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FULL PAPER

Tyrosinases in organic chemistry: a versatile tool for the α-arylation of β-dicarbonyl compounds

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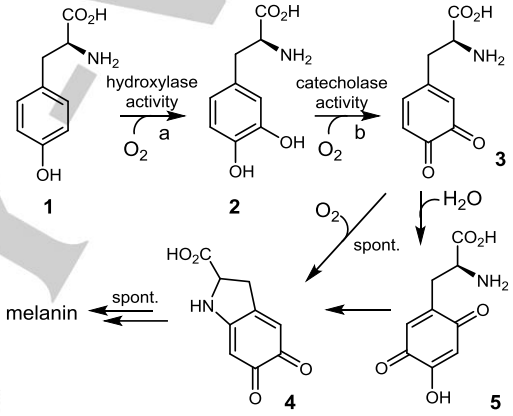
Abstract: A tyrosinase-mediated arylation towards a variety of different building blocks is presented. Utilizing phenol or simple substituted phenols, the corresponding quinones are synthesized in a two-step procedure by an enzyme-catalyzed oxidation (tyrosinase from *Aspergillus oryzae*). The activated intermediates undergo a 1,4-addition with selected β-dicarbonyl compounds. Starting from phenol, yields of isolated product for the hydroxylation-oxidation-arylation sequence range from 43-77%, while substituted acceptors provided 9-55%, only. Different substitution patterns on phenol revealed that electron donating functionalities are preferentially accepted to electron withdrawing ones, whereas *ortho*-substituted phenols are not accepted at all.

Introduction

Tyrosinases, alongside with laccases, belong to the enzyme class of oxidoreductases. These type III binuclear copper oxidases are present in all domains of life, e.g., in fungi, plants and bacteria.[1] The fact that they use molecular oxygen for their oxidation on phenolic compounds accompanied with the release of water makes them an interesting and versatile tool as catalysts in organic chemistry. In nature, tyrosinases are responsible for browning of fruits and vegetables and are essential for melanisation. The latter comes along with UV protection, which is also related to pathogenesis and defensive roles in microorganisms.[2, 3] Based on the work of Raper, Mason and Lerner,[4] the proposed melanin biosynthesis starts with the amino acid tyrosine. Since tyrosinases - per definition - exhibit the capability to perform a hydroxylation of phenolic compounds (phenolase activity) followed by a subsequent oxidation (catecholase activity), the initial steps of the melanin synthesis are the hydroxylation of L-tyrosine (**1**) to L-3,4-dihydroxyphenylalanine (**2**) (L-DOPA) followed by its oxidation to the corresponding *o*-dopaquinone (**3**). Interestingly, later investigations on the biosynthesis by Cánovas and co-workers revealed a strong dependence on the pH whether the *o*-dopaquinone (**3**) is transformed to melanin *via* dopachrome (**4**) intermediates (under physiological pH values) or if it proceeds *via* a *p*-topaquinone (**5**) intermediate (pH < 4). Thereby, the synthesis of *o*-dopaquinone (**3**) is irrespective of the pH value.[5]



Scheme 1. Proposed biochemical pathway towards melanin.

The fact that the tyrosinase is able to perform two subsequent reactions turns them into an attractive tool also from a chemical point of view, since oxidation of aromatic compounds using molecular oxygen as only stoichiometric reagent would seem ecologically beneficial.[6] More conventional alternatives have been reported: Bogle and co-workers showed a few examples of arylation reactions with a polymer supported periodate as oxidant for substituted phenols.[7] Another example is given in the synthesis of epicolactone in which the catechol derivatives are oxidized by potassium ferrocyanide.[8] Moreover, the application of electrochemical tools for the oxidation of catechols and methylcatechols in presence of benzoylacetonitrile has recently been reported as well.[9] As alternative, "green" oxidants, laccases and tyrosinases have already found their way into the fields of organic chemistry.[10, 11] Laccases are known to oxidize catechols to quinones, which may act as Michael acceptor in 1,4-addition reactions. For instance, Beifuss *et al*. provided an application of the laccase from *Agaricus bisporus* to mediate the reaction of diverse pyrazolones with catechols yielding benzofuranones.[11a] Furthermore, Tozzi *et al*. presented a tyrosinase catalysed reaction towards dibenzoazocanes.[11b] Relating to these biocatalysts, our institute presented a variety of arylation

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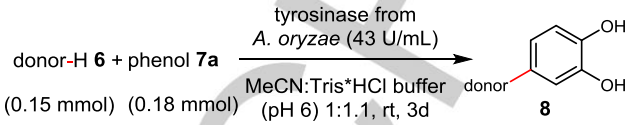
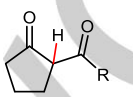
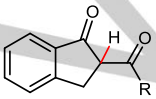
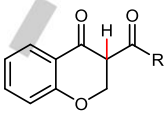
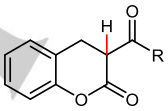
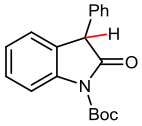
reactions with different laccases.[12] The current paper presents an update on the latter mentioned laccase mediated arylation reactions being subject in several publications in the past few years.[12] The thereby synthesised products, possessing a quaternary stereogenic center, represent building blocks for a variety of biologically interesting compounds.[12, 13] Instead of laccases and catechols, we herewith present an alternative starting from phenols as precursor by using the purified (trypsin activated) fungal tyrosinase melB from *Aspergillus oryzae*.[14] The advantage is obvious, since the tyrosinase can perform the necessary hydroxylation towards the catechols and subsequently perform the oxidation to the desired *o*-quinones, which undergo the previously mentioned 1,4-addition reaction. With this biocatalyst in hand, it is possible to start from readily available and cheap precursors. Compared to the laccase, we are able to perform two steps instead of one. We hereby present our recent results on tyrosinase mediated arylation reactions, touching the subject of substrate scope and limitations.

