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Laccase-mediated multi-step homo- and heteromolecular reactions of *ortho*-dihydroxylated aromatic compounds and mono- or diaminated substances resulting in C-C, C-O and C-N bonds

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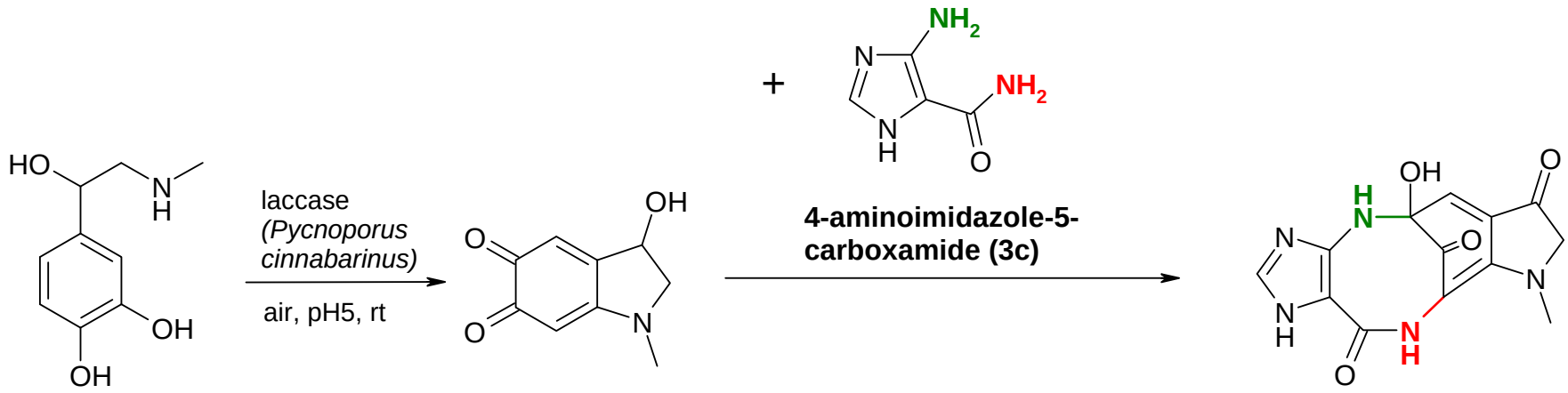
Graphical Abstract



epinephrine (1d) **7** **8a** yield = 15 %

Highlights

- The laccase-mediated multi-step reactions resulted in homo- or heteromolecular cyclization products as well as monoaminated quinonoid compounds.
- The mild and environmentally friendly reaction conditions are advantagous properties for different applications with laccase as green catalyst.

Abstract

Three types of reactions involving oxidation, inter- and intramolecular Michael-addition as well as homo-intermolecular dimerization, catalyzed by laccase [E.C.1.10.3.2] of *Pycnoporus cinnabarinus* in the presence of oxygen, resulted in the formation of dimeric, trimeric and cyclic products with yields up to 44% (non-optimized reactions). For heteromolecular reactions a number of different aminated five- and six-membered aromatic compounds were used. The first reaction type involved the oxidation of catechol, 3- or 4-methylcatechol to *o*-quinone and subsequent Michael-addition of the amino compounds with C-N bond formation. Conclusive analytical data (UV–vis data, MS spectra) for the respective *o*-quinone are provided. The second type of reaction included a homo-intermolecular dimerization probably with C-C and C-O bond formation to the proposed dibenzofuran derivatives. The heteromolecular reaction with the amino compounds yielded trimers consisting of homomolecular dimers and the amino compound connected via a C-N bond. The third reaction type started from epinephrine (adrenaline) which was oxidized by laccase and underwent an intramolecular Michael-addition to adrenochrome. The subsequent heteromolecular reaction resulted in the unusual substance class of cyclooctenes (diazocines). Differences between the reaction types in regard to the kind of *o*-hydroquinone, amino compound used and the types of product recovered are discussed.

Keywords: oxidoreductase; quinone; benzene-1,2-diol; cascade / domino reaction; aminobenzoic acid

1. Introduction

The formation of novel heterocyclic compounds is becoming more and more important not only as a source of building blocks for synthetic organic chemistry but also for the development of new drugs. In this respect laccases [E.C. 1.10.3.2, benzenediol:dioxygen oxidoreductase] possess numerous advantages. Laccases use oxygen as co-factor, water is the only by-product and mild environmentally friendly reaction conditions (atmospheric pressure, pH usually at 5-7, room temperature) enable a "green" synthesis of various compounds. Laccases contain four copper atoms responsible for the catalysis of the oxidation reaction resulting in radicals which may undergo non-enzymatic reactions.

So far, laccase-mediated reactions have been used for the synthesis of dimers and trimers as well as of higher polymers. The dimeric products can be homomolecular consisting of two identical molecules connected via a C-C bond (such as salicylic esters) [1] or a C-O bond as described for 2-hydroxydibenzofuran [2]. For heteromolecular products - formed between two or more different reaction partners C-C [3,4], C-O [5] and also C-N [6,7] or C-S [8,9] bonds are possible. Some of the products may be biologically active e.g. antibacterial [10,11], antifungal [12], antioxidative [13,14] or anticancer [9].

In recent years the synthesis of heterocyclic compounds by laccase-mediated domino reactions has been described and this is a very promising approach since it permits a "one-pot" reaction without the need for protecting groups. Different types of heterocycles may be formed during laccase-catalyzed reactions. In this way five-membered rings such as benzofurans [15,16], dibenzofurans [17-21], thiazoles [22] as well as six-membered rings such as phenoxazinone chromophores [23-25] have been synthesized.

Recently, we used laccase to produce additional heterocycles including cycloheptenes (diazepines), cyclooctenes (diazocines), diazaspiro cyclohexenes, and phenazines using *para*-dihydroxybenzoic acid derivatives and aromatic or heteroaromatic amines [26].

The aim of this study was to describe the product formation - in particular of the cyclic products - generated using the *ortho*-dihydroxylated substances catechol, 3-methylcatechol, 4-methylcatechol, and epinephrine (adrenaline). For heteromolecular reactions a number of aminated azoles and benzoic acid derivatives were used. The formation of diazocines was of particular interest since the chemical synthesis of these substances involves harsh reaction conditions as which involve heating at 110°C with ethyl acetate [27] as well as the use of acids or metals such as palladium [28].

