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C–N coupling of 3-methylcatechol with primary amines using native and recombinant laccases from *Trametes versicolor* and *Pycnoporus cinnabarinus*

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ABSTRACT

Five laccase genes from *Pycnoporus cinnabarinus* and *Trametes versicolor* encoding for different isoenzymes have been cloned, recombinantly expressed and characterized. Following C–N coupling of primary linear, branched-chained and cyclic amines to 3-methylcatechol was mediated by native and recombinant laccases yielding the corresponding secondary amines. Formation of C5-monoaminated *ortho*-methylquinones occurred within 1–2 h; prolonged incubation led to the formation of high-molecular mass products. No difference between the use of native or recombinant isoenzymes from *P. cinnabarinus* or *T. versicolor* was observed. Optimization of the reaction conditions included variation of amine donor ratios, pH, amount and type of enzyme preparations. The formation of by-products could be suppressed at pH values corresponding to the enzymes optima (pH 4–5). A total of 10 secondary amines were synthesized with product formations of up to 80%. Furthermore, all purified secondary amines were characterized by NMR-, LC–MS- and HRMS-analysis and log *P* values were determined.

1. Introduction

The synthesis of fine chemicals using biocatalysts represents an efficient alternative to conventional chemical routes and the investigation of various enzyme classes lead to widespread applications in industry.[1] Laccases (*p*-diphenol:dioxygen oxidoreductases, EC 1.10.3.2) catalyze the oxidation of aromatic compounds, especially of substituted phenols and anilines, whilst molecular oxygen is reduced and water formed without the need of a cofactor and harsh reaction conditions.[2,3] Many laccases have been studied and they exhibit a broad temperature and pH range (usually pH 4–9),[4] are stable in organic solvents[5] and towards proteolytic enzymes as well.[6]

In principle laccases can be easily obtained by cultivation of wild-type organisms, but often a mixture of isoenzymes is received, which might be problematic for a biocatalytic application as it was described for pig liver esterase isoenzymes.[7] At the same time high amounts of enzymes are needed for application in large-scale synthesis and therefore enzymes are commonly produced by recombinant expression. Thus, recombinant production of laccases is important. Indeed they could already be successfully expressed in a range of heterologous hosts, for example, fungal laccases in *Pichia pastoris*, *Saccharomyces cerevisiae*, *Aspergillus* sp. or *Trichoderma* sp., and bacterial laccases in *Escherichia coli*.[8] However, fungal laccases were not expressed in *E. coli* so far and known recombinant expression systems are not sufficient or still too expensive for industrial applications.

The substrate scope of laccases is very broad. Beside activity towards *para*-dihydroxylated substrates, they also convert *ortho*-dihydroxylated compounds, monophenols and nonphenolic substances with a wide range of substituents.[6a,9] In the presence of oxygen, laccases mediate an oxidation of the substrate molecule generating reactive radicals, which usually undergo homomolecular coupling generating polymers.[10] More interesting for synthesis is the heteromolecular coupling, where an acceptor molecule reacts with the radical forming new C–C-, C–O-, C–N- or C–S-coupling products in a non-enzymatic step.[11]

In the present study, we investigated the laccase-mediated heteromolecular coupling of an *ortho*-dihydroxylated substrate molecule, 3-methylcatechol (**1**), with primary linear amines with alkyl chain lengths ranging from C4 (*n*-butylamine) to C9 (*n*-nonylamine) (**2a**–**f**), the branched-chained compounds 2-ethyl-1-hexylamine (**2g**), (*R*)-2-aminohexane (**2h**), 2-amino-5-methylhexane (**2i**), and the cyclic (*R*)-(+)-bornylamine (**2j**) to obtain the pharmaceutically

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valuable secondary amines **3a**–**j**. These reactions were performed using native and recombinant laccases from the white-rot fungi *Pycnoporus cinnabarinus* or *Trametes versicolor* and various reaction parameters were studied.

