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Laccase-induced derivatization of unprotected amino acid L-tryptophan by coupling with *p*-hydroquinone 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide

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Summary. We have studied the enzymatic derivatization of amino acids by use of the polyphenol oxidase laccase. Derivatization of L-tryptophan was achieved by enzymatic crosslinking with the laccase substrate 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide. The main product (yield up to 70%) was identified as the quinoid compound 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid and demonstrates that laccase-catalyzed C–N-coupling occurred on the amino group of the aliphatic side chain. These enzyme based reactions provide a simple and fast method for the derivatization of unprotected amino acids.

Keywords: Laccase – Cross-coupling – Derivatisation – Tryptophan – Unprotected amino acid – *Pycnoporus cinnabarinus*

Introduction

Amino acids are of basic biological concern as the most important building blocks for proteins. Furthermore, in nature they and their derivatives play roles as hormones and co-factors or inhibitors of enzymes (Gander-Coquoz and Seebach, 1988) or as toxic amino acid antagonists like L-mimosin or L-canavarin (Steinegger and Hänsel, 1992). Their different characteristics permit their wide application e.g. as agricultural chemicals, feed supplements, cosmetics, food additives and pharmaceuticals. The annual world production of amino acids increases continuously and is currently estimated at 2.5 million metric tons per year. Around 20% of the total is manufactured for various medical applications (Mueller and Huebner, 2003). During the last years the potential of amino acids and their derivatives as active ingredients for pharmaceuticals was the object of many investigations and this has resulted in numerous patent applications for derivatisation of amino acids. Among these are amino acid derivatives directed against neurodegenerative disorders such as epilepsy, Alzheimer's disease, depression, Parkinson's disease, AIDS dementia or against diabetes or cancer (Bryans et al., 2002; Konradi et al., 2005; Pfizer et al., 2004; Seko and Kato, 2001; Stürzebecher et al., 2004). D-phenylglycine and *p*-hydroxy-D-phenylglycine have been widely applied as side chains for β-lactam antibiotics (Rozzell and Wagner, 1992); alicyclic β-amino acids in medical chemistry (Kuhl, 2005).

For drug development, the screening of synthetic peptides or amino acid analogues is important to determine metabolic stability as well as to maximize biological response while minimizing toxicity. Furthermore, amino-acid based polymers including polypeptides have been developed as biocompatible materials and for other uses (Erhan, 1997; Kaplan, 1998). Yamamoto and co-workers investigated chemical cross-linking using bifunctional cross-linking reagents, photoinduced crosslinks, and enzymatic methods to produce such synthetic co-polypeptides, (Ohkawa et al., 2001). Beside lysyl oxidase (Smith-Mungo and Kagan, 1998) and transglutaminase (Piacentini et al., 2000) the use of tyrosinases (Michon et al., 1998; Oudgenoeg et al., 2001) and peroxidases (Burzio and Waite, 2000; Jee et al., 2000) have been studied intensively. The last two enzymes are known to oxidize the phenol moiety of tyrosine in proteins which is then subjected to crosslinking by formation of carbon–carbon or carbon–oxygen bonds.

Because of the low affinity of laccases (EC 1.10.3.2) to the monophenolic compound tyrosine or the other amino acids, this polyphenol oxidase seemed not to be useful for the derivatization or cross-coupling reactions of these compounds. Figueroa-Espinoza and Rouau (1999) reported that crosslinking of feruloylated arabinoxylans to proteins with a fungal laccase was not successful.

In this study we show that L-tryptophan, a pure amino acid without any protected groups, can be derivatized by a laccase catalyzed reaction.

Materials and methods

Chemicals

L-tryptophan was purchased from Reanal Finechemical Co. (Budapest, Hungary). 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide was obtained from Sigma-Aldrich Fine Chemicals (Taufkirchen, Germany); N-acetyl-tryptophan from Bachem AG (Weil am Rhein, Germany).

Fungal strain

Pycnoporus cinnabarinus SBUG-M 1044 was isolated from an oak tree in northern Germany. The white rot fungus is deposited at the strain collection of the Department of Biology of the University of Greifswald (SBUG).

Cultivation of Pycnoporus cinnabarinus *SBUG-M 1044*

P. cinnabarinus was initially cultivated on malt agar plates that were incubated for 7 days at 30 °C and then kept at 4 °C. The culture was prepared by inoculating a nitrogen-rich medium containing 5 g glucose, 1 g KH_2PO_4, 0.52 g L-asparagine, 0.5 g yeast extract, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 50 ml mineral salt solution and 50 ml $FeSO_4$ solution ($0.2\,g \cdot l^{-1}$) with three 1 cm^2 agar culture fragments. The mineral salt solution contained 1 g $Ca(NO_3)_2 \cdot 4H_2O$, 0.06 g $CuSO_4 \cdot 5H_2O$, and 0.04 g $ZnSO_4 \cdot 7H_2O$ per liter (modified according to Braun-Lüllemann, 1997). Incubation was performed without shaking at 30 °C for 7 days. A uniform inoculum was obtained by homogenization of this culture with an Ultra-Turrax homogenizer T25 (IKA Labortechnik, Staufen, Germany) at 8000 rpm. For the production of the ligninolytic enzyme laccase, 40 ml medium inoculated with 2 ml of the homogenized pre-culture was incubated in 100 ml Erlenmeyer flasks for 7 days with 3,4-dimethoxybenzyl alcohol (10 mM), a known inducer of laccase. Cultures were shaken in a water bath (GFL model 1092, Burgwedel, Germany) at 30 °C and 158 rpm.

Crude preparation of laccase

Pycnoporus cinnabarinus SBUG-M 1044 was cultivated as above. The culture medium was filtered through a glass fiber filter in a Büchner funnel to separate the medium from whole cells. The cell-free culture medium was stirred with DEAE-Sephacel (Sigma, Steinheim, Germany) for 1 h and the adsorbed enzymes were eluted from the DEAE-Sephacel with 20 mM sodium acetate buffer (pH 5). This enzyme extract was desalted using a Sephadex G-25 Superfine column (Pharmacia, Freiburg, Germany).

Measurement of laccase activity

The activity of laccase was determined spectrophotometrically at 420 nm with ABTS (2,2-azino-bi-(3-ethyl-benzthiiazolin-sulfonate)) as substrate (Bourbonnais and Paice, 1990) using the method described by Jonas et al. (1998).

Experimental procedures

For analytical experiments the substrates L-tryptophan (1 mM) and 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (1 mM) were incubated with laccase (activity 287 $nmol\,ml^{-1}\,min^{-1}$) in 20 mM sodium acetate buffer, pH 5. The reaction mixture was incubated in 1.7-ml-brown-glass vials with 1-ml-sample volumes at room temperature with agitation at 200 rpm.

Analytical HPLC

For routine analysis, the reaction mixtures were analyzed using an HPLC system (Hewlett-Packard GmbH, Bad Homburg, Germany) on an end-capped, 5-µm, LiChroCart 125-4 RP 18 column (Merck, Darmstadt, Germany) at a flow rate of 1 ml/min. A solvent system consisting of methanol (eluent A) and 0.1% phosphoric acid (eluent B), starting from an initial ratio of 10% A and 90% B and reaching 100% methanol within 14 min, was used.

Isolation of transformation product by solid phase extraction

A RP18 silicagel column (polypropylene 6 ml, 2000 mg adsorbent material, Merck, Darmstadt, Germany) was charged with the reaction mixture.