Results and Discussion

In order to establish the application of the tyrosinase from *A. oryzae* for the presented arylation, we chose the commercially available cyclopentanone carboxylate **6a** (R = OMe; Scheme 2) and phenol (**7a**) as substrates for initial studies and optimizations of the reaction conditions. The following basics of the published reaction conditions[12] for laccases were applied for comparison: a) Performance of the reaction at room temperature and maximum duration of three days. b) The use of acetonitrile as co-solvent and buffer was adopted (here in a ratio of 1:1.1 instead of the published 1:2 ratio and a change of KP_i to Tris-HCl buffer). Since the initial experiments with the tyrosinase (43 U/mL) resulted in a full conversion of the ester **6a** into the product **8a**, this volumetric activity was kept for all further experiments. In case of the exemplarily chosen cyclopentanone methyl ester **6a** the maximum duration of three days resulted in a decrease of product yield and presumably increase of polymerization products. Interestingly, this compound seems to be far more reactive than the other products. Already after 3.5 h full conversion was achieved and 65% of the desired product **8a** could be isolated after purification via silica chromatography (Scheme 2, entry 1). This result depends also on the applied equivalents of the starting materials. Initial experiments with equimolar ratios of both starting materials or in case of an excess of the Michael donor **6** did not lead to full conversion. Not until the phenol (**7a**) was used in excess, the desired product yields could be accomplished. However, the yield of isolated product could not be increased further, presumably due to the formation of unknown polymeric product visible at the baseline of the TLC and column chromatography, respectively. Confident that the established reaction conditions are adequate for the tyrosinase, since the published arylation product **8a** was obtained in the same yield of 66% with the laccase from *P. ostreatus*,[12a] further variations on the donor molecule were investigated (Scheme 2). Unfortunately, changes on the ester functionality of the cylopentanone derivative resulted in a slight decrease in yield. Despite several attempts, the ethyl ester **6b** did not exceed the 55% (Scheme 2, entry 1). The same is true for the *tert*-butyl ester **6c** (51%, Scheme 2, entry 1). In a next step, indanone esters **6d** and **6e** were investigated. The *tert*-butyl derivative **6e** gave 43% yield of compound **8e** whereas the ethyl ester **6d** resulted in the formation of arylated product **8d** in moderate yield (61%; Scheme 2, entry2).



entry	donor **6**		arylation product **8** (yield)
1		**6a-c**	**8a** R = OMe (65%)[a] **8b** R = OEt (55%) **8c** R = O*t*Bu (51%)
2		**6d-e**	**8d** R = OEt (61%) **8e** R = O*t*Bu (43%)
3		**6f**	**8f** R = OMe (77%)[a]
4		**6g-h**	**8g** R = OEt (70%)[a] **8h** R = Me (77%)
5		**6i**	**8i** R = OMe (53%)[a]

Scheme 2: Tyrosinase-mediated 1,4-additions of donor substrates **6a-6i** to phenol (**7a**) providing highly substituted compounds **8a-i** [[a] full conversion of the donor after 3.5 h].

Changing to isocoumarins **6f** and coumarins **6g**,**h** as donors did also proof possible and the desired products **8f-h** could be isolated in 70-77% yield, respectively (Scheme 2, entry 3 and 4). This is in accordance with the published results using the laccase from *A. bisporus* for the corresponding oxidation-arylation-sequence using catechols (63-71% yield).[12d] Last, also conversion of oxindole **6i** was shown to be a suitable starting material and resulted in 53% yield of the desired arylation product **8i** along with the formation of structurally unassigned side-products (Scheme 2, entry 5).

Next to these initial experiments, comparative experiments in cases of substrate **6b** were performed in the presence of catechol **9a**, to investigate whether the tyrosinase accepts preferentially the phenol or catechol (Scheme 3). Furthermore, the effect of an additional amount of H_2O_2 as well as a change in buffer were tested.

Additional comparative kinetics emphasize the experimental results revealing that the tyrosinase from *A. oryzae* shows a higher reactivity towards catechol **9a** than in presence of phenol

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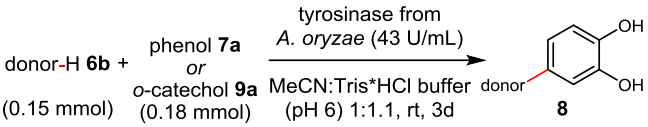
7a as substrate**,** demonstrated by the over 500 times higher turnover number k_{cat} (Fig. 1, A). Likewise, the application of catechol **9a** in the arylation reaction resulted in an increase of yield about 16% (Scheme 3, entry 3) compared to the application of phenol (Scheme 3, entry 1). Furthermore, the K_M-value in MeCN and Tris-HCl (1:1), when catechol **9a** is the substrate, is approximately 30 times higher than in presence of the phenol **7a**. Thus, demonstrating that the phenol is indeed rather accepted by the enzyme, presumably due to its similarity towards the natural substrate, but catechol is more readily oxidized.



entry	acceptor	additives	arylation product **8b** (yield)
1	**7a**	-	(53%)
2	**7a**	5 mol% H_2O_2	(56%)
3	**9a**	-	(69%)

Scheme 3: Comparative experiments towards arylated product **8b** using either phenol (**7a**), *o*-catechol (**9a**) and/or H_2O_2 as additive

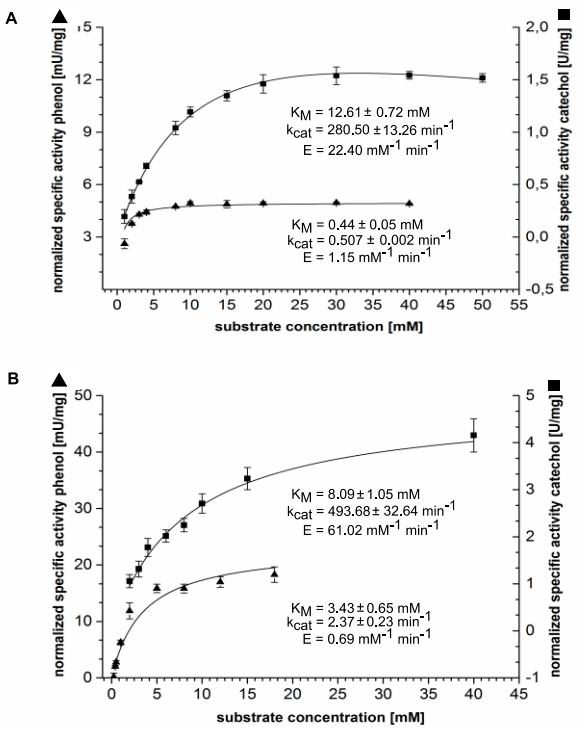
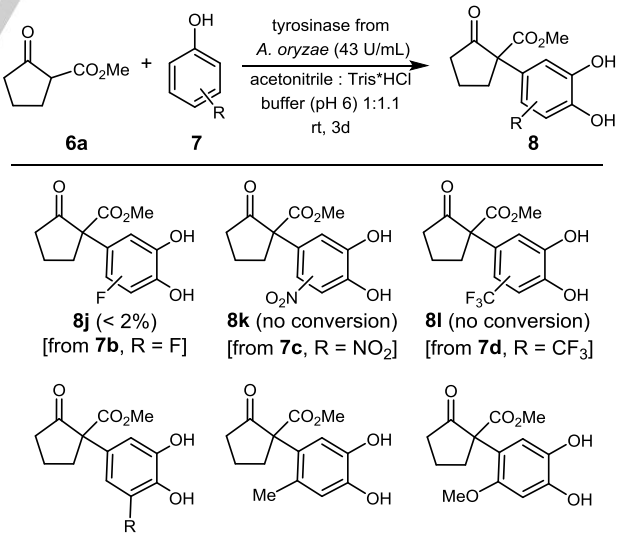


