivatives with product titers up to 5.2 g L^{-1}, a STY of up to 0.19 g L^{-1} h^{-1} and close to perfect ee (>97%). The key to success was the in situ removal of inhibiting phenolic reaction intermediates during the course of the cascade.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b02129.

Catalyst preparation, cascade optimization, and analytical data (HPLC traces, LC-MS, ^{1}H-, ^{13}C-, and ^{19}F-NMR) for isolated and purified compounds (PDF)

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Notes

The authors declare no competing financial interest.

General reaction conditions: KPi buffer (0.1 M; pH 8.0), 12 U mL^{-1} GDH, 400 μM NADP^{+}, 4 μM P450 BM3 variant M2 (R47S, Y51W, I401M; cell-free lysate), 40 μM PLP, 180 mM NH_4^{+}, 40 mM substrate **1a**−**f**, 1% (v/v) DMSO (cosolvent), 1.18 g of D-glucose, 660 mg of pyruvate, 1200 U ml^{-1} catalase, and 90 mg of TPL mutant M379V;[22] (cell-free lysate). Conversions were done at 30 mL scale with 400 rpm stirring at RT and with pH stat control using 0.5 M KOH for 48 h.

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