The products of single incubation of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide with laccase (hereafter denoted as homomolecular products) were eluted with 10% methanol in water. The cross-coupling product was eluted with 100% methanol. For nuclear magnetic resonance (NMR) spectroscopy the isolated product was dried by lyophilization.

Characterization

The product was characterized by liquid chromatography/mass spectrometry (LC/MS). Atmospheric pressure ionization (API) mass spectrometry experiments were performed using an Agilent Series 1100 HPLC system and an Agilent 1946C quadrupole mass spectrometer (Waldbronn, Germany). The MS was used with both, atmospheric pressure chemical ionization (APCI) and electrospray ionization (API-ES) sources.

APCI/API-ES conditions (positive and negative ion mode) were as follows: nebulizer and drying gas, nitrogen; nebulizer pressure, 30 psig; drying gas flow, 10 l/min; vaporizer temperature (for APCI), 350 °C; drying gas temperature, 250 °C; capillary voltage, 4 kV; corona current (for APCI), 4 µA; LC-MS eluent (MeOH) flow rate, 0.5 ml/min.

HPLC-MS separation was performed on a LiChroCART® 125-4, LiChrosphere® 100 RP-18e column (Merck, Darmstadt, Germany) at 25 °C at a flow rate of 1 ml/min within a 14-min gradient from 10 to 100% methanol in 0.1% aqueous formic acid.

All FT-ICR MS high-resolution mass spectrometry experiments were performed on a Bruker Daltonics APEX III FT-ICR mass spectrometer (Karlsruhe, Germany) equipped with a 7.0 T tesla shielded superconducting magnet. The vacuum was maintained by means of a sliding vane rotary pump followed by turbomolecular pumps in two different regions; ion source ($\sim 4.3 \times 10^{-7}$ mbar) and cell region ($< 3.5 \times 10^{-10}$ mbar). The flow rate for the eluent (H_2O/ACN/HCOOH 49/49/2; all HPLC-grade) was 2 µl/min, using a syringe pump (Cole-Palmer 74900 series). The ions were generated from an external electrospray ionization source (Bruker Apollo ESI-Source) with nebulizing gas pressure at 20 psi, heated drying gas at 20 psi and 150 °C, and a capillary entrance voltage of −4500 V negative ion mode and +4500 V positive ion mode (Marshall and Schweikhard, 1992; Amster, 1996; Marshall et al., 1998). Mass spectra were acquired with both, positive and negative, ion modes with broadband detection (32 scans each experiment) from 100 to 2.000 Da using 1,024 K data points. All experimental sequences, including scan accumulation and data processing, were performed with XMASS 6.1.2 on Windows 2000.

The ^1H nuclear magnetic resonance (NMR) spectra were recorded on a Bruker ARX 300 instrument (Karlsruhe, Germany) at 300 MHz

Table 1. ^{1}H-NMR data of the substrates 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (**1**) and L-tryptophan (**2**) and the product (**3**) formed due to the coupling by laccase; with MeOH-d_4 as solvent

Product **3**		Substrate **1**		Educt **2**		Proton assignment (**product**; substrate/educt)
δ [ppm]	J [Hz]	δ [ppm]	J [Hz]	δ [ppm]	J [Hz]	
3.14	^{2}J = 14.5 ^{3}J = 9.6			3.14	^{2}J = 15.25 ^{3}J = 9.5	**dd**, **1H**, **CH_2**, **C3**
3.44	^{3}J = 5.5	3.44	^{3}J = 5.4			**t**; m, **2H**, **CH_2**, **$\underline{CH_2}$NH**
3.54	^{2}J = 14.5 ^{3}J = 3.8			3.52	^{2}J = 15.1 ^{3}J = 4.1	**dd**, **1H**, **CH_2**, **C3**
3.66	^{3}J = 5.5	3.62	^{3}J = 5.4			**t**, **2H**, **CH_2**, **$\underline{CH_2}$OH**
5.39	^{2}J = 9.6 ^{3}J = 3.8			3.86	^{2}J = 9.4 ^{3}J = 4.1	**dd**, **1H**, **CH**, **C2**
		6.75	^{3}J = 8.8			**d**, **1H**, **CH**, (**C4″–C_6H_2**; C3–C_6H_3)
6.11	^{3}J = 10.2					
		6.9	^{3}J = 8.9			**d**; dd, **1H**, **CH**, (**C5″–C_6H_2**; C4–C_6H_3)
6.40	^{3}J = 10.2		^{4}J = 2.6			
/	/					/
		7.01	^{4}J = 2.7			d(s), 1H, CH, C6–C_6H_3
7.01	^{3}J = 7.5 ^{4}J = 1.0			7.04	^{2}J = 7.45 ^{3}J = 1.0	**dd**, **1H**, **CH**, **C5′**
7.09	^{3}J = 7.5 ^{4}J = 1.0			7.12	^{2}J = 7.22 ^{3}J = 1.3	**dd**, **1H**, **CH**, **C6′**
7.17	/			7.19	/	**s**, **1H**, **CH**, **C2′**
7.31	^{3}J = 10.2			7.36	^{3}J = 8.0	**d**, **1H**, **CH**, **C7′**
7.54	^{3}J = 10.2			7.70	^{3}J = 7.8	**d**, **1H**, **CH**, **C4′**

in MeOH-d_4 and on a Bruker Avance 600 instrument (Karlsruhe, Germany) at 600 MHz in $CDCl_3$. Tetramethylsilane was used as an internal standard.

Table 2. ^{1}H-NMR data of product **3** formed due to the coupling by laccase; with $CDCl_3$ as solvent

δ [ppm]	J [Hz]		Proton assignment
3.31	^{2}J = 14.4 ^{3}J = 9.4	dd	1H, CH_2 am C3
3.57		m	3H
	^{3}J = 3.8		1H, CH_2, am C3
	^{3}J = 14.4		
	/		2H, CH_2, $\underline{CH_2}$NH
	/		
3.77	^{3}J = 5.6	m	2H, CH_2 am CH_2OH
	/		
3.91	^{3}J = 5.6	t	1H, OH, $\underline{CH_2}$OH
5.37	^{3}J = 6.3	m	1H, CH am C2
	^{3}J = 9.4		
	^{3}J = 3.8		
6.35	^{3}J = 10.1	d	1H, CH am C5″
6.59	^{3}J = 10.2	d	1H, CH am C4″
7.13	^{3}J = 8.1	dd	1H, CH am C5′
	^{3}J = 7.5		
7.19	^{3}J = 8.1	m	1H, CH am C6′
	^{3}J = 7.5		
7.24	/	s	1H, CH am C2′
7.34	^{3}J = 8.1	d	1H, CH am C7′
7.62	^{3}J = 8.1	d	1H, CH am C4′
8.29	/	s	1H, NH, aromatic
9.96	^{3}J = 5.6	t	1H, NH, $NHCH_2CH_2OH$
12.70	^{3}J = 6.3	d	1H, NH, $NHCH(COOH)CH_2$

FT-IR spectra of starting materials and the product 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid were recorded as KBr pellets on a Nicolet Magna 550 spectrometer.

2-[2-(2-Hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid

This compound was recovered as a transformation product of L-tryptophan and the quinonoid form of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide. Isolation from the reaction mixture was achieved by use of a RP18-silicagel column. Purification was carried out as described before: yield 0.186 mg ml^{-1} (about 15%). After optimization the yield was more than 70%. 300 MHz ^{1}H NMR (MeOH-d_4) see Table 1; 600 MHz ^{1}H NMR ($CDCl_3$) see Table 2.