Figure 1: Michaelis-Menten kinetics of the tyrosinase from *A. oryzae* [**A** in presence of phenol **7a** (grey) or catechol **9a** (black) as substrate; in a 1:1 ratio of MeCN and Tris*HCl buffer; **B** same kinetics in MeCN and KP_i-buffer (1:1). For comparison, specific activities for phenol **7a** and catechol **9a** have been normalized to the specific activity for the natural substrate L-tyrosine; further comparative kinetics between commercially available and purified tyrosinase are shown in the supporting information]

The lower catalytic efficiency in case of phenol **7a** emphasizes the assumption that the hydroxylation is the rate-determining step in the reaction sequence. Consequentially, the oxidation of catechol **9a** towards the corresponding *o*-quinone is preferred. With regard to the kinetics in KP_i-buffer systems one can see similar relations (Fig. 1, B; for further details on the kinetics, see supporting information). Remarkably, the oxidation of the catechol is even faster in KP_i-buffer, leading to the assumption that the Tris-HCl buffer might inhibit the reaction.[15] Despite this known fact, the choice of buffer does not affect the outcome in yield of the desired arylation products.

In a last step, after variation of the donors, the substrate acceptance of the tyrosinase concerning different phenolic acceptors was investigated. Therefore, substituted phenols **7b**-**f** were used, bearing either electron withdrawing or donating groups (Scheme 3). The electronic effects have a distinct impact on the catalyzed reactions. The tyrosinase from *A. oryzae* accepts electron-donating groups on the phenolic derivatives rather than electron-withdrawing ones. In general, it becomes clear that the introduction of a substituent, irrespective of its position, comes along with a decrease of product formation. Electron-withdrawing groups, such as a nitro- (**7c**) or a trifluoromethyl (**7d**) group in *ortho*, *meta*, or *para* position, did not result in any product formation. Only the simple fluorinated derivative *o*-, *m*- and *p*-**7b** showed traces of a product **8j**, but with the low yield of the crude product (~1-2%) it was not worth investigating or analysing it in more detail (Scheme 3). In order to verify, whether it is a single fact of our tyrosinase from *A. oryzae*, which does not accept these compounds, the commercially available mushroom tyrosinase (from Sigma Aldrich[16]) was tested and did also not show any conversion of one of the above mentioned substrates (**7b-d**) bearing electron-withdrawing groups.



Scheme 4: Oxidation and arylation of cyclopentanone **6a** with phenols **7b**-**7f**.

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In contrast, electron-donating groups, e.g., such as in cresols (**7e**) and methoxy phenols **7f** showed results that are more promising. Although the *ortho* position is still not favored and thus does not lead to any detectable products *o*-**8m** and *o*-**8n**, the methylated phenolic compounds *m*-**7e** and *p*-**7e** could be transformed into the desired product *m*-**8m** successfully. In comparison to the *meta*-substrate *m*-**7e**, phenol *p*-**7e** gave higher yields (26%, Scheme 4). It is worth emphasizing that *m*-**7e** and *p*-**7e** (same for *m*-**7f** and *p*-**7f**) resulted in the same regioisomeric product. Consequently, the conversion of the starting materials differs because of the substitution pattern, thus resulting in different yields of arylation product. For the methoxy-compounds, again the *para*-substituted phenol *p*-**7f** gave the highest yield (55%) of product *m*-**8n**.

Conclusions

To sum up, we successfully established a tyrosinase-initiated (using purified and trypsin activated tyrosinase from *A. oryzae*) one-pot hydroxylation-oxidation-arylation sequence forming products (**8a**-**8i**), all possessing a quaternary carbon atom.[12] HPLC measurement of **8a** revealed no selectivity coming from the tyrosinase (chromatogram see supporting information). Introducing substituted phenolic compounds revealed that the tyrosinase from *A. oryzae* is able to convert phenols with electron donating substituents (OMe, Me) rather than electron withdrawing ones (F, NO_2, CF_3). In both cases the *ortho*-substituted phenols are not accepted. This might be due to steric hindrance in the active site of the tyrosinase. Since the phenolic compounds have to enter the active site in case of the tyrosinase to reach the binuclear copper center the substituents in *ortho* position might have a steric influence. The – albeit limited - acceptance of the fluorinated substrate *o*-**7b** appeared to be an exception. However, since fluorine is obviously relatively small, it might fit better into the pocket. This fact is different to laccases, which lack the phenolase activity, and they are tunnelling the electrons from the substrates to the corresponding copper atoms utilizing the oxidase (catecholase) activity.[17] The enzymatic kinetics revealed that the hydroxylation is the rate-determining step in the reaction sequence towards the arylation products. Therefore, the present tyrosinase can be also considered as alternative oxidizing enzyme, since catechols are converted in a higher catalytic efficiency. Mild conditions and low-cost as well as readily accessible starting materials makes this procedure an appealing alternative to more conventional methods.

Experimental Section

Chemicals and Methods

All chemicals used were purchased from Sigma Aldrich, Alfa Aesar, VWR International/Merck and Fischer Scientific. The acetonitrile, used for the enzymatic reactions, was purchased from Fischer Scientific as HPLC grade. Further solvents were either distilled or taken from the solvent purification system (MBraun, SPS-800). Thin layer chromatography (TLC) was conducted on POLYGRAMÔ SIL G/UV_{254} plates with fluorescence indicator. Detection occurred either by UV absorption or treatment with ceric ammonium molybdate solution followed by heating. NMR spectra were recorded on a Bruker Advance/DRX 600 instrument (^{1}H at 600 MHz; ^{13}C at 151 MHz). Chemical shifts (δ) are reported relative to deuterated chloroform (^{1}H: 7.26 ppm; ^{13}C: 77.2 ppm) or acetone (^{1}H: 2.05 ppm; ^{13}C: 29.8 ppm). Infrared spectra were recorded using a Perkin–Elmer SpectrumOne IR spectrometer. Melting points were measured with a Büchi melting point B-540 device.