In contrast, the laccase based reaction is suitable for a more environmentally friendly synthesis of these compounds. To the best of our knowledge this is the first study concerning the laccase-mediated synthesis of such epinephrine derivatives.

2. Experimental

2.1 Chemicals

Catechol and 2-aminobenzoic acid were purchased from Merck (Hohenbrunn, Germany). 3-Methylcatechol, 4-methylcatechol, epinephrine, 2-aminobenzamide and 4-aminoimidazole-5-carboxamide hydrochloride were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 3-Aminopyrazole-4-carboxamide hemisulfate was purchased from Tyger Scientific Inc. (Ewing Township, New Jersey, USA).

2.2 Enzymes

The laccase used was obtained from *Pycnoporus cinnabarinus* SBUG-M 1044. This white rot fungus was isolated from an oak tree in northern Germany, and is deposited at the strain collection of the Department of Biology of the University of Greifswald (SBUG). Cultivation of *Pycnoporus cinnabarinus* SBUG-M 1044 and the preparations of the crude laccase were carried out as reported previously [2]. This enzyme preparation contains isoenzymes of laccase, but no other enzymes and was always used in 20 mM sodium acetate buffer (SAB) pH 5.0, at its pH optimum [2,29].

2.3 Measurement of laccase activity

The activity of laccase was determined spectrophotometrically at 420 nm with ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) as substrate [30] using the method described by Jonas et al. [2]. 1 U is defined as the turnover of 1 $\mu mol \cdot ml^{-1} \cdot min^{-1}$.

2.4 Experimental procedures

For analytical experiments the dihydroxylated compounds (1 mM, 2 mM) and amines (1 mM, 2 mM) were incubated with 0.5 U of laccase.
For reactions with epinephrine 16 mM of this compound and 16 mM tartaric acid (Lachema/Chemapol, Brno/Prague, Czech Republic) were gently mixed in SAB and used after one day for biotransformation with an end concentration of 1 mM epinephrine.
In controls the respective compounds were incubated in SAB without laccase. Reaction mixtures were incubated with agitation at 200 rpm at 23 °C in the dark.

2.5 Analytical HPLC (high-performance liquid chromatography)

For routine analysis, the reaction mixtures and isolated products were analyzed using an HPLC system LC-10AT VP (Shimadzu, Germany) consisting of a FCV-10AL VP pump, SPD-M10A VP diode array detector, and a SCL-10A VP control unit controlled by Class-VP version 6.12 SP5. Substances were separated on an endcapped, 5-µm, LiChroCART® 125-4 RP18 column (Merck, Darmstadt, Germany) run at a flow rate of 1 ml/min. The solvent system used consisted 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.

2.6 Structural characterization of products by LC/MS (liquid chromatography/mass spectrometry) and NMR (nuclear magnetic resonance) spectrometry

The reaction mixtures and isolated products were characterized using a LC/MS system. The atmospheric pressure ionization (API) mass spectrometry experiments were performed on an Agilent Series 1200 HPLC system with diode array detector and an Agilent 6120 quadrupole mass spectrometer (Waldbronn, Germany). The MS was run with the electrospray ionization (API-ES) source in positive mode (dry and nebulizer gas: nitrogen; nebulizer pressure: 45 psig; drying gas flow: 10 l/min; drying gas temperature: 350°C; capillary voltage: 4 kV; fragmentor voltage: 75 V). HPLC separation was performed on a Zorbrax SB-C18 (2.1x50mm, 1.8µm) column (Agilent, Waldbronn, Germany), at a flow rate of 0.08 ml/min (excepting reactions with epinephrine: 0.12 ml/min). The solvent system consisted of acetonitril (eluent A) and 0.1% aqueous ammonium formate (eluent B), starting from an

initial ratio of 10% A and 90% B and reaching 100% methanol within 7 min. Elution with methanol was continued for a further 6 minutes.

NMR spectra were obtained at 600 MHz (^{1}H, HSQC, HMBC) and at 150 MHz (^{13}C) in deuterated methanol or dimethyl sulfoxide on a Bruker Avance 600 instrument (Rheinstetten, Germany).

2.6.1 2-(3,4-Dihydroxyphenylamino)-benzamide (4a)

Synthesis and isolation as described above. Red solid. Yield 43.5 % (10.7 mg). ^{1}H NMR: δ (600 MHz, DMSO-d_6) 6.42 (m, *J* = 2.2, *J* = 8.4, 1H, H-6'), 6.56 (d(s), *J* = 2.2, 1H, H-2'), 6.62 (m, *J* = 7.9, 1H, H-5), 6.68 (d, *J* = 8.4, 1H, H-5'), 6.96 (d, *J* = 8.5, 1H, H-3), 7.20 (m, *J* = 8.5, 1H, H-4), 7.64 (d, *J* = 7.9, 1H, H-6), 9.69 (s, 1H, NH). ^{13}C NMR: δ (150 MHz, DMSO-d_6) 110.7 (C-2'), 113.3 (C-3), 113.6 (C-6'), 115.8 (C-5), 116.1 (C-5'), 129.1 (C-6), 132.1 (C-4). HMBC correlations: (DMSO-d_6) NH (C-1, (C-1')*, (C-2), (C-2'), C-3, C-6'), H-2' ((C-1'), (C-3'), C-4', C-6'), H-3 (C-1, C-5, (C-7)), H-4 (C-2, C-6), H-5 (C-1, ((C-2))**, C-3, ((C-4)), ((C-6))), H-5' (C-1', C-3', (C-4')), H-6 (C-2, C-4, C-7), H-6' (C-2', C-4'). R_f (HPLC) 7.08 min, R_f (LC/MS) 8.773 min, UV-vis λ_{max} 209, 290, 492 nm. MS *m/z* (rel. intensity) AP-ESI: pos. ion mode $[M+H]^+$ 243.1 (100), $[2M+Na]^+$ 507.1 (10).