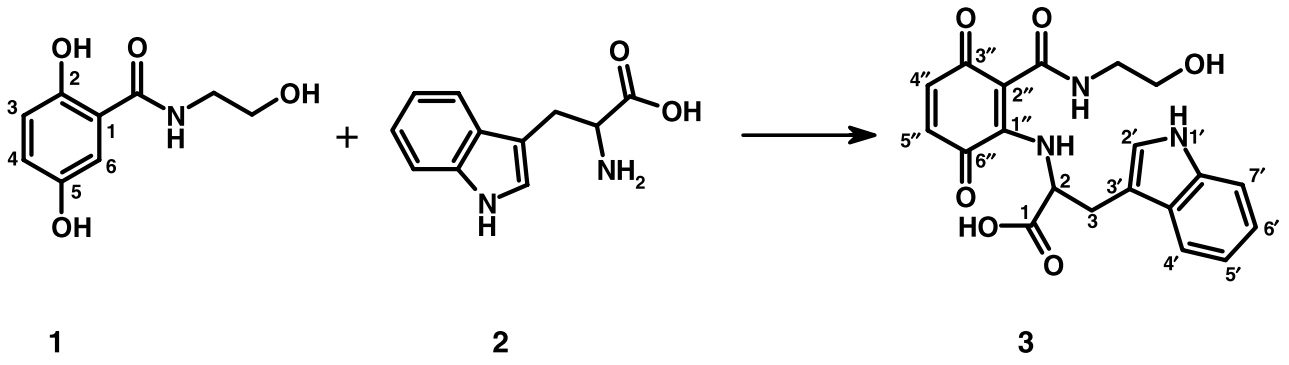
MS (LC/MS): *m/z* (relative intensity): APCI, pos. ion mode 398 ($[M+H]^+$, 14), 352 ($[M–C_2H_4OH]^{\bullet+}$,100); APCI, neg. ion mode 352 ($[M–C_2H_4OH]^{\bullet-}$, 100); API-ES, pos. ion mode 420 ($[M+Na]^+$, 100), 398 ($[M+H]^+$, 19), 352 ($[M–C_2H_4OH]^{\bullet+}$, 23), 337 ($[M–NHC_2H_4OH]^{\bullet+}$, 20); API-ES, neg. ion mode 352 ($[M–C_2H_4OH]^{\bullet-}$, 100).

HRMS for 3 ($C_{20}H_{19}N_3O_6$): calcd: 397.1274; found: 397.1275.

FT-IR (KBr): 3404 (s), 2929 (m), 1720 (m), 1685 (m), 1638 (s), 1543 (vs), 1458 (s), 1422 (s), 1339 (s), 1231 (s), 1127 (m), 1066 (m), 848 (m), 745 (s) cm^{-1}.

Results

By incubating the reaction mixture containing equimolar amounts of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and L-tryptophan (substrates **1** and educt **2** each at 1 mM, Fig. 1) with a crude preparation of laccase the initially colorless mixture changed to red within 20 min.



1 **2** **3**

Fig. 1. 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (substrate **1**), L-tryptophan (educt **2**) and the product 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid (**3**) formed due to a laccase catalyzed reaction

The analysis of the reaction mixture with high-performance liquid chromatography (HPLC) demonstrated the decrease of the two starting substances and simultaneously four products were formed. Comparison of the results with those of an assay with 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide as the only substrate showed that three of the products (retention times $t_R = 2.51$ min; $t_R = 3.81$ min; $t_R = 5.63$ min) arose from the homomolecular transformation of substrate **1** by laccase. Only one product ($t_R = 9.82$ min) resulted from crosslinking to L-tryptophan.

After an incubation time of 90 min 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide was no longer present in the reaction mixture, whereas L-tryptophan was still detectable (Fig. 2a).

The major part of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (around 75%) was transformed by laccase into homomolecular products and consequently not available for the cross-coupling reaction with L-tryptophan.

After formation of the product a large fraction of the L-tryptophan was still present and the product yield was only 15% (Fig. 2b). To enhance the consumption of L-tryptophan and to increase the product yield, the substrate 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide was added stepwise to the reaction mixture during incubation (0.25 mM every 20 min). The yield of cross-coupling product was increased by more then a factor of 4 (product yield 70%) after an incubation time of two hours.

Separation and purification of the coupling product was performed by solid phase extraction. The isolated product was dried by lyophilization. Comparison of proton–proton spin systems of the ^{1}H-NMR-spectra and mass spectra of the compound **3** with those of the substrates **1** and educt **2** showed structural similarities and led to the later identification of product **3** as 2-[2-(2-hydroxy-ethylcarbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid (Fig. 1).

The identification of the cross-coupling product is based on the following data:

The HPLC-MS analyses of compound **3** specifies it unambiguously as a quinonoid structure, arising from oxidation and coupling of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and tryptophan. In the API-ES positive ion mode (Fig. 3b), the anticipated quasi-molecular ions $[M + H]^+$ at $m/z = 398$ and $[M + Na]^+$ at $m/z = 420$ (most intense signal) were found. Furthermore, fragment ions like $[M–C_2H_4OH]^{\bullet+}$ ($m/z = 352$) and $[M–NHC_2H_4OH]^{\bullet+}$ ($m/z = 337$) were present with a moderate relative intensity. The appendant spectra in negative ion mode (Fig. 3a) showed only the signal of the fragment ion $[M–C_2H_4OH]^{\bullet-}$ at $m/z = 352$ with a relative intensity of 100%. Additional measurements with atmospheric pressure chemical ionization (APCI) in positive ion mode showed the fragment ion $[M–C_2H_4OH]^{\bullet+}$ ($m/z = 352$) as the 100% intensity signal, whereas the $[M + H]^+$ ion at $m/z = 398$ was detected with less than 15% relative intensity (Fig. 3d). The measurements in APCI negative ion mode showed some interesting characteristics of the examined quinonoid compound. Besides the fragment ion $[M–C_2H_4OH]^{\bullet-}$ which is the strongest signal in this spectrum (Fig. 3c), there are three more significant signals which can not directly be associated with the structure of compound **3**. Due to the detailed mass spectrometric investigations (Figs. 4, 5a–d) of the corresponding hydroquinonoid form of compound **3**, the signals $m/z = 380$ (40%) and $m/z = 398$ (19%) can be assigned to the hydroquinone form. The occurrence of the latter signals must be the result of an intra mass spectrometer reduction process caused by the conditions during APCI negative ion mode measurements.