Starting materials

The starting materials **6a** and **6b** are commercially available. Compound **6f** has been prepared as reported previously[12b] starting from the commercially available chromone-3-carboxylic acid. In a second step, compound **6f** was reduced according to the published method using the H-Cube from Thales Nano. The same procedure was applied in case of compounds **6g-h**,[12c] starting from the commercially available ethyl coumarin-3-carboxylate and 3-acetylcoumarin.

Synthesis of *tert*-butyl 2-oxocyclopentane-1-carboxylate (6c): i) According to Bunnage *et al*.[18] of adipoyl chloride (600 mg, 3.3 mmol, 1 eq) in Et_2O (1.3 mL) was added dropwise to a stirred solution of *tert*-butanol (790 mg, 10.7 mmol, 3.25 eq) and *N,N*-dimethylaniline (1.25 g, 10.3 mmol, 3.15 eq) in Et_2O (2.5 mL) at 0 °C. After complete addition, the reaction was stirred at room temperature for 16 h. Afterwards the reaction mixture was diluted with H_2O and the organic phase was extracted five times with a 3 M HCl solution, two times with $NaHCO_3$ and once with brine. It was dried ($MgSO_4$) and purified via column chromatography. The desired product (colourless oil) was isolated in 70% yield (590 mg, 2.3 mmol). R_f (80/20 PE/EtOAc): 0.62. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.44 [s, 18 H, 2 C(CH_3)$_3$], 1.60 (m, 4 H), 2.22 (m_c, 4 H). ii) NaH (31 mg, 60% oil suspension) is stirred in toluene (1.5 mL) and heated to 60 °C. To the suspension, 8 mg (total amount: 200 mg, 0.8 mmol) of the di-*tert*-butyladipoyl ester from i) were added and stirred for 30 min. The remaining starting material was added and the reaction mixture heated for 3 h at 100 °C. Compound **6c** could be isolated in 64% yield (90 mg, 0.5 mmol). R_f (80/20 PE/EtOAc): 0.50. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.46 [s, 9 H, (CCH_3)$_3$], 1.79-1.87 (m, 1 H, 3-H or 4-H), 2.07-2.13 (m, 1 H, 3-H or 4-H), 2.22-2.29 (m, 4 H, 5-H + 3-H or 4-H), 3.03 (t, J = 8.6 Hz, 1 H, 1-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] δ 21.1 (C-3 or C-4), 27.6 (C-5), 28.2 [C(CH_3)$_3$], 38.2 (C-4 or C-3), 55.9 (C-1), 81.8 [C(CH_3)$_3$], 168.9 (C-1‘ or C-2‘), 213.0 (C-2‘ or C-1‘). HRMS: *m/z* = 183.1028 $[M-H]^+$, calculated for $C_{10}H_{15}O_3^-$: 183.1027. All analytical data are in full agreement to those published.[18]

Synthesis of ethyl 1-oxo-2,3-dihydro-1*H*-indene-2-carboxylate (6d): Referring to a protocol of Brown *et al*.,[19] indanone (1.03 g, 7.8 mmol) in diethyl carbonate (27.6 g, 234 mmol, 30 eq) was added to a suspension of NaH (623 mg, 15.6 mmol, 60% suspension in oil, 2 eq) in diethyl carbonate (27.62 g, 234 mmol, 30 eq) via syringe. The mixture was heated to reflux for 5 min (formation of a green solid). Another 12.5 mL of diethyl carbonate was added and heated to reflux for further 15 min. The mixture was allowed to cool to room temperature before it was acidified with 1 M HCl until pH 1. The mixture was extracted four times with EtOAc and the organic phases dried over $MgSO_4$. Purification occurred via column chromatography (90/10 PE/EtOAc). Compound **6d** could be isolated as brown oil in 53% yield (812 mg, 4.0 mmol). R_f (80/20 PE/EtOAc): 0.52. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.31 (t, J = 7.1 Hz, 3 H, 3‘-H), 3.38 (dd, J = 17.2, 8.2 Hz, 1 H, 3-H_a), 3.56 (dd, J = 17.2, 4.1 Hz, 1 H, 3-H_b), 3.71 (dd, J = 8.3, 4.1 Hz, 1 H, H-2), 4.25 (q, J = 7.2 Hz, 2 H, 2‘-H), 7.40 (dd, J = 7.7 Hz, 1 H, 6 or 7-H), 7.50 (d, J = 7.7 Hz, 1 H, 5 or 8-H), 7.62 (dd, J = 7.4 Hz, 1 H, 7 or 6-H), 7.78 (d, J = 7.7 Hz, 1 H, 8 or 5-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] δ 14.4 (C-3‘), 30.5 (C-3), 53.5 (C-2), 61.9 (C-2‘), 124.8 (C-5 or C-8), 126.7 (C-8 or C-5), 127.9 (C-6 or C-7), 135.4 (C-4_a or C-4_b), 135.5 (C-7 or C-6) 153.8 (C-4_b or C-4_a), 169.3 (C-1‘), 199.7 (C-1).

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HRMS: *m/z* = 205.0859 $[M+H]^+$, calculated for $C_{12}H_{13}O_3$: 205.0859. All analytical data are in full agreement to those published.[19]

Synthesis of *tert*-butyl 1-oxo-2,3-dihydro-1*H*-indene-2-2carboxylate (6e): To the solution of indanone (300 mg, 2.3 mmol) in THF (2.3 mL), 109 mg of NaH (60% oil suspension, 4.5 mmol) in THF (9 mL) were added at room temperature. The mixture was refluxed under the addition of pyrrol-*tert*-butyl-carboxylate (775 mg, 4.5 mmol) in THF (1.1 mL). After completion of the reaction, the mixture was cooled to 0 °C and quenched with 1 N HCl. The solution was extracted four times with Et_2O and washed one with NaCl. Two column chromatography afforded 11% of the desired product **6e** (58 mg, 0.2 mmol). R_f (80/20 PE/EtOAc): 0.64. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.49 [s, 9 H, C(CH_3)$_3$], 3.33 (dd, *J* = 17.1, 8.2 Hz, 1 H, 3-H_a), 3.49 (dd, 17.2, 4.0 Hz, 1 H, 3-H_b), 3.62 (dd, *J* = 8.2, 4.0 Hz, 1 H, 2-H), 7.34 (dd, *J* = 7.4 Hz, 1 H, 6-H or 7-H), 7.49 (d, *J* = 7.7 Hz, 1 H, 5-H or 8-H), 7.60 (dd, *J* = 7.4 Hz, 1 H, 7-H or 6-H), 7.75 (d, *J* = 7.7 Hz,1 H, 8-H or 5-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] δ 28.15 [C(CH_3)$_3$], 30.46 (C-3), 54.40 (C-2), 82.17 [*C*(CH_3)$_3$], 124.68 (C-5 or C-8), 126.64 (C-8 or C-5), 127.79 (C-6 or C-7), 135.33 (C-7 or C-6), 135.59 (C-4_a or C-4_b), 153.80 (C-4_b or C-4_a), 168.45 (C-1 or C-1'), 200.13 (C-1 or C-1'). All analytical data are in full agreement to those published.[20]