* () signals with low intensity

** (()) signals with very low intensity

2.6.2 Product 5b

R_f (HPLC) 13.26 min, R_f (LC/MS) 18.942 min, UV-vis λ_{max} 205, 398 nm. MS *m/z* (rel. intensity) AP-ESI: pos. ion mode $[M+H]^+$ 243.0 (100), $[2M+Na]^+$ 507.1 (5).

2.6.3 Product 6h

Synthesis and isolation as described above. Brown green solid. Yield 9.4 % (16.1 mg). ^{1}H NMR: δ (600 MHz, DMSO-d_6) 1.63 (s), 1.66 (s), 1.67 (s), 1.74 (s), 1.80 (s), 1.89 (s), 1.97 (s), 2.08 (s), 2.11 (s), 6.64 (s), 6.65 (s), 7.23 (s), 7.74 (s), 7.84 (s), 7.94 (s), 8.00 (s), 8.81 (s). ^{13}C NMR: δ (150 MHz, DMSO-d_6) 9.4, 15.8, 15.9, 16.5, 21.5, 102.7, 127.6, 128.4, 130.5, 135.7, 135.8, 137.6. HMBC correlations: (DMSO-d_6) 1.63 (109.8, 132.7, 143.5), 1.66 (108.5, 132.7, 143.9), 1.67 (108.5, 132.7, 143.9), 1.74 (128.4, 135.1, 184.2), 1.80 (127.6, 135.1, 184.2), 1.89 (172.1), 1.97 (138.2, 183.1), 2.08 (121.6, 124.8, 143.9), 2.11 (129.9, 137.2, 183.9), 6.64 ((15.9)*, (113.1), (145.2), 184.2), 6.65 ((15.9), (113.1), (145.2), 184.2), 7.23 (112.8, 132.5, 143.4), 7.74 (123.4, 147.5), 7.84, 7.94, 8.00 ((144.3)), 8.81. R_f (HPLC) 6.60 min, R_f (LC/MS)

14.437 min, UV-vis λ_{max} 232, 267, 293, 463 nm. MS *m/z* (rel. intensity) AP-ESI: pos. ion mode $[M+H]^+$ 367.0 (100).
* () signals with low intensity

2.6.4 Epinephrine derivative (8a)

Synthesis and isolation as described above. Yellow solid. Yield 14.5 % (13.0 mg). ^{1}H NMR: δ (600 MHz, DMSO-d_6) 3.06 (s, 3H, H-5), 4.15 (m, *J* = 18.8, 2H, H-4), 5.04 (s, 1H, H-1), 7.20 (s, 1H, NH), 7.34 (s, 1H, NH, H-16), 7.82 (s, 1H, H-12), 11.09 (s (broad), 1H, OH), 13.31 (s, 1H, NH, H-13). ^{13}C NMR: δ (150 MHz, DMSO-d_6) 32.2 (C-5), 60.0 (C-4), 81.3 (C-1), 137.5 (C-12). HMBC correlations: (DMSO-d_6) H-1 (C-2, C-3, C-7, C-8, C-9), H-4 (C-2, C-3, C-5, C-6, C-7), H-5 (C-4, C-6), H-12 (C-11, C-14, C-15), H-13 (C-11), H-16 (C-7, C-8, C-9, C-14, C-15). R_f (HPLC) 5.18 min, R_f (LC/MS) 1.156 min, UV-vis λ_{max} 204, 272, 296, 354, 414 nm. MS *m/z* (rel. intensity) AP-ESI: pos. ion mode $[M+H]^+$ 302.0 (100).

Experimental methods; UV–vis data and MS spectra for all compounds (Tab. S1-34); ^{1}H-NMR, ^{13}C-NMR, HSQC, HMBC spectra of product **4a-c**, **8a,b** (Tab. S5-7, S33, 34); ^{1}H-NMR, HSQC, HMBC spectra of product **6h** (Tab. S27) is available in the electronic supplementary file.

3. Results and discussion

The laccase is able to oxidize substrates to form reactive radicals which can undergo reactions with each other or with additional reaction partners like amino compounds. In these reactions multiple bond formations are possible resulting e.g. in cyclic products. We demonstrate the product formation during homomolecular reaction of *o*-dihydroxylated aromatic substances as well as the heteromolecular reaction of these products with amino compounds.

3.1 First reaction type: In the laccase-catalyzed homomolecular reactions catechol (**1a**), 3-methylcatechol (**1b**) or 4-methylcatechol (**1c**) were completely transformed within 20 minutes and the corresponding *o*-quinones (**2a-c**) were formed (Fig. 1). The HPLC analysis showed that the retention time of **2a** (R_f 3.30 min; Tab. 1) was lower than that of the catechol **1a** (R_f 5.50 min). Similarly, Albarran et al. [31] described the appearance of the *ortho*-quinone

before the catechol in HPLC analysis of the reaction products of a chemically-catalyzed reaction with hexachloroiridate(IV). In addition, the UV-vis spectrum of the *ortho*-benzoquinone showed an absorption maximum at 389 nm [31] which corresponds exactly to **2a**. The *ortho*-benzoquinone has a large dipole moment and hence high polarity which may explain the low retention time [31]. Albarran et al. [31] described for the LC/MS (API-ES) analyses the formation of cation(s) and anion(s) with *m/z* 109 for the *ortho*-benzoquinone. The mass *m/z* 109 for an anion was explained by the formation of a hydride adduct. In contrast, our LC/MS (API-ES positive mode) analysis of **2a** resulted in the detection of fragment ions at *m/z* (rel. intensity) 126.1 $[M+NH_4]^+$ (100) and 131.0 $[M+Na]^+$ (25) (Suppl. Mat. Tab. S2). The *m/z* 109 $[M+H]^+$ for a cation was detected with a low relative intensity of 6. In addition, API-ES negative mode studies resulted in *m/z* 109 of an anion but the ionization was considerably less than in positive mode (data not shown). The MS analyses of the oxidation products of **1b** and **1c** showed again ammonium adducts as well as the molecular ion peak at *m/z* 123.0 $[M+H]^+$ confirming the *o*-quinone formation (Suppl. Mat. Tab. S3,4). The oxidation of *o*-hydroquinones to *o*-benzoquinones via laccase has been well known [32-34] but detailed analyses about the benzoquinones formed are missing to date. Despite the lack of an authentic sample and limited analytical data in the literature, the *ortho*-benzoquinone (**2a**) as well as its methylated derivatives (**2b,c**) were clearly identified by MS analyses.