Due to the occurrence of the hydroquinone form 2-[3,6-dihydroxy-2-(2-hydroxy-ethylcarbamoyl)-phenylamino]-3-(1H-indol-3-yl)-propionic acid of product **3** in the course of the HPLC-MS measurements (Fig. 4), a direct comparison

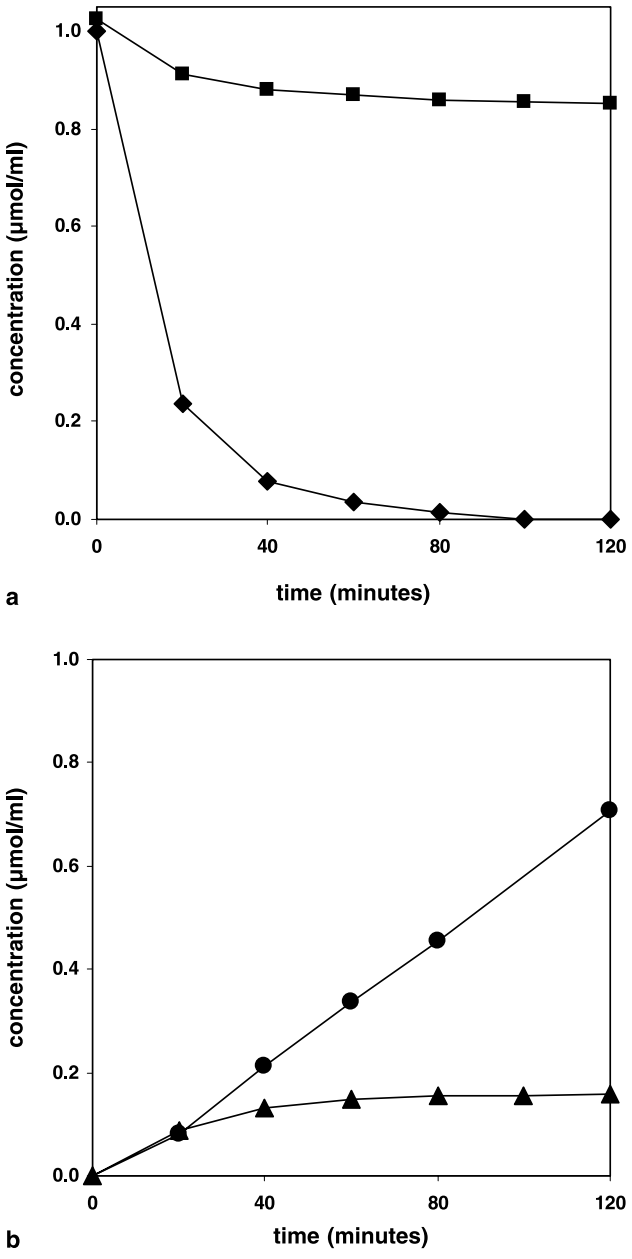


Fig. 2. **a** Kinetics of substrate depletion, 2,5-dihydroxy-N-(2-hydroxy-ethyl)-benzamide ◆ (substrate **1**), L-tryptophan ● (educt **2**) during the laccase catalyzed coupling reaction, substrates in equimolar amounts (1 mM). **b** Kinetics of formation of the product 2-[2-(2-hydroxy-ethyl-carbamoyl)-3,6-dioxo-cyclohexa-1,4-dienylamino]-3-(1H-indol-3-yl)-propionic acid (**3**) during the laccase catalyzed coupling reaction with substrates in equimolar amounts (1 mM) ▲; formation of the product **3** when 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide was added stepwise ● (every 20 min 0.25 mM) to reaction mixture

of MS data between both structural variations was possible. In contrast to the quinonoid product **3**, the most intense signals in the API-ES modes are the deprotonated $[M–H]^-$ ($m/z = 398$) respectively the protonated $[M + H]^+$ ($m/z = 400$) quasi-molecular ions (Fig. 5a, b). Fragment ions like $[M–C_2H_4OH]^{\bullet+}$ ($m/z = 354$) and $[M–N_HC_2H_4OH]^{\bullet+}$ ($m/z = 339$) were detected with an increased relative intensity compared with the corresponding fragment ions shown in Fig. 3b. The measurements in APCI mode showed a similar pattern (Fig. 5c, d). Moreover, all fragments and quasi-molecular ions were directly assignable to the hydroquinone structure. No evidence was found for an intra mass spectrometer conversion, for example partial oxidation of the hydroquinone functionality or comparable changes. All measurements are in full accordance with the expected values and reinforced the definite identification of product **3**.

High-resolution mass spectrometry of product **3** in the positive ion mode showed a signal at $m/z = 398.1358$ for the quasi-molecular ion $[M + H]^+$, which was in good conformity with $m/z = 398.1352$ as the calculated value for $[M + H]^+$ of the expected product **3** ($C_{20}H_{19}N_3O_6$). Furthermore, the strongest signal in the FT-ICR spectrum was at $m/z = 420.1167$ and is typically related to the $[M + Na]^+$ ion, with a calculated value of $m/z = 420.1172$. All results were within a deviation of less than 2 ppm to the calculated values and are therefore in excellent accordance to the proposed structure. The negative ion mode was not informative for the high-resolution mass spectrometric analysis of the desired product.

^{1}H-NMR spectral analysis of compound **3** documented the existence of 12 proton signals (Table 1, MeOH-d_4 as solvent; amino and hydroxyl protons were not analyzed with the method used). Comparison of ^{1}H-NMR spectral data of product **3** with those of the starting compounds **1** and **2** revealed the loss of one proton signal of the three proton signals of the aromatic moiety of the substrate 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide (C3, C4, C6). Only two proton signals (formerly C3, C4; now C4″, C5″) could be detected. Therefore it is highly probable that coupling with L-tryptophan occurred at carbon atom 6 of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide. The chemical shift of the proton located at carbon 2 of L-tryptophan to the lower field gave evidence for changes around this proton.

By ^{1}H-NMR analysis with $CDCl_3$ as solvent (Table 2), all of the three amino protons and one aliphatic hydroxyl proton were detected. The two aromatic hydroxyl protons were not visible confirming the formation of quinonoid product structure. Due to the lower solubility of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide in $CDCl_3$ as solvent a comparison of the data with those of compound **3** was not possible. After 14 h at room temperature the same ^{1}H-NMR spectrum of the product were detected.

^{1}H-NMR analysis with DMSO-d_6 as solvent was carried out for educt **2** and product **3** to make N-bound protons