Synthesis of *tert*-butyl 2-oxo-3-phenlindoline-1-carboxylate (6i): i) The synthesis of **6i** was performed according to a published procedure by Hamashima *et al*.[21] To a solution of isatin (1.0 g, 6.8 mmol) in THF (30.0 mL) at -40 °C, a solution of PhMgBr in THF (1 M, 13.6 mL) was added. The mixture was allowed to warm to room temperature. The reaction mixture was diluted with diethyl ether, cooled to 0 °C, and quenched with 1 M HCl. The aqueous phase was extracted four times with ether and the combined organic layers were washed once with water and brine. Then, dried over $MgSO_4$. After removal of the solvent under reduced pressure, the crude mixture was directly used in the second step. ii) The crude mixture as obtained in i) (theoretical amount: 1.5 g, 10.2 mmol) was diluted in CH_2Cl_2 (100 mL). After addition of DMAP (124 mg, 1.0 mmol) and Boc_2O (2.5 eq, 5.56 g, 25.0 mmol) the reaction was stirred for 3 h at room temperature. The mixture was diluted with NH_4Cl and CH_2Cl_2 and the aqueous phase was extracted four times with CH_2Cl_2 and washed once with brine. The organic phase was dried over $MgSO_4$ and the solvent evaporated under reduced pressure. The product (white, crystalline) was isolated after two column chromatographic purification steps (PE/EtOAc 90/10) in 22% yield (648 mg, 1.5 mmol, over 2 steps). R_f (PE/EtOAc 70/30): 0.67. Mp: 135-136 °C. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.38 [s, 9 H, C(CH_3)$_3$], 1.61 [s, 9 H, C(CH_3)$_3$], 7.24 (t, *J* = 7.5 Hz, 1 H, 6-H or 7-H), 7.29-7.34 (m, 6 H, Ph + 5-H or 8-H), 7.45 (t, *J* = 7.9 Hz, 1 H, 7-H or 6-H), 7.98 (d, *J* = 8.2 Hz, 1 H, 8-H or 5-H). ^{13}C-NMR (151 MHz, CDCl3) [ppm] δ 27.7 [C(CH_3)$_3$], 28.2 [C(CH_3)$_3$], 81.8 (C-3), 84.1 [*C*(CH_3)$_3$], 84.7 [*C*(CH_3)$_3$], 115.6 (C-5 or C-8), 124.2 (C-8 or C-5), 125.1 (C-6 or C-7), 126.9 (C-Ph), 127.5 (C-$4_{a/b}$ or C-Ph), 128.7 (C-Ph), 129.3 (C-Ph), 130.5 (C-7 or C-6), 136.1 (C-$4_{a/b}$ or C-Ph), 140.8 (C-$_{4a/b}$ or C-Ph), 149.3 (C-2 or C-1' or C1''), 151.1 (C-2 or C-1' or C1''), 171.7 (C-2 or C-1' or C1''). IR $\tilde{\nu}$ 2981, 1799, 1747, 1730, 1611, 1471, 1396, 1371, 1346, 1314, 1284, 1247, 1147, 1113, 1101, 1087, 1036, 1003, 971, 940, 924, 907, 854, 839, 800, 771, 760, 744, 716, 693 cm^{-1}. HRMS: *m/z* = 448.1731 $[M+Na]^+$, calculated for $C_{24}H_{27}O_6NNa^+$: 448.1731; 443.2180 $[M+NH_4]^+$, calculated for $C_{24}H_{27}O_6NNH_4^+$: 443.2177. iii) The product (150 mg, 0.4 mmol) from ii) was dissolved in methanol (7.1 mL) and Pd/C (71 mg, 0.7 mmol) was added to the solution. The mixture was stirred at room temperature under a H_2-atmosphere (balloon) for 5 h. Afterwards the reaction mixture was filtrated over celite to remove the catalyst and washed with ether. After removal of the solvent under reduced pressure, the desired product (white solid) could be isolated via column chromatography in 57% yield (62 mg, 0.2 mmol). R_f (PE/EtOAc 70/30): 0.66. Mp: 102-106 °C. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.64 [s, 9 H, C(CH_3)$_3$], 4.73 (s, 1 H, 3-H), 7.16-7.17 (m, 2 H, Ar-H or Ph-H), 7.19-7.21 (m, 2 H, Ar-H or Ph-H), 7.29-7.28 (m, 4 H, Ar-H or Ph-H), 7.94 (d, *J* = 8.2 Hz, 1 H, Ar-H or Ph-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] δ 28.2 [C(CH_3)$_3$], 52.7 (C-3), 84.6 [*C*(CH_3)$_3$], 115.3 (C-Ar), 124.7 (C-Ar), 125.2 (C-Ar), 127.5 (C-$4_{a/b}$ or C-Ph), 128.0 (CH), 128.8 (C-Ph), 129.1 (C-Ph), 136.4 ($C_{quart.}$ C-$4_{a/b}$ or C-Ph), 140.6 (C-$4_{a/b}$ or C-Ph), 149.5 (C-1'), 174.0 (C-2). IR $\tilde{\nu}$ 2982, 1768, 1725, 1608, 1479, 1464, 1394, 1370, 1346, 1288, 1252, 1202, 1146, 1089, 1048, 1024, 990, 845, 758, 723, 697, 675 cm^{-1}. HRMS: *m/z* = 309.1438 $[M+H]^+$, calculated for $C_{19}H_{20}NO_3$: 309.1438. All analytical data are in full agreement to those published.[21]

Arylation products

The compounds **8a**-**n** have been synthesized according to the following general procedure. The analytical data of products **8a**,**b**,**e** and *m*-**8m**[12a] as well as **8f**[12b] and **8g**.[12c] are in full agreement to those published.