The *o*-benzoquinone (**2a-c**) formation was a prerequisite for the heteromolecular reaction with 2-aminobenzamide (**3a**) resulting in heteromolecular dimers (**6a-c**) with yields of 40-44%. The coupling of one molecule of the catechol and one molecule of the amino compound were confirmed by the presence of all carbon atoms of the catechols **1a-c** and of the amino partner **3a** in the ^{13}C NMR spectra of the products **4a-c**. Two signals in the range of 140 - 150 ppm indicated a hydroquinonoid character of the products (e.g. for product **4a** 141.7 ppm (C-4') and 145.8 ppm (C-3')). No signals were detected in the range of 180 ppm - the range of carbonylic groups. Furthermore, all proton signals of each of the two reactants, as well as the proton of the C-1', were detectable in the ^1H NMR spectra of the products **4a-c**. The number of CH proton signals of the dihydroxylated phenyl rings changed from two - in the reactant **1a** - to three signals in the product **4a** and from three - in the reactants **1b-c** - to two signals in the products **4b** and **4c**. The multiplicity of the signals indicated a further substituent at the C-1' position of **4a-c**. The HMBC spectrum of **4a** showed correlations between the aliphatic amine proton (9.69 ppm) and the C-1', C-2' and C-6' of the hydroquinonoid ring on the one hand and on the other with the C-1, C-2 and C-3 of the amino partner. These facts show **4a** to be

aminated at the C-1' position of the hydroquinone ring. The HMBC included also correlations between the H-2', H-5' and H-6' protons of the hydroquinone ring and the carbons C-3' and C-4' which are substituted by hydroxyl groups, confirming the hydroquinonoid character. Nevertheless, the LC/MS (API-ES positive mode) analyses of **4a-c** resulted in molecular ion peaks (e.g. for **4a**: *m/z* (rel. intensity) 243.0 $[M+H]^+$ (100)) for monoaminated *ortho*-quinonoid compounds. In addition, the UV-vis spectra (**4a**: 204, 273, 497 nm; **4b**: 208, 300, 505 nm; **4c**: 202, 293, 495 nm) resemble those of aminated *para*-quinonoid products with two absorption maxima between 200-300 nm and a weak maximum around 500 nm [6]. Depending on the measurement used we can postulate hydroquinonoid or quinonoid structures, but with all methods we could demonstrate the coupling of one molecule of catechol and one molecule of the amino compound (Fig. 1).

Further reactions of **1a-c** with 2-aminobenzoic acid (**3b**), 4-aminoimidazole-5-carboxamide (**3c**) and 3-aminopyrazole-4-carboxamide (**3d**) resulted also in the formation of heteromolecular dimers (**4d-l**) which were structurally characterized by LC/MS (Suppl. Mat. Tab. S8-16). The products consist of one molecule of the oxidized catechol and one molecule of the benzoic acid or aminated azole, respectively. It was not possible to isolate **4d-l** due to complex reaction kinetics and instability of some of these products.

The formation of heteromolecular dimers for the reaction of *ortho*-dihydroxylated aromatic acids with amino-β-lactam antibiotics was reported by Mikolasch et al. [33]. The products presented by Mikolasch et al. [33] had a quinonoid structure in contrast to our identified products **4a-c** with hydroquinonoid structure.

For **1b** the regioselective formation of catechol thioethers was described by Abdel-Mohsen et al. [35] with a nucleophilic attack at C4 or C5 (C4 favoured with approx. 66%). In contrast, for **1a** and **1c** only a Michael-addition at C5 was described by Abdel-Mohsen et al. [35]. These results are in accordance with our experiments for **1a** and **1c** and confirm C5 as the favoured position for a nucleophilic attack at catechols.

We note that in some reactions also trimeric products consisting of one molecule of the oxidized catechol and two molecules of the amino compound were detected in accordance with other laccase-mediated reactions [36,37]. However in the experiments reported here only traces of such trimers were formed because of the use of equimolar concentrations of reactants and the strong competing homomolecular reaction.

3.2 Second reaction type: The reactions of the catechols (**1a-c**) described above are not straightforward kinetics to the *o*-quinones. Instead these reactions resulted not only in the

formation of the respective *o*-quinones (**2a-c**) but also in the production of homomolecular dimers (**5a-c**) which were formed only in small amounts (according to the peak area at 254 nm; Fig. 2).

The LC/MS (API-ES positive mode) analyses of **5a-c** resulted in the detection of fragment ions at *m/z* (rel. intensity) 215.0 $[M+H]^+$ (100; Suppl. Mat. Tab. S17-19) implying an connection of two molecules **1a-c**, respectively. The products may consist of two quinones connected via one C-C bond or may be formed by one C-C and one C-O bond. According to Hajdok et al. [19,20] the first step of the reaction process is the laccase catalyzed oxidation of one molecule of catechol (**1a-c**) with O_2 to *o*-benzoquinone (**2a-c**). The *o*-benzoquinones can undergo an intermolecular 1,4-addition with a second molecule of **1a-c** acting as a nucleophile to yield a dihydroxy intermediate (I) that cannot be isolated, but that can be further transformed. On one hand two laccase catalyzed oxidation steps can result in **5a$_1$-c$_1$**. On the other hand side one laccase catalyzed oxidation step followed by a second 1,4-addition and a second laccase catalyzed oxidation step can result in the intramolecular formation of **5a$_2$-b$_2$**. Due to the methyl group of **1c** the formation of a **5c$_2$** structure is not possible. However, a comparable product to **5a-b** could be analyzed for **1c**, which is why this third probable pathway must be considered. One laccase catalyzed oxidation step followed by a 1,3-addition and a second laccase catalyzed oxidation step may result in the formation of **5a$_3$-c$_3$**. In contrast to the homomolecular reactions of **1a-c** described herein, Hajdok et al. described the formation of products comparable to **5a$_2$-b$_2$** but for heteromolecular reactions of cyclohexane-1,3-diones [19] or 1,3-dicarbonyls [20] with catechols.