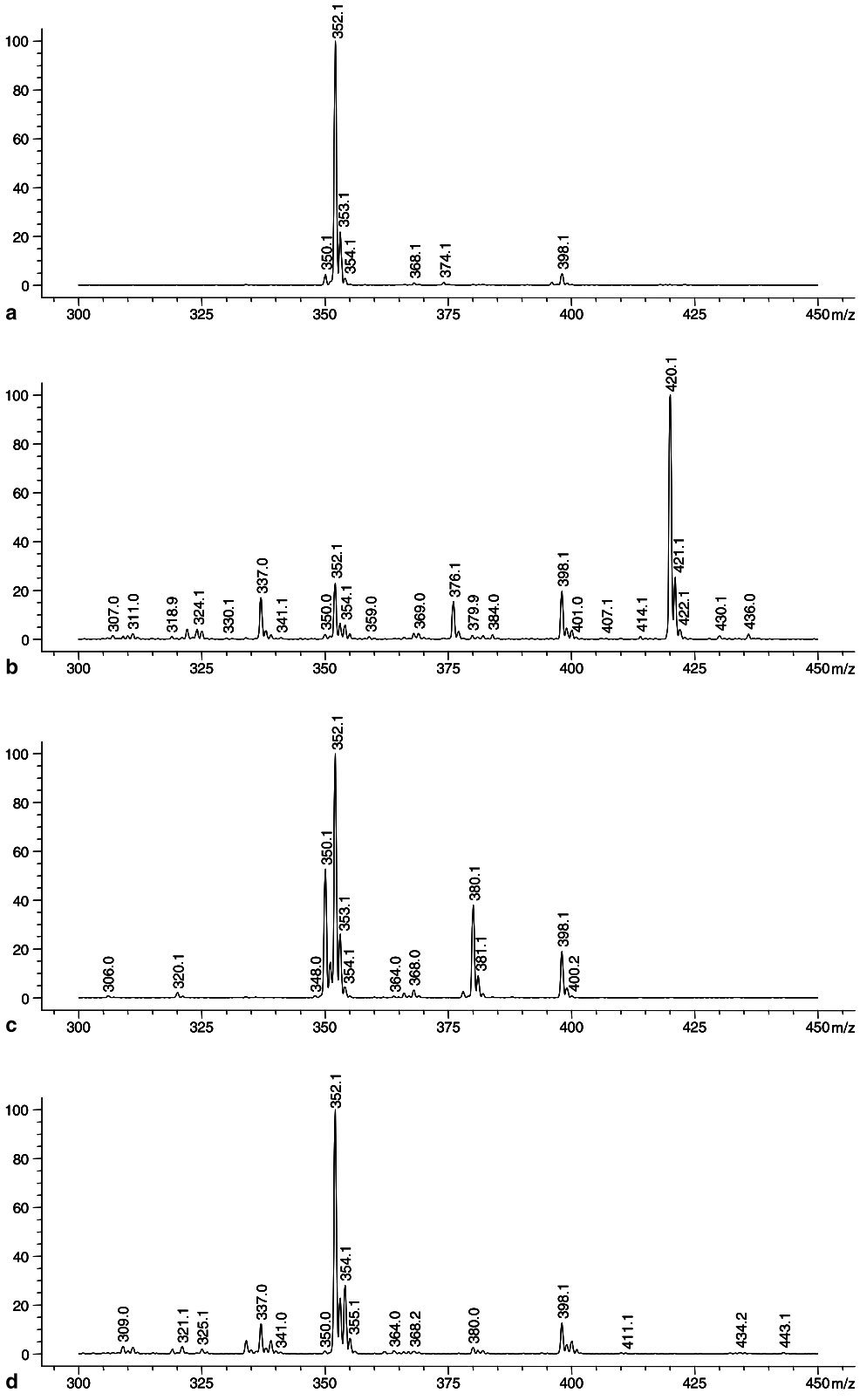


Fig. 3. Mass spectra of product **3**: measurement in **a** API-ES negative; **b** API-ES positive; **c** APCI negative; **d** APCI positive ion mode

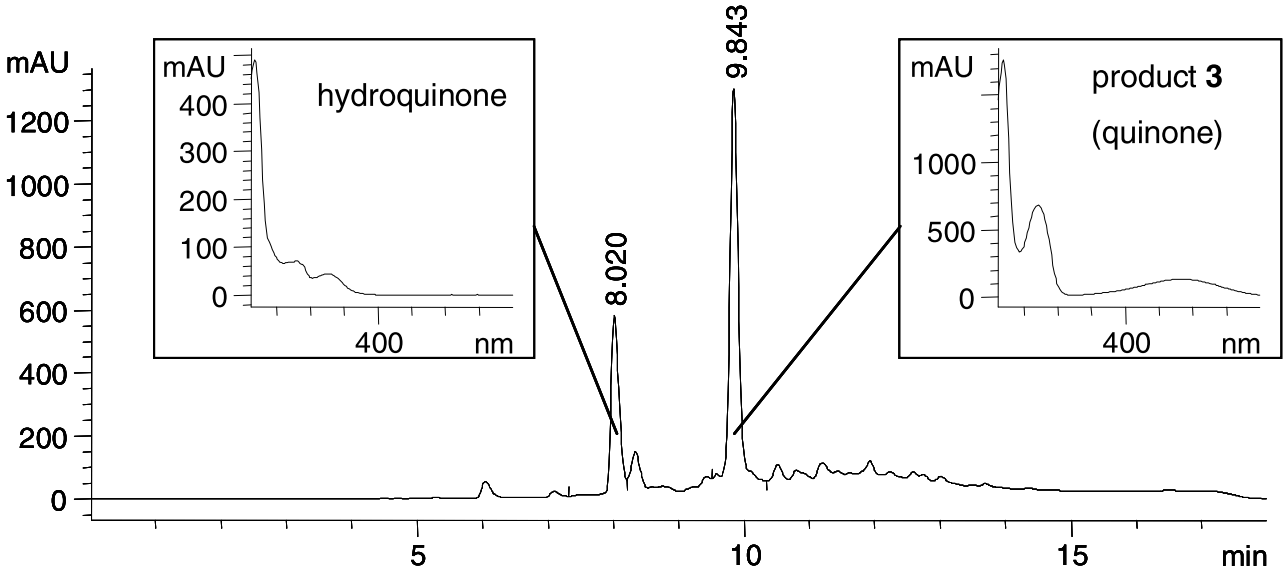


Fig. 4. HPLC-chromatogram of product **3** dissolved in methanol after 1 h: Rt = 9.84 min coupling product **3** and Rt = 8.02 min hydroquinone form 2-[3,6-dihydroxy-2-(2-hydroxy-ethylcarbamoyl)-phenylamino]-3-(1H-indol-3-yl)-propionic acid; appendant UV-spectra

visible and to identify where the coupling took place on the L-tryptophan molecule. Both spectra showed the same signal for the proton located at N1 whereas a proton signal at N2 was only detectable for the substrate L-tryptophan.

^{13}C-NMR analysis or other NMR experiments were not possible, because of the low stability of highly concentrated solutions of compound **3** in methanol and its insufficient solubility in chloroform.

Coupling of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide at the amino group of L-tryptophan was confirmed by use of N-acetyl-tryptophan in the coupling reaction. This L-tryptophan derivative contains an amino group protected by an acetyl residue and in consequence no comparable cross-coupling reaction occurred.

After lyophilization compound **3** retained its dark-red colour and was stable for more than ten months at +4 °C. The coupling product was completely soluble in methanol and tetrahydrofuran, incompletely soluble in water, acetonitrile and chloroform. In chloroform the solubilized product was relatively stable for about 20 hours; in the other solvents the coupling product **3** transformed into the corresponding hydroquinonoid form 2-[3,6-dihydroxy-2-(2-hydroxy-ethylcarbamoyl)-phenylamino]-3-(1H-indol-3-yl)-propionic acid (Figs. 4, 5a–d).

Discussion

The results show in case of L-tryptophan that pure amino acids can be derivatized by crosslinking reactions catalyzed by laccase. Structure and chemical data imply that the derivative is formed by nucleophilic attack of the radical cation formed from the *para*-dihydroxylated phenolic compound by laccase. Substrate oxidations by laccase are one-electron reactions, which create radicals (Ciecholewski et al., 2005; Bourbonnais et al., 1995; Potthast et al., 1996; Thurston, 1994; Heinzkill et al., 1998). These radicals can be transformed into quinones. In this way oxidation of the diphenols resulted in the formation of quinones, which can undergo nucleophilic attack by substances having amino groups (Figueroa et al., 1998). As a result C–N-coupled quinonoid products are generated. Such laccase-induced aminobenzoquinones were described recently with 4-aminobenzoic acid as amino partner (Manda et al., 2005). Until now laccases are known to catalyze polymerization reactions, and have been used for pollutant detoxification, textile dye transformation, biosensing and diagnostics (Xu, 1999, Tominaga et al., 2004; Young and Qing, 2004; Chakar and Ragauskas, 2004; Gianfreda et al., 1999; Mayer and Staples, 2002; Balakshin et al., 2001). Only a limited number of applications in organic synthesis have been described (Agematu et al., 1993a, 1993b; Schaefer et al., 2001; Anyanwutaku et al., 1994; Potthast et al., 1996; Fritz-Langhals and Kunath, 1998; Mikolasch et al., 2002; Manda et al., 2005; Baiocco et al., 2003).