General procedure for the arylation reaction with the tyrosinase from *A. oryzae*: In a 10 mL flask, 0.15 mmol of compound **6a-6i** and 0.18 mmol of phenol **7a-7f** (1.2 eq) were diluted in 1.0 mL acetonitrile. 1100 µL of the purified and activated enzyme solution in 10 mM Tris*HCl buffer pH 6 (43 U/mL; see *activation of tyrosinase*) were added to the reaction mixture and stirred at room temperature up to three days. Afterwards, the reaction mixture was acidified with a few drops of 1 M HCl, diluted with dH_2O and EtOAc and the aqueous phase was extracted four times with EtOAc. All combined organic phases were washed once with sat. NaCl solution and dried over $MgSO_4$. Purification *via* silica column chromatography (PE/EtOAc 70/30 – 80/20) resulted in the desired products **8a-8n**. Problems to remove solvent residues kept in the, to some extent, oily and resinous products have been overcome with several "washing steps" with deuterated chloroform.

General procedure for the arylation reaction with the commercially available tyrosinase (from mushroom): In a 10 mL flask, 0.15 mmol of compound **6a** and 0.18 mmol of the substituted phenol *p*-**7b-7d** (1.2 eq) were diluted in 1.0 mL acetonitrile. After addition of 1.1 mL Tris*HCl buffer (10 mM, pH 6), 0.17-0.28 mg (2687 U/mg) of the commercially available tyrosinase[16] were added to the reaction mixture and stirred at room temperature up to three days. Since no conversion was detected (TLC), the reaction mixture was discarded.

***tert*-Butyl 1-(3,4-dihydroxyphenyl)-2-oxocyclopentane-1-carboxylate (8c)** was obtained as slightly yellow oil; 51% (22 mg, 75 µmol). R_f (70/30 PE/EtOAc): 0.17. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] δ 1.42 [s, 9 H, C(CH_3)$_3$], 1.90-2.01 (m, 2 H, 3-H, 4-H or 5-H), 2.29-2.35 (m, 1H, 3-H, 4-H or 5-H), 2.44-2.50 (m, 2 H, 3-H, 4-H or 5-H), 2.75 (m_c, 1 H, 3-H, 4-H or 5-H), 5.55 (s, O*H*), 5.80 (s, O*H*), 6.77-5.80 (m, 2 H, Ar-H), 6.96 (s, 1 H, Ar-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] δ 19.5 (C-3, C-4 or C-5), 28.0 [C(CH_3)$_3$], 35.0 (C-3, C-4 or 5), 37.8 (CH_2), 65.2 (C-1), 82.7 [*C*(CH_3)$_3$], 115.1 (C-Ar), 115.3 (C-Ar), 120.1 (C-Ar), 128.8 (C-6), 143.5 (C-8 or C-9), 143.5 (C-9 or C-8), 170.4 (C-2 or C-1'), 213.0 (C-2 or C-1'). IR $\tilde{\nu}$ 3414, 2957, 2927, 2856, 2358, 2339, 1724, 1602, 1457, 1369, 1258, 1151, 1124, 1074, 743. 309 cm^{-1}. HRMS: *m/z* = 315.1203 [M+Na], calculated for $C_{16}H_{20}O_5Na$: 315.1208; 293.1383 $[M+H]^+$, calculated for $C_{16}H_{21}O_5^+$: 293.1384.

Ethyl 2-(3,4-dihydroxyphenol)-1-oxo-2,3-dihydro-1H-indene-2-carboxylate (8d) was obtained as colorless solid, 61% yield (29 mg, 92 µmol). R_f (70/30 PE/EtOAc): 0.10. Mp.: 142-143 °C. ^{1}H-NMR (600 MHz, Aceton-d6) [ppm] δ 1.15 (t, *J* = 7.1 Hz, 3 H, 3'-H), 3.67 (d, *J* = 17.4 Hz, 1 H, 3-H_a), 4.07 (d, *J* = 17.4 Hz, 1 H, 3-H_b), 4.14 (q, *J* = 7.1 Hz, 2 H, 2'-H), 6.76 (s, 2 H, Ar-H), 6.97 (s, 1 H, Ar-H), 7.47 (dd, *J* = 7.4 Hz, 1 H, 6-H or 7-H), 7.62 (d, *J* = 7.8 Hz, 1 H, 5-H or 8-H), 7.70-7.74 (m, 2 H, Ar-H). ^{13}C-NMR (151 MHz, Aceton-d6) [ppm] δ 14.3 (C-3'), 41.1 (C-3), 62.1 (C-2'),

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65.3 (C-2), 115.8 (C-Ar), 116.1 (C-Ar), 119.8 (C-Ar), 125.1 (C-5, C-6, C-7 or C-8), 127.4 (C-5 or C-8), 128.8 (C-6 or C-7), 130.8 (C-$4_{a/b}$ or C-Ar), 135.9 (C-$4_{a/b}$ or C-Ar), 136.3 (C-5, C-6, C-7 or C-8), 145.4 (C-Ar), 145.6 (C-Ar), 153.1 (C-$4_{a/b}$ or C-Ar), 171.5 (C-1'), 200.7 (C-1). IR ṽ 3448, 3261, 2984, 1713, 1686, 1604, 1590, 1517, 1469, 1441, 1365, 1328, 1264, 1214, 1190, 1158, 1139, 1118, 1083, 1050, 1027, 1013, 957, 921, 881, 863, 845, 812, 788, 766, 742, 692, 678, 659 cm^{-1}. HRMS: *m/z* = 313.1073 $[M+H]^+$, calculated for $C_{18}H_{17}O_5^+$: 313.1071.