In the heteromolecular reaction of **1a-c** with **3a-c** heteromolecular trimers (**6a-l**) were detected consisting of one homomolecular dimer (**5a$_1$-c$_1$, 5a$_2$-c$_2$** or **5a$_3$-c$_3$**) and one molecule of the respective amino compound **3a-d** (LC/MS data for **6a-l**: Suppl. Mat. Tab. S20-31). The products **6a-l** were formed in small amounts and thus only one product (**6h**) could be isolated at a yield of 9% (Fig. 3).

The LC/MS (API-ES positive mode) analysis for **6h** resulted in a molecular ion peak at *m/z* (rel. intensity) = 367.1 (100). The NMR analyses revealed the formation of at least three structurally different products. The structure 4-[(4-hydroxy-3,8-dimethyl-6,7-dioxodibenzofuran-2-yl)amino]-1H-imidazole-5-carboxamide is proposed for one of these products (Tab. 2). The structure of 4-[(4-hydroxy-3,8-dimethyl-6,7-dioxodibenzofuran-2-yl)amino]-1H-imidazole-5-carboxamide was confirmed by the presence of all carbon atoms of two catechol molecules and of one molecule of the amino partner in the ^{13}C NMR spectrum of the product **6h**. A signal in the range of 180 ppm indicated a quinonoid character of ring A.

Two signals in the range of 140 - 150 ppm suggested that a furanic structure is part of the product (143.4 ppm (C-9a) and 145.2 ppm (C-9b)). The HMBC included correlations between the H-14 and C-9a (143.4 ppm) on one hand and between H-13 and C-11 (184.2 ppm) on the other, confirming the hydroquinonoid character of ring B and the quinonoid character of ring A. The singulet character of the proton signal H-14 indicated a further substituent at the C-7 position. The chemical shift to lower field of the C-7 carbon (143.9 ppm) compared with the C-8 carbon (132.7 ppm) demonstrated the presence of an electron-withdrawing group such as the amino partner **3c**.

According to the NMR analyses of **6h** the homomolecular dimers **5a$_3$-c$_3$** could be postulated to be dibenzofuran derivatives.

As described previously the dibenzofurans resulted from homo-intermolecular dimerizations involving C-C and C-O bond formation. Descriptions of the laccase-mediated formation of C-C and C-O bonds in a one-pot reaction with only one compound are very rare. Thus, Nicotra et al. [38] described the formation of a dehydrodimer during laccase-catalyzed reaction of resveratrol. The formation of a heteroaromatic five-membered furan ring within a dibenzofuran system has been described for the laccase-mediated homo-intramolecular reaction of 3,5-dichloro-2-hydroxybiphenyl by Kordon et al. [39].

In contrast to the homomolecular reactions described by Nicotra et al. [38], Kordon et al. [39] and in our study, the formation of heteroaromatic five-membered rings resulting from heteromolecular reactions of *ortho*-hydroquinones have been repeatedly studied [17-22].

In some reactions, such as with **1c**, the homomolecular product (**5c**) was hydroxylated (data not shown) probably as a result of an attack by water possibly after the formation of **2c** or **5c**. Furthermore, this hydroxylated product can undergo a coupling reaction with **3c** or **3d** as described for the heteromolecular reaction of 2,5-dihydroxy-N-(2-hydroxyethyl)-benzamide and morpholine [12].

In general morpholines and azoles (the last are elements of products **4** and **6**) are building blocks for antimicrobial drugs [40-42]. In addition, dibenzofurans may also possess biological activity as is the case with usnic acid derived from lichens that exerts antibacterial [43] and anticancer [44] activity.

3.3 Third reaction type: The laccase-mediated reaction of epinephrine (**1d**) resulted in the formation of one sole product (**7**). The HPLC analysis showed that **7** (R_f 1.7 min) had a

similar retention time than **1d** (R_f 1.3 min). The UV-vis spectrum of **7** showed absorption maxima at 219, 301, 487 nm which corresponds to those found in the literature for adrenochrome (220, 305, 490 nm; [32]). The LC/MS (API-ES positive mode) analysis resulted in fragment ions at *m/z* (rel. intensity) 180.0 $[M+H]^+$ (100) and 381.1 $[2M+Na]^+$ (19) confirming an intramolecular cyclization to form adrenochrome.

In the heteromolecular reactions of **1d** with **3c,d** only one product (**8a,b**) was formed, respectively. The products were detected within 20 min and their concentrations increased over at least 24 hours. The LC/MS (API-ES positive mode) analyses for the products resulted in molecular ion peaks at *m/z* (rel. intensity) 302.0 (100) for **8a** and 302.1 (100) for **8b** (Suppl. Mat. Tab. S32-33). We measured various NMR sprectra – ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and ^{1}H^{1}H COSY – and from all these spectra we tentatively propose the structures of **8a** and **8b** as shown in Fig. 4.

The LC/MS (API-ES positive mode) analysis for the product **8c** of the reaction from **1d** with **3a** resulted in molecular ion peaks at *m/z* (rel. intensity) 312.0 (100) (Suppl. Mat. Tab. S34) confirming a similar structure as shown for **8a,b**.

The first step for the formation of **8a-c** is the postulated oxidation of **1d** to *o*-quinone followed by a nucleophilic attack of the side chain nitrogen atom to the C5-position of the ring resulting in leukoadrenochrome which is oxidized to adrenochrome [45].

The oxidation of epinephrine (adrenaline) by enzymes such as laccase [46,47] or tyrosinase [48] is used for the development of methods/sensors for the detection of this neurotransmitter in blood and plasma.

The formation of **7** was the prerequisite for the heteromolecular reaction with the amino partners **3a,c,d**. Thus, the cyclization - and not the 1,4-addition forming a dimer as described for products **4a-l** - is essential for the formation of a stable product. We described previously [26] the reaction of *para*-dihydroxylated benzoic acid derivatives with **3c,d** resulting in cyclization after 1,4-addition of the azole and subsequent intramolecular 1,2-addition forming cycloheptenes (diazepines) or addition-elimination-reaction forming cyclooctenes (diazocines).

We propose a similar scheme for the formation of **8a-c**. At first, the 1,4-addition starts from the amino group of the carboxamide of **5a-c** (Fig. 4) and the resulting non-detectable heteromolecular dimer undergoes a 1,3-addition to form the derivatives **8a-c**. As expected, the reaction of **1d** with **3b** leads to no heteromolecular product due to the lack of two amino groups which are required for cyclooctene formation.