In a previously published example, a structural analogue compound, the secondary amine 4-butylamino-5-methyl-*ortho*-quinone (MLTQ), acting as a model cofactor for lysyl oxidase[12] and as a cross-linking inhibitor on osteolathyrism induction,[13] was synthesized using 4-methylcatechol and *n*-butylamine and the chemical coupling mediator sodium iodate.[14] Furthermore Burzio and Waite examined the synthesis and structural characterization of mushel adhesive proteins and their cross-linking mediates by tyrosinase and byssal catechol oxidase in mixtures containing 4-methyl- and 4-ethylcatechol with several amino acids.[15] In order to design potential biomaterials against the background of mushel adhesive proteins, Mikolasch et al. investigated the laccase-catalyzed cross reaction between L-lysine or lysine-containing peptides and dihydroxylated aromatics, for example, *ortho*-dihydroxylated hydrocaffeic acid.[16] Besides this, novel cephalosporins, penicillins and carbacephems, inhibiting the growth of several Gram positive bacterial strains, as the methicillin-resistant *Staphylococcus aureus* strains and vancomycin-resistant Enterococci, were synthesized by amination of 3- and 4-methylcatechol with amino-β-lactams using laccase from *Trametes* sp.[5b] *ortho*-Quinones are known for their biological activity, like acting as oxidants generating oxidative stress and as electrophiles forming covalent adducts with cellular macromolecules and partially intercalate with DNA, and thus a range of pharmacokinetic effects had been discussed.[17,18] In addition, secondary amines and arylalkylamine structures are motifs in various drugs like β-blockers[19] and hallucinogens[20] and were applied in medication of neuropathic pain causing antidepressant effects.[21] Therefore, a further part of this work deals with the determination of log *P* values of products **3a**–**f** to estimate their distribution in biomembranes or cytosolic areas for potential applications in the pharmaceutical area.

2. Results and discussion

2.1. Biochemical properties of native and recombinant laccases

First, four different laccase encoding genes from *T. versicolor* SBUG-M 1050 and one laccase gene from *P. cinnabarinus* SBUG-M 1044 were isolated, cloned and sequenced. The four isoenzymes from *T. versicolor* (TvL) showed 68–76% amino acid sequence similarity and were identical to the isoenzymes described recently (Table 1).[22] Unfortunately, only one laccase isoenzyme gene could be isolated from *P. cinnabarinus*, although several protein-, DNA- and mRNA-sequences with 72–99% homology are known from literature.[23]

All five genes were cloned into pET22b(+) without their native signal peptide for a recombinant expression in *E. coli*. Regrettably, soluble expression of active laccase could not be achieved despite the use of various *E. coli* strains, co-expression with different chaperones (Takara), or fusion-tags (data not shown). Therefore all laccase genes were cloned for a constitutive expression in pGAP-Zalpha_B, using the alpha secretion factor of *S. cerevisiae*, instead of their native signal peptide, for a secretion into the culture supernatant. TvL5, TvL10 and PcL35 could be expressed in sufficient amounts at 30 °C and with an addition of 0.3 mM $CuSO_4$ (Table 1). When adding 0.3 mM $CoSO_4$ to the culture media, 75% and without any addition, still 50% of laccase activity compared to the addition of $CuSO_4$ (100%) could be obtained. In comparison to the group of Urlacher[22] the activity of TvL5 could not be increased by using its native signal peptide, but by cultivation at 20 °C a 25-fold increase was reached. The same observation was made in the recombinant expression of laccase PcL35, while in case of TvL10 expression was lowered (Table 1). In case of TvL13 and TvL20 only marginal laccases activities could be observed, which were only detectable by activity staining with ABTS in native polyacrylamide gel electrophoresis. An explanation might be the assumed lower protein level of laccases due to the constitutive expression in comparison to an induced and controlled expression using the *AOX*3 promotor. Another reason can be seen in the three amino acid substitutions for both isoenzymes in comparison to the known protein sequences as shown in Table 1.

Table 1
Summary of isolated laccase isoenzymes, their comparison with published sequences and expression levels in *Pichia pastoris* as secreted enzyme, using the alpha factor of *S. cerevisiae* as signal sequence into the culture supernatant containing 0.3 mM $CuSO_4$ at 20 °C or 30 °C

Laccase isoenzyme	Alternative name[22]	Analogous GenBank accession no.	Amino acid substitutions	Enzyme activities [mU $mg^{-1}_{protein}$]	
				30 °C	20 °C
TvL5	Lccβ	CAA77015	Asp75Asn Asp519Tyr	16	423
TvL10	Lccα	AAW2942	Phe350Leu Asp360Asn	728	168
TvL13	Lccγ	Q12717[a] or AAC49829	Ala155Pro Gly249Asp Asp520Gly	n.d.	n.d.
TvL20	Lccδ	Q12719 or CAA59161	Ile71Val Ile186Val Thr499Ala	n.d.	n.d.
PcL35	—	AAF13052.1 or AAG13724.1	Ala285Val	15	364

n.d.: not detectable.
[a] Protein sequence of Q12717 has 99% identity to BAA23284, which corresponds to the described Lccg. Q12717 and BAA23284 differ additionally in three amino acids, while Q12717 consists of 527 amino acid and BAA23284 of 526.

Further characterization of laccase isoenzymes was only performed with TvL5, TvL10 and PcL35, since the activity of the other laccases was too low. The pH optima of native (laccase isoenzyme mixture) and recombinant laccases from *T. versicolor* and *P. cinnabarinus* were very similar. All of them showed two optima, one at pH 2 using phosphate buffer and one at pH 4 in acetate buffer, in which activities of approx