In this study we show the enzymatic derivatization of a typical, natural occurring proteinogenic amino acid with a *p*-hydroquinone by use of laccase, to form a quinonoid C–N-dimer as product. Chemical structure and functional

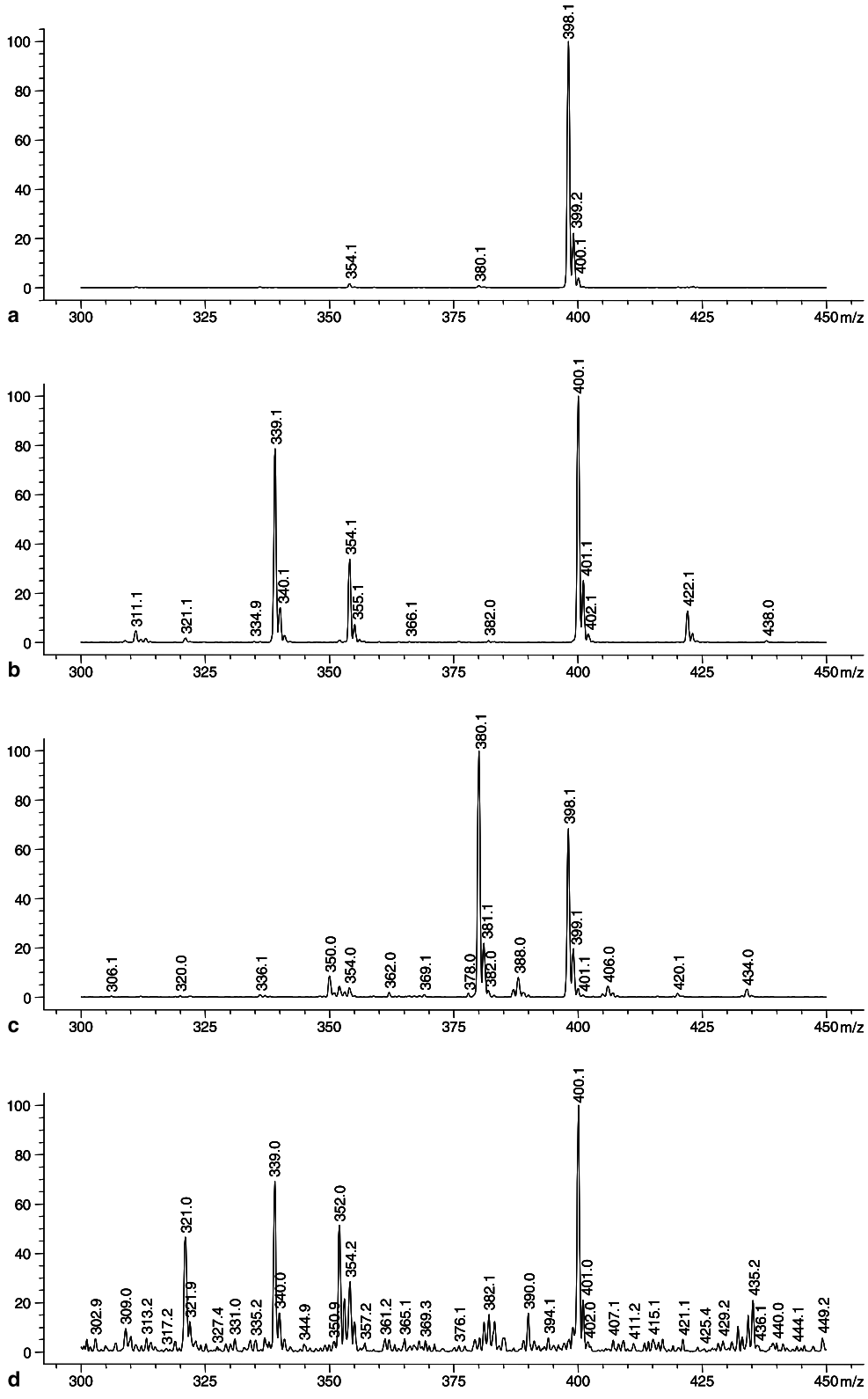


Fig. 5. Mass spectra of hydroquinone 2-[3,6-dihydroxy-2-(2-hydroxy-ethylcarbamoyl)-phenylamino]-3-(1H-indol-3-yl)-propionic acid of the quinonoid product **3**: measurement in **a** API-ES negative; **b** API-ES positive; **c** APCI negative; **d** APCI positive ion mode

groups of quinonoid compounds define their physicochemical properties, which are important for many biological processes. Natural benzoquinone derivatives like vitamin K and plastoquinone are well-known (Vaccaro and Scola, 1999) and quinonoid compounds such as mitomycins, anthracyclines, lapachols etc. are approved anticancer drugs (Brunmark and Cadenas, 1989). In the past much effort has been expended in the synthesis of derivatives of quinones like aminobenzoquinones (Kehrmann and Cordone, 1913; Trautner and Roberts, 1950; Nithiandandam and Erhan, 1998; Vaccaro and Scola, 1999; Kallmayer, 1978; Vaideeswaran, 1999; Machocho et al., 2003). Such quinones modified with amino-compounds are of interest for example as anti-corrosive coatings (Nithiandandam and Erhan, 1998; Vaccaro and Scola, 1999) or adhesion promoters (Vaccaro and Scola, 1999). However, derivatives of quinones and amino acids have also received attention. Most of the aminobenzoquinones are synthesised chemically. This usually involves harsh conditions, for example with sodium periodate (Nithiandandam and Erhan, 1998), with peracetic acid or high temperatures (Vaccaro and Scola, 1999; Kallmayer, 1978). Coupling of amino acids with quinones were described also in cold alcoholic solutions (Fischer and Schrader, 1910). In this case the chemically synthesized product of *p*-benzoquinone and glycine ethyl ester was identified as a 2,5-diamino-1,4-benzoquinone-derivative. The chemical synthesis of quinones containing an amino acid ester have also been reported (Cranwell and Haworth, 1970). Glycine and alanine or their esters can react with *p*-benzoquinone to form disubstituted quinonyl derivatives (Osman et al., 1995).

In contrast to chemical synthesis, the use of enzymes has advantages in the production of aminobenzoquinones. Laccase as a biocatalyst distinguishes itself by high stability and substrate selectivity. In addition, all reactions take place under mild conditions, at room temperature, without high pressure and in aqueous solution. Because of the use of nontoxic substances it offers an environmentally friendly possibility to create aminobenzoquinones.

However, some examples are given for the formation of coupling-products of quinones and amino acids during enzymatic reaction. The formation of intensely coloured products of catechol and glycine with tyrosinase was reported (Trautner and Roberts, 1950). Also tyrosinase-catalyzed formation of Dopa-Leu-containing quinones was described (Rosei et al., 1999). Furthermore, experiments on the coupling of amino acids and phenolic compounds with laccase in the context of amino acid linkage to lignin in soil have been described (Liu et al., 1985). Only attempts to isolate hybrid products of amino acid esters with protocatechuic acid or syringic acid have been reported; but isolation of products from nonderivatized amino acids was not possible. Likewise attempts to catalyse the reactions of amino- and tyrosyl groups of proteins with ferulic acid using laccase of *Pycnoporus cinnabarinus* were not successful (Figueroa et al., 1998). Our results show that enzymatic derivatization of a pure amino acid with *p*-hydroquinone compound by use of laccase is possible and can be performed with good yields.