3.4 Additional remarks

Some products were detected in small quantities also in the respective controls without addition of laccase. We described such autocatalytic reactions previously for the dimeric products of *para*-dihydroxylated substances and amino acids [36]. The concentration of the products (**4b**, **4c**, **4e**, **4f**) was, however, very small. Thus, within 24 h only 3% of **4c** was formed autocatalytically in comparison to the amount formed in 20 minutes in the laccase-catalyzed reaction. Consequently, the autocatalytic reaction is negligible.

3.5 Summary

In summary, for the reactions of **1a-c** with **3a-d** the formation of dimers (**4a-l**) takes place in the order (from large to low quantities; Fig. 5): **1a-c** with **3a** (products **4a-c**) = **1a-c** with **3b** (products **4d-f**) > **1a-c** with **3c** (products **4g-i**) > **1a-c** with **3d** (products **4j-l**). In contrast, for **6a-l** the amino compound **3c** (with products **6g-i**) gave the highest yield. Thus, **3a** and **3b** were the most effective nucleophiles resulting in fast and efficient Michael-addition forming dimeric products (**4a-f**) whereas for the reaction involving the homomolecular products **5a-c** the azole **3c** was most efficient. In the reactions of **1a-c** with **3a** or **3b** (for products **6a-f**) the formation of homomolecular products, beyond the *o*-benzoquinone, is strongly suppressed due to a fast reaction of the amino compound with the catechols enabling an almost completely consumption of the two reaction partners to one heteromolecular product (**4a-f**). In contrast, the reactions with the azoles **3c** or **3d** and **1a-c** are not as fast as with **3a,b** allowing both reaction types simple amination via Michael-addition resulting in products **4g-l** and a reaction including homomolecular formation of potential dibenzofuran derivatives and only then reaction with **3c,d** forming **6g-l**.

Beyond this, for the products **8a-c** the reaction of **1d** to **7** is the only possibility for the reaction with **3a,c,d** resulting in cyclooctene derivatives.

The yield of the products for the non-optimized reactions was between 9 and 44%. For reactions of *para*-dihydroxylated aromatic substances and amino compounds yields of 20-80% are achievable [12] and for optimized reactions a yield of 96% is possible [6].

4. Conclusions

The three identified reaction types between *ortho*-dihydroxylated aromatic substances and amino compounds result in Michael-addition forming heteromolecular dimers or included homomolecular dimerization with amination and ring closure mechanisms.
The catalytic action of the enzyme laccase allows multi-step or domino reactions and thereby enlarges the application possibilities. Furthermore, the reactions demonstrated here enable the use of laccase for the production of new substances not only for fine chemical synthesis but also for novel antimicrobial or anticancer pharmaceuticals.

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Figure Captions

Fig. 1 Laccase-mediated reactions of catechol (**1a**), 3-methylcatechol (**1b**) or 4-methylcatechol (**1c**) with 2-aminobenzamide (**3a**)

Fig. 2 Laccase-catalyzed reaction of catechols (**1a-c**) resulting in the tentatively proposed products **5a-c**

Fig. 3 Laccase-mediated reaction of 3-methylcatechol (**1b**) with 4-aminoimidazole-5-carboxamide (**3c**)

Fig. 4 Laccase-mediated reaction of epinephrine (**1d**) with 4-aminoimidazole-5-carboxamide (**3c**) or 3-aminopyrazole-4-carboxamide (**3d**)

Fig. 5 Concentration of products **4a-l** (white columns) and **6a-l** (black columns) formed in reactions of **1a-c** with **3a-d** (2:2 mM)

Tables

Table 1 Structural data of homomolecular products (**2a-c**) formed during laccase-catalyzed reaction of **1a-c**

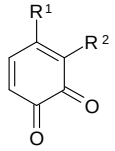
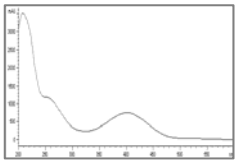
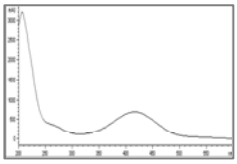
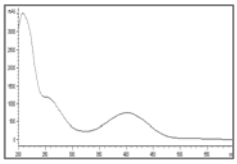
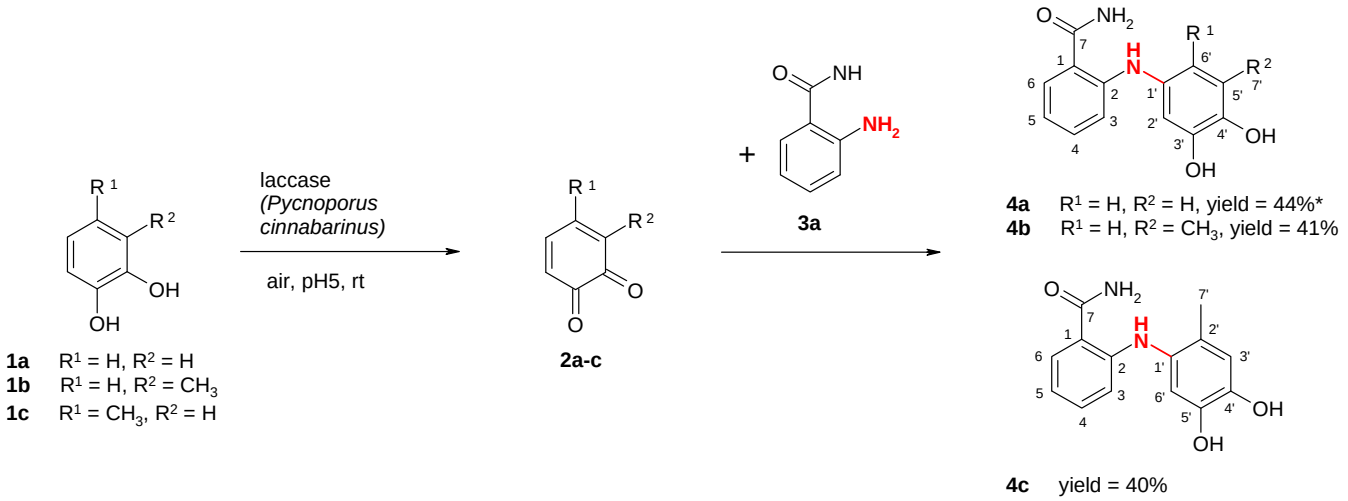
	Reaction of	**1a**	**1b**	**1c**
***o*-Benzo-quinone**		**2a**	**2b**	**2c**
 **2a** R^1=H, R^2=H **2b** R^1=H, R^2=CH_3 **2c** R^1=CH_3, R^2=H				
	R_f (HPLC)	3.30 min	5.34 min	5.10 min
	UV-vis λ_{max}	205, 389 nm	203, 414 nm	208, 399 nm
2a [M] 108.10 **2b,c** [M] 122.12	MS (AP-ESI pos. ion mode): *m/z* (%)	$[M+H]^+$ 109.1 (6), $[M+NH_4]^+$ 126.1 (100), $[M+Na]^+$ 131.0 (25)	$[M+H]^+$ 123.1 (27), $[M+NH_4]^+$ 140.1 (100)	$[M+H]^+$ 123.1 (100), $[M+NH_4]^+$ 140.1 (68)