3-Acetyl-3-(3,4-dihydroxyphenyl)chroman-2-one (8h) was obtained as colourless solid, 77% yield (34 mg, 115 µmol). R_f (70/30 PE/EtOAc): 0.12. Mp: 242-243 °C (turned black from 220 °C on). ^{1}H-NMR (600 MHz, acetone-d6) [ppm] *δ* 2.03 (s, 3 H, 2'-H), 3.60 (d, *J* = 16.3 Hz, 1 H, 3-H_a), 3.75 (d, *J* = 16.3 Hz, 1 H, 3-H_b), 6.68 (dd, *J* = 8.3, 2.4 Hz, 1 H, 14-H), 6.79 (d, *J* = 2.4 Hz, 1 H, 10-H), 6.82 (d, *J* = 8.3 Hz, 1 H, 13-H), 6.98 (d, *J* = 8.1 Hz, 1 H, 8-H), 7.08 (dd, *J* = 7.5 Hz, 1 H, 6-H), 7.24 (dd, *J* = 7.7 Hz, 1 H, 7-H), 7.33 (d, *J* = 7.6 Hz, 1H, 5-H), 8.00 (bs, O*H*), 8.13 (bs, O*H*). ^{13}C-NMR (151 MHz, aceton-d6) [ppm] *δ* 27.0 (C-2'), 32.3 (C-3), 63.6 (C-2), 115.6 (C-10),116.5 (C-13), 116.6 (C-8), 119.8 (C-14), 123.1 (C-4_a or C-4_b), 125.4 (C-6), 126.5 (C-9), 129.2 (C-7), 129.4 (C-5), 146.2 (C-11 or C-12), 146.3 (C-12 or C-11),152.1 (C-4_a or C-4_b), 168.0 (C-1' or C-1), 203.7 (C-1 or C-1'). IR ṽ 3462, 3211, 1762, 1683, 1607, 1542, 1533, 1488, 1457, 1437, 1360, 1339, 1284, 1256, 1234, 1299, 1182, 1144, 1108, 1084, 1030, 979, 947, 916, 869, 834, 809, 795, 774, 761, 680 cm^{-1}. HRMS *m/z* = 299.0915 $[M+H]^+$, calculated for $C_{17}H_{15}O_5^+$: 299.0914; 321.0735 [M+Na], calculated for $C_{17}H_{14}O_5Na$: 321.0739.

***tert*-Butyl 3-(3,4-dihydroxyphenyl)-2-oxo-3-phenylindoline-1-carboxylate (8i)** was obtained as slightly yellow solid, 53% yield (32 mg, 77 µmol). R_f (70/30 PE/EtOAc): 0.28. Mp: 87-88 °C. ^{1}H-NMR (600 MHz, acetone-d6) [ppm] *δ* 1.61 [s, 9 H, C(C*H*$_3$)$_3$], 5.50 (bs, O*H*), 5.75 (bs, O*H*), 6,55 (dd, *J* = 8.3, 2.2 Hz, 1 H, 14-H), 6.69 (d, *J* = 8.4 Hz, 1 H, 13-H), 6.76 (d, *J* = 2.2 Hz, 1 H, 10-H), 7.13-7.19 (m, 4 H, Ar-H), 7.21-7.32 (m, 4 H, Ar-H), 7.87 (d, *J* = 8.2 Hz, 1 H, Ar-H). ^{13}C-NMR (151 MHz, aceton-d6) [ppm] *δ* 28.2 [C(*C*H$_3$)$_3$], 62.5 (C-3), 85.1 [*C*(CH$_3$)$_3$], 115.2 (C-13), 115.4 (C-Ar), 116.2 (C-10), 121.5 (C-14), 124.9 (C-Ar), 126.3 (C-Ar), 127.6 (C-Ar), 128.6 (2 C-Ar), 128.6 (2 C-Ar), 132.1 (C-Ar), 133.8 (C-Ar), 138.9 (C-Ar), 141.9 (C-Ar), 143.6 (C-Ar), 149.4 (C-2 or C-1'), 177.0 (C-1' or C-2). IR ṽ 3393, 2980, 2930, 1781, 1733, 1603, 1517, 1495, 1478, 1464, 1434, 1395, 1370, 1341, 1309, 1284, 1251, 1143, 1115, 1092, 1034, 1001, 947, 909, 856, 837, 813, 753, 729, 696 cm^{-1}. HRMS: *m/z* = 418.1649 $[M+H]^+$, calculated for $C_{25}H_{24}NO_5^+$: 418.1649; 440.1468 [M+Na], calculated for $C_{25}H_{23}NO_5Na$: 440.1468.

Methyl 1-(4,5-dihydroxy-2-methoxyphenyl)-2-oxocyclopentane-1-carboxylate (*m*-8n) was obtained as slightly yellow oil, 55% yield (23 mg, 82 µmol). R_f (70/30 PE/EtOAc): 0.07. ^{1}H-NMR (600 MHz, $CDCl_3$) [ppm] *δ* 1.84-1.91 (m, 1 H, 3-H or 4-H or 5-H), 1.95-2.02 (m, 1 H, 3-H or 4-H or 5-H), 2.27 (m, 1 H, 3-H or 4-H or 5-H), 2.43-2.50 (m, 2 H, 3-H or 4-H or 5-H), 2.88 (m_c, 1 H, 3-H or 4-H or 5-H), 3.67 (s, 3 H, 2'-H or 7-H), 3.74 (s, 3 H, 7-H or 2'-H), 6.44 (s, 1 H, 8-H or 11-H), 6.49 (s, 1 H, 11-H or 8-H). ^{13}C-NMR (151 MHz, $CDCl_3$) [ppm] *δ* 19.9 (C-3 or C-4 or C-5), 35.6 (C-3 or C-4 or C-5), 38.7 (C-3 or C-4 or C-5), 53.1 (C-2' or C-7'), 56.1 (C-2' or C-7'), 64.4 (C-1), 101.3 (C-8 or C-11), 115.6 (C11 or C-8), 119.8 (C-6, C-7, C-9 or C-10), 136.4 (C-6, C-7, C-9 or C-10), 144.7 (C-6, C-7, C-9 or C-10), 151.4 (C-6, C-7, C-9 or C-10), 171.3 (C-2 or C-1'), 215.1 (C-1' or C-2). IR ṽ 3398, 2955, 1719, 1614, 1574, 1515, 1456, 1429, 1402, 1352, 1306, 1231, 1194, 1171, 1104, 1077, 1036, 1014, 986, 937, 912, 866, 832, 728, 647, 609, 563, 519. HRMS: *m/z* = 279.0863 $[M-H]^+$, calculated for $C_{14}H_{15}O_6^-$: 279.0874; 281.1020 $[M+H]^+$, calculated for $C_{14}H_{17}O_6^+$: 281.1020.

Methods in Biology

Salts for the different buffer systems were purchased from VWR Chemicals, AppliChem Panreac ITW Companies and Carl ROTH®. Trypsin from bovine pancreas as well as trypsin inhibitor were purchased from Sigma Aldrich. Donor organism for the used plasmid, containing the desired tyrosinase, was *Aspergillus oryzae*. The accession number of the tyrosinase gene is BD165761, DNA Data Bank of Japan, Mishima-shi. No synthetic gene was used. For further specific details, see ref. 14. Acceptor organism is *E. coli* BL21 (DE3). The initial vector was a pET-30(+) vector from Novagen. Complete description of the final GVO is: *E.coli* BL21/pETHmelB.[14a, c] Expression of the tyrosinase in *E. coli* was performed in 1 L *Fernbach* flasks that were tempered and shaken in a New Brunswick™ Innova® 42 shaker. Harvesting of the cells by centrifugation was performed *via* the RC5B centrifuge from Thermo Scientific. Cell disruption was performed *via* a *Sonoplus* or *Sonoplus RC6 plus* from *Bandelin electronic GmbH & Co.KG,* Berlin. pH values of the different buffer systems were adjusted *via* the Mikroprozessor-pH-Meter from *Knick*.