The yield of product formed in the presence of equimolar concentrations of the substrates was at first roughly 15%. The major part of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide was transformed by laccase into homomolecular products and consequently not available for the cross coupling reaction with L-tryptophan. Homomolecular coupling of the hydroxylated coupling partner was also described for a hybrid dimer formation from 3,4-dichloroaniline and syringic acid (Tatsumi et al., 1994) as well as for various cross-coupling reactions with hydrocaffeic acid (Mikolasch et al., 2002). These undesirable reactions diminished product yields up to 50%.

By courtesy of the stepwise addition of the substrate 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide to the reaction mixture during the incubation, the consumption of L-tryptophan could be enhanced and the product yield increased up to 70%.

In case of laccase-catalyzed reactions the present data show for the first time the contemporaneous presence of a quinone and its corresponding hydroquinone form of a coupling product in one solution. The occurrence of both forms can be directly associated with the solvent used and the consequential stability of the product in it. Up to now, all published studies stated only the exclusive occurrence of either the quinonoid (Leonowicz et al., 1984; Bhalerao et al., 1994; Manda et al., 2005) or the hydroquinonoid (Anyanwutaku et al., 1994; Mikolasch et al., 2002; Nicotra et al., 2004) form.

During the last few years the potential of amino acid derivatives as active ingredients for pharmaceuticals has been recognized and considerable growth can be predicted. Further studies shall provide additional data and clarify if all proteinogenic amino acids and also free groups of peptides can react in a similar manner.

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References

Agematu H, Kominato K, Shibamoto N, Yoshioka T, Nishida H, Okamoto R, Shin T, Murao S (1993a) Transformation of 7-(4-hydroxyphenylacetamido) cephalosporanic acid into a new cephalosporin antibiotic,7-(1-oxaspiro(2.5) octa-6-oxo-4,7-diene-2-carboxamido)cephalosporanic acid, by laccase. Biosci Biotechnol Biochem 57: 1387–1388

Agematu H, Tsuchida T, Kominato K, Shibamoto N, Yoshioka T, Nishida H, Okamoto R, Shin T, Murano S (1993b) Enzymatic dimerization of penicillin-x. J Antibiot 46: 141–148

Amster IJ (1996) Fourier-transform mass spectrometry. J Mass Spectrom 31: 1325–1337

Anyanwutaku IO, Petroski RJ, Rosazza JPN (1994) Oxidative coupling of mithramycin and hydroquinone catalyzed by copper oxidases and benzoquinone. Implications for the mechanism of action of aureolic acid antibiotics. Bioorg Med Chem 2: 543–551

Baiocco P, Barreca AM, Fabbrini M, Galli C, Gentili P (2003) Promoting laccase activity towards non-phenolic substrates: a mechanistic investigation with some laccase-mediator systems. Org Biomol Chem 1: 191–197

Balakshin M, Capanema E, Chen CL, Gratzl J, Kirkman A, Gracz H (2001) Biobleaching of pulp with dioxygen in the laccase-mediator system – reaction mechanisms for degradation of residual lignin. J Mol Catal B Enzym 13: 1–16

Bhalerao UT, Muralikrishna C, Rani BR (1994) Laccase enzyme-catalyzed efficient synthesis of 3-substituted-1,2,4-triazolo(4,3-B)(4,1,2)benzothiadiazine-8-ones. Tetrahedron 50: 4019–4024

Bourbonnais R, Paice MG (1990) Oxidation of non-phenolic substrates-an expanded role for laccase in lignin biodegradation. FEBS Lett 267: 99–102

Bourbonnais R, Paice MG, Reid ID, Lanthier P, Yaguchi M (1995) Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2′-azinobis(3- ethylbenzthiazoline-6- sulfonate) in kraft lignin depolymerization. Appl Environ Microbiol 61: 1876–1880

Braun-Lüllemann A, Majcherezyk A, Hüttermann A (1997) Degradation of styrene by white-rot fungi. Appl Microbiol Biotechnol 47: 150–155

Brunmark A, Cadenas E (1989) Redox and addition chemistry of quinoid compounds and its biological implications. Free Radical Biol Med 7: 435–477

Bryans JS, Williams SC, Blakemore DC (2002) Alkyl amino acid derivatives useful as pharmaceutical agents. European Patent Application, EP 1 178 034 A1

Burzio LA, Waite JH (2000) Cross-linking in adhesive quinoproteins: studies with model decapeptides. Biochemistry 39: 11147–11153

Chakar FS, Ragauskas AJ (2004) Biobleaching chemistry of laccase-mediator systems on high-lignin-content kraft pulps. Can J Chem 82: 344–352

Ciecholewski S, Hammer E, Manda K, Bose G, Nguyen VTH, Langer P, Schauer F (2005) Laccase-catalyzed carbon-carbon bond formation: oxidative dimerization of salicylic esters by air in aqueous solution. Tetrahedron 61: 4615–4619

Cranwell PA, Haworth RD (1970) Humic acid – VI: The reaction of a-amino acid esters with quinones. Tetrahedron 27: 1831–1837

Erhan S (1997) Development of novel materials from proteins. In: Arshady R (ed) Desk reference of functional polymers, syntheses and applications. ASM, Washington DC, pp 261–270

Figueroa-Espinoza M-C, Morel M-H, Rouau X (1998) Effect of lysine, tyrosine, cysteine, and glutathione on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. J Agric Food Chem 46: 2583–2589

Figueroa-Espinoza MC, Rouau X (1999) Effect of cysteinyl caffeic acid, and L-dopa on the oxidative cross-linking of feruloylated arabinoxylans by a fungal laccase. J Agric Food Chem 47: 497–503

Fischer E, Schrader H (1910) Verbindungen von Chinon mit Aminosäureestern. Ber Dtsch Chem Soc 43: 525–529

Fritz-Langhals E, Kunath B (1998) Synthesis of aromatic aldehydes by laccase-mediator assisted oxidation. Tetrahedron Lett 39: 5955–5956

Gander-Coquoz M, Seebach D (1988) Synthesis of enantiometrically pure, alpha-alkylated lysine, ornithine, and tryptophan derivatives. Helv Chem Acta 71: 224–236

Gianfreda L, Xu F, Bollag J-M (1999) Laccases: a useful group of oxidoreductive enzymes. Bioremediation J 3: 1–25

Heinzkill M, Bech L, Halkier T, Schneider P, Anke T (1998) Characterization of laccases and peroxidases from wood-rotting fungi (Family Coprinaceae). Appl Environ Microbiol 64: 1601–1606

Jee JG, Park SJ, Kim HJ (2000) Tyrosinase-induced cross-linking of tyrosine-containing peptides investigated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 14: 1563–1567

Jonas U, Hammer E, Schauer F, Bollag J-M (1998) Transformation of 2-hydroxy dibenzofuran by laccases of the white rot fungi *Trametes versicolor* and *Pycnoporus cinnabarinus* and characterization of oligomerisation products. Biodegradation 8: 321–328