Table 2 ^{1}H and ^{13}C assignments and HMBC correlations for **6h**[a]

^{13}C	**^{1}H**	**^{1}H-^{13}C correlations**	**Rings**
9.4 (C-16)	1.66 (s, H-16)	108.5, 132.7 (C-8), 143.9 (C-7)	B
15.9 (C-15)	1.74 (s, H-15)	128.4 (C-13), 135.1 (C-12), 184.2 (C-11)	A
102.7 (C-14)	7.23 (s, H-14)	112.8 (C-13b), 132.5 (C-8), 143.4 (C-9a)	B
128.4 (C-13)	6.64 (s, H-13)	(15.9 (C-15))*, (113.1 (C-13[a])), (145.2 (C-9b)), 184.2 (C-11)	A
137.6 (C-2)	7.74 (s, H-2)	123.4 (C-5), 147.5 (C-4)	C

[a] Chemical shifts are expressed in δ (ppm) calibrated on the resonances of the residual nondeuterated solvent DMSO. *() Signals with low intensity

Figure 1



1a R^1 = H, R^2 = H
1b R^1 = H, R^2 = CH_3
1c R^1 = CH_3, R^2 = H

4a R^1 = H, R^2 = H, yield = 44%*
4b R^1 = H, R^2 = CH_3, yield = 41%

4c yield = 40%

* Yields refer to isolated products.

Figure 2

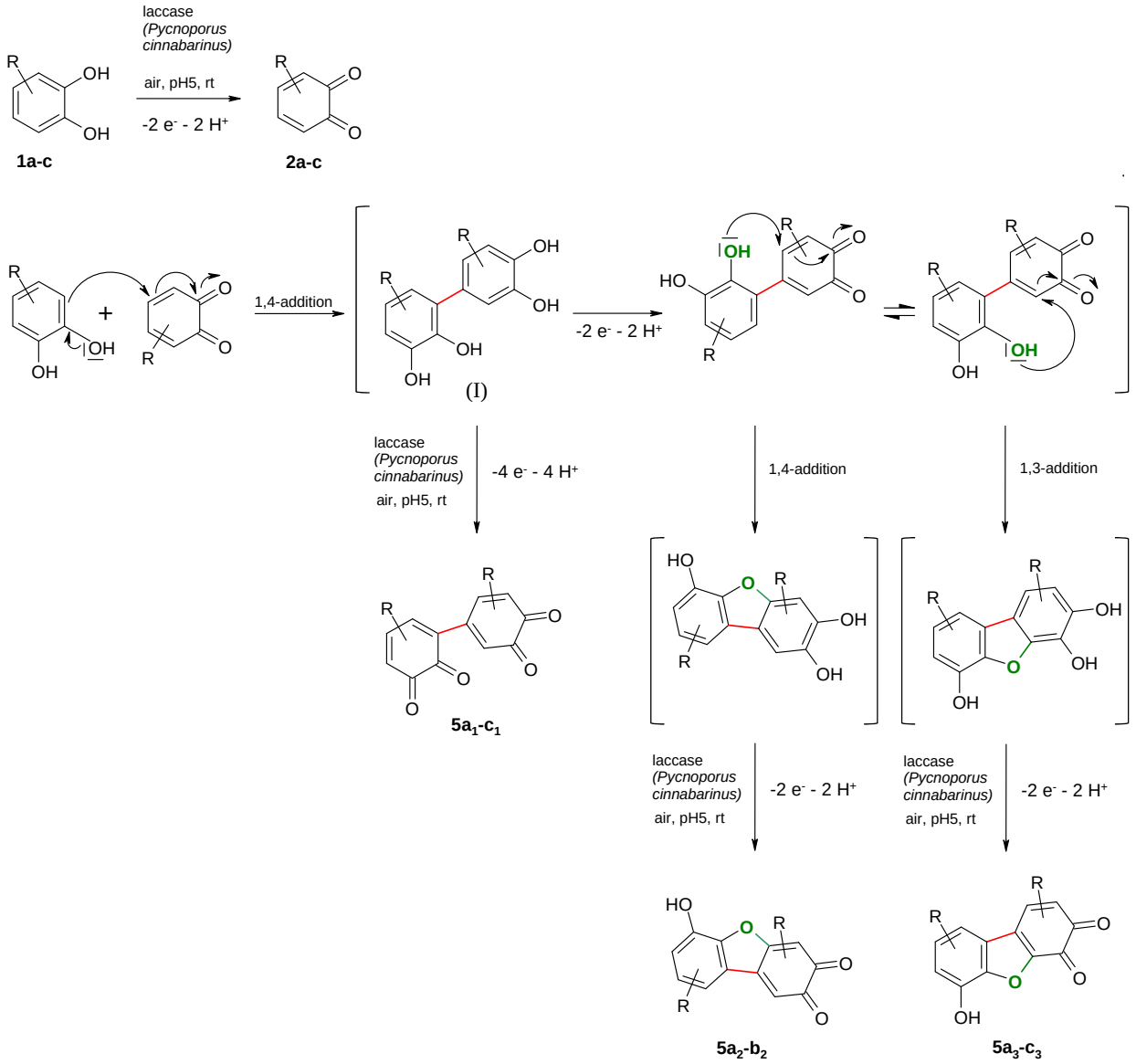
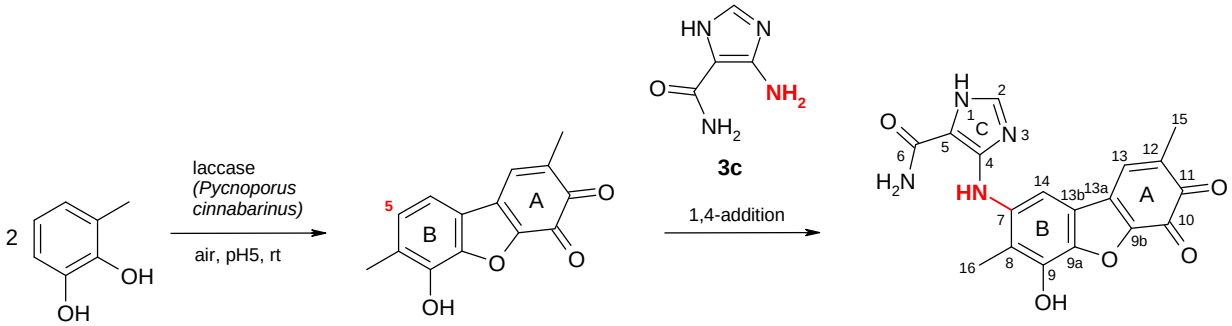
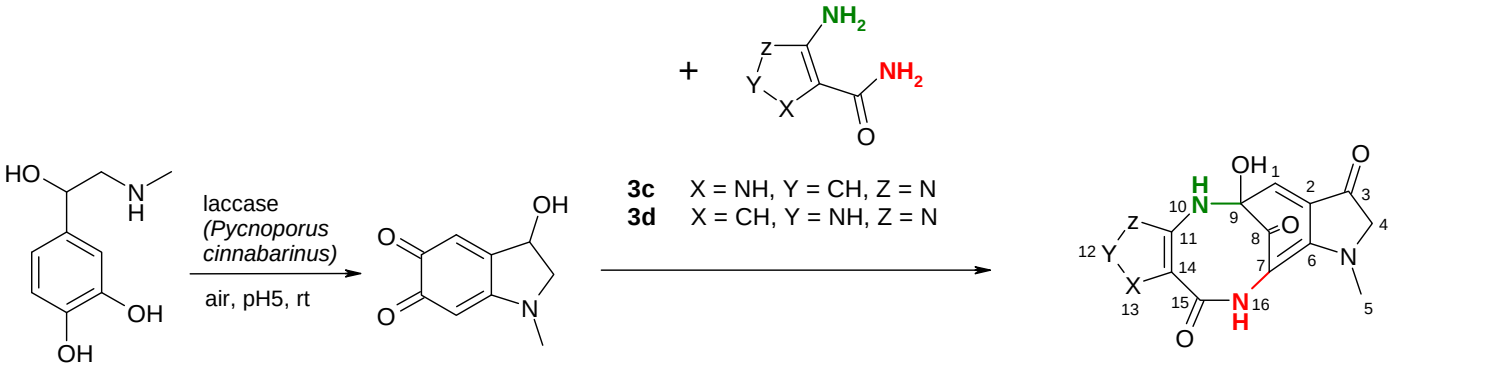