Transformation* of *E. coli BL21 with the pETH/melB plasmid was performed by putting 3 µL of the plasmid (negative control with 3 µL of sterilised water) onto competent cells keeping them for 30 min on ice. Heat shock occurred for 90 sec at 42 °C. Afterwards 1 mL of LB medium was added to the cells and incubated for 1 h at 37 °C. After centrifugation, the supernatant was discarded and the pellet was re-suspended with the holdover of the supernatant and distributed onto kanamycin agar plates, which were incubated over night at 37 °C.

Preparatory cultures were prepared by picking one single *E. coli* BL21/pETHmelB colony and transferring it into 5 mL LB medium with 5 µL kanamycin (KAN). Incubation continued over night at 37 °C.

Expression was performed in 1 L *Fernbach* flasks. One preparatory culture was added to 1 L TB media [Terrific-Broth media from Carl ROTH®; containing casein 12 g/L, yeast extract 24 g/L, K_2HPO_4 12.54 g/L and KH_2PO_4 2.31 g/L, pH ~7.2; 1 mL KAN (50 mg/mL) and 1 M (1.2 mL) $CuSO_4$]. The expression started at 29 °C for 1 h, followed by 4 h at 36 °C and again 1 h at 29 °C before induction with IPTG (100 µL, 10 mg/mL). The culture was further shaken (120 rpm) over night at 21 °C. The cells were harvested by centrifugation at 4 °C, 12.000 rpm (7084 rcf) for 20 min. Afterwards the pellets were suspended with 0.85% NaCl solution and centrifuged a second time before they were stored until further use at -20 °C.

Purification of the enzyme occurred *via* a peristaltic pump P-1 from *Pharmacia* and a Ni-NTA column. Following buffer systems were used for protein purification [binding buffer: 0.3 M NaCl, 10 mM imidazole, 10% glycerol; 20 mM Bis-Tris, pH 7.2; elution buffer I: 0.3 M NaCl, 0.1 M imidazole, 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 20 mM Bis-Tris, pH 7.2; elution buffer II: 0.3 M NaCl, 0.5 M imidazole, 0.5 mM TCEP, 20 mM Bis-Tris, pH 7.2]. 5 g of the cell pellet were suspended ad 20 mL of the binding buffer and disrupted with the sonotrode for three times 5 min on ice. The Ni-NTA column was washed with binding buffer (10 mL) before the crude enzyme solution was put on it in flow for 30 min. Afterwards the column was washed again with 5-10 mL of the binding buffer to elute unbound enzyme. In two steps (15 mL each) the enzyme was eluted from the column using elution buffer I+II. Purification of the Ni-NTA occurred with a 2 M imidazole solution, followed by washing it with dH_2O and stored in 20% EtOH.

For ***concentration*** of the protein solutions, *Vivaspin 20* (10 kDa MWCO) or *Vivacell 250* from *Sartorius Stedim* were used. For buffer changes, PD-10 columns from *GE-Healthcare* were used (Tris-HCl buffer: 10 mM Tris-HCl, 10 mM NaCl, 10% glycerol, pH 6). Therefore, the PD-10 columns were loaded each with 2.5 mL enzyme solution and eluted with 3.5 mL of the Tris-HCl buffer.

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DNA- and protein ***concentrations*** were measured *via Nano Drop 2000c from Thermo Scientific Corp.*, MA, USA. Determination of the protein concentration occurred by using the Bradford assay. As a calibration curve, a BSA standard curve was taken. Incubation of the enzyme solution with the Bradford dye took 10 min.

Activity measurements were performed on the *Shimadzu UV-1800* photometer from *Shimadzu*, Duisburg. According to literature, the pro-tyrosinase is activated by the hydrolytic enzyme trypsin, which digests the pro-tyrosinase: A C-terminal fragment, covering the copper-centre, is separated (decrease in molecular weight of about 20 kDa; detected via MALDI-TOF/MS).[14a] Activation of the tyrosinase occurred *via* trypsin (from bovine pancreas) which was added as solution in citrate buffer (100 mM, pH 6). After 30 min of incubation, trypsin inhibitor (from soybean) was added. To a 1 mM solution (992 µL) of tyrosine in citrate buffer (100 mM, pH 6), 8 µL of the activated enzyme solution (turned slightly red) was added and the reaction rate was monitored at 475 nm.

Activation of the tyrosinase for the arylation reaction was also performed with trypsin but this time in Tris-HCl buffer (10 mM; pH 6). In order to minimize the dilution of the tyrosine during the process of activation *via* trypsin and inactivation of the protease, the additives were diluted in a ten-fold higher concentration so that less volume would be needed for the activation procedure. Example: 900 µL of the tyrosinase [43 U/mL; 2-3.2 U/mg (a range of specific activities is given which depends on the charge of purified enzyme); turnover number: 204-330 min^{-1} (referring to a molecular mass of 102 kDa, after digestion)] in Tris-HCl buffer were activated *via* the addition of trypsin (100 µL of a ten-times concentrated solution in Tris-HCl) for 30 min at room temperature. Afterwards trypsin inhibitor was added to stop the protease (100 µL of a ten-times concentrated solution in Tris-HCl) resulting in a total volume of 1100 µL for the arylation reactions.

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Keywords: 1,4-addition • arylation • biocatalysis • oxidation • tyrosinase

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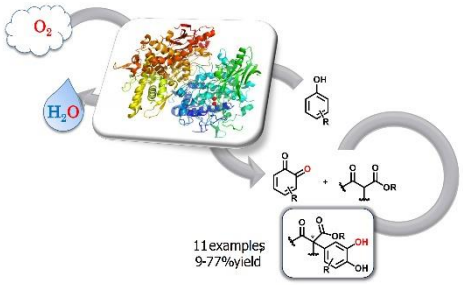
FULL PAPER

A tyrosinase mediated arylation reaction is presented. Starting from a variety of substituted phenols, the enzyme generates activated o-quinones which subsequently can undergo a 1,4-addition towards the desired arylation products, processing a quaternary center.



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Tyrosinases in organic chemistry: a versatile tool for the α-arylation of β-dicarbonyl compounds

Key Topic

Domino Reaction

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