Kallmayer HJ (1978) Reactions between 2-Methyl-1,4-naphthochinone und 4-Aminophenol. Arch Pharm 312: 230–239

Kaplan DL (1998) Biopolymers from renewable resource. Springer, Berlin

Kehrmann F, Cordone M (1913) Über Anilino-chinone und Azin-Derivate derselben. Ber Dtsch Chem Soc 46: 3009–3014

Konradi AW, Pleiss MA, Thorsett ED, Ashwell S, Welmaker GS, Kreft A, Sarantakis D, Dressen DB, Grant FS, Semko C, Xu YZ (2005) Alpha amino acid derivatives-inhibitors of leukocyte adhesion mediated by VLA-4. United States Patent Application, US 2005/0085459 A1

Kuhl A, Hahn MG, Dumic M, Mittendorf J (2005) Alicyclic β-amino acids in medical chemistry. Amino Acids 29: 89–100

Leonowicz A, Edgehill RU, Bollag J-M (1984) The effect of pH on the transformation of syringic and vanillic acids by the laccases of *Rhizoctonia praticola* and *Trametes versicolor*. Arch Microbiol 137: 89–96

Liu S-Y, Freyer AJ, Minard RD, Bollag J-M (1985) Enzyme-catalyzed complex-formation of amino-acid esters and phenolic humus constituents. Soil Sci Soc Am J 49: 337–342

Machocho AK, Win T, Grinberg S, Bittner S (2003) Reaction of benzoquinones and naphthoquinones with 1,8-diamino-3,6-dioxanonane and with 1,11-diamino-3,6,9-trioxaundecane. Tetrahedron Lett 44: 5531–5534

Manda K, Hammer E, Mikolasch A, Niedermeyer T, Dec J, Jones AD, Benesi AJ, Schauer F, Bollag J-M (2005) Laccase-induced cross-coupling of 4-aminobenzoic acid with *para*-dihydroxylated compounds 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and 2,5-dihydroxybenzoic acid methyl ester. J Mol Cat B 35: 86–92

Marshall AG, Schweikhard L (1992) Fourier-transform ion-cyclotron Resonance mass-spectrometry – technique developments. Int J Mass Spectrom Ion Proc 118: 37–70

Marshall AG, Hendrickson CL, Jackson GS (1998) Fourier transform ion cyclotron resonance mass spectrometry: a primer. Mass Spectrom Rev 17: 1–35

Mayer AM, Staples RC (2002) Laccase: new functions for an old enzyme. Phytochem 60: 551–565

Michon T, Wang W, Ferrasson E, Gueguen J (1998) Wheat prolamine crosslinking through dityrosine formation catalyzed by peroxidases: Improvement in the modification of a poorly accessible substrate by "indirect" catalysis. Biotechnol Bioeng 63: 449–458

Mikolasch A, Hammer E, Jonas U, Popowski K, Stielow A, Schauer F (2002) Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines using laccase. Tetrahedron 58: 7589–7593

Mueller U, Huebner S (2003) Economic aspects of amino acid production. Biochem Eng Biotechnol 79: 137–170

Nicotra S, Cramarossa MR, Mucci A, Pagnoni UM, Riva S, Forti L (2004) Biotransformation of resveratrol: synthesis of trans-dehydrodimers catalyzed by laccases from Myceliophtora thermophyla and from Trametes pubescens. Tetrahedron 60: 595–600

Nithianandam VS, Erhan S (1998) Quinone-amine polymers: 18 – A novel method for the synthesis of poly(alkyl aminoquinone)s. Polymer 39: 4095–4098

Ohkawa K, Fujii K, Nishida A, Yamauchi T, Ishibashi H, Yamamoto H (2001) Lysyl oxidase-catalyzed cross-linking and insolubilization reactions of lys-containing polypeptides and synthetic adhesive proteins. Biomacromolecules 2: 773–779

Osman AM, El-Maghraby MA, Khalil ZH, Hassan KM (1975) Egypt. J Chem 18: 993–999

Oudgenoeg G, Hilhorst R, Piersma SR, Boeriu CG, Gruppen H, Hessing M, Voragen AGJ, Laane C (2001) Peroxidase-mediated cross-linking of a tyrosine-containing peptide with ferulic acid. J Agric Food Chem 49: 2503–2510

Pfizer Ltd, Maw GN, Rawson DJ, Thompson LR (2004) Therapeutic use of aryl amino acid derivatives. International Patent Application, PCT/WO 2004/014357 A2

Piacentini M, Rodolfo C, Farrace MG, Autuori F (2000) "Tissue" transglutaminase in animal development. Int J Dev Biol 44: 655–662

Potthast A, Rosenau T, Chen CL, Gratzl JS (1996) A novel method for the conversation of benzyl alcohols to benzaldehydes by laccase-catalyzed oxidation. J Mol Cat A 108: 5–9

Rosei MA, Coccia R, Foppoli C, Blarzino C, Cini C, Schinina E (1999) Cysteinyldopaenkephalins: synthesis, characterization and binding to bovine brain opioid receptors. Biochim Biophys Acta 1478: 19–29

Rozzell JD, Wagner F (1992) Biocatalytic production of amino acids and derivatives. Carl Hanser, Munich

Schaefer A, Specht M, Hetzheim A, Francke W, Schauer F (2001) Synthesis of substituted imidazoles and dimerization products using cells and laccase from *Trametes versicolor*. Tetrahedron 57: 7693–7699

Seko T, Kato M (2001) Amino acid derivatives and drugs containing the same as the active ingredient. European Patent Application, EP 1 097 929 A1

Smith-Mungo LI, Kagan HM (1998) Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 16: 387–398

Steinegger E, Hänsel R (1992) Pharmakognosie, 5th ed. Springer, Berlin Heidelberg New York, pp 440–443

Stürzebecher J, Steinmetzer T, Schweinitz A, Stürzebecher A, Uhland K (2004) N-sulfonylierte Aminosäurederivate, Verfahren zu ihrer Herstellung und ihre Verwendung. German Patent Application, DE 103 22 191 A1

Tatsumi K, Freyer A, Minard RD, Bollag J-M (1994) Enzymatic coupling of chloroanilines with syringic acid, vanillic acid and protocatechuic acid. Soil Biol Biochem 26: 735–742

Thurston CF (1994) The structure and function of fungal laccase. Microbiology 140: 19–26

Tominaga J, Michizoe J, Kamiya N, Ichinose H, Maruyama T, Goto M (2004) Factors affecting the oxidative activity of laccase towards biphenyl derivatives in homogeneous aqueous-organic systems. J Biosci Bioeng 98: 14–19

Trautner EM, Roberts EAH (1950) The chemical mechanism of the oxidative deamination of amino acids by catechol and polyphenolase. Austr J Sci Res Ser B3: 356–380

Vaccaro E, Scola DA (1999) New applications of polyaminoquinones. Chemtech 29: 15–23

Vaideeswaran K, Bell JP, Nikles DE (1999) Quinone-amine polyurethanes: novel corrosion-inhibiting coupling agents for bonding epoxy to steel. J Adh Sci Techn 13: 477–499

Xu F (1999) Laccase. In: Fleckinger MC, Drew SW (eds) Encyclopedia of bioprocess technology. J Wiley, New York, pp 1545–1554

Young SK, Qing XL (2004) Fungal laccase-catalyzed degradation of hydroxy polychlorinated biphenyls. Chemosphere 56: 23–30

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