laccase
(Pycnoporus
cinnabarinus)
air, pH5, rt
-2 e- - 2 H+
1a-c
2a-c
1,4-addition
(I)
-2 e- - 2 H+
laccase
(Pycnoporus
cinnabarinus)
air, pH5, rt
-4 e- - 4 H+
1,4-addition
1,3-addition
5a1-c1
laccase
(Pycnoporus
cinnabarinus)
air, pH5, rt
-2 e- - 2 H+
laccase
(Pycnoporus
cinnabarinus)
air, pH5, rt
-2 e- - 2 H+
5a2-b2
5a3-c3

Figure 3



1b **5b$_3$** **6h** (NMR), yield = 9 %

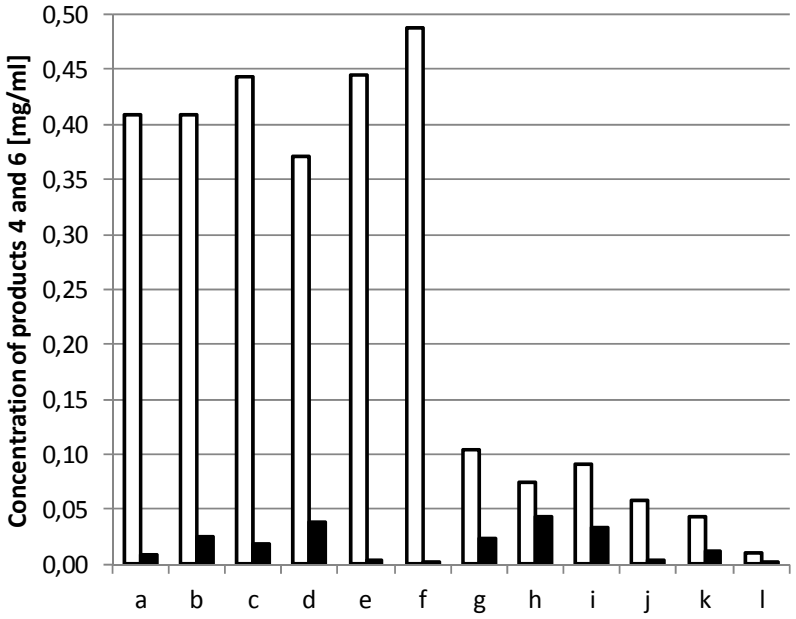
Figure 4



1d **7**

8a X = NH, Y = CH, Z = N (NMR), yield = 15 %
8b X = CH, Y = NH, Z = N (NMR), yield = 11 %

Figure 5


Concentration of products 4 and 6 [mg/ml]
0,50
0,45
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
a
b
c
d
e
f
g
h
i
j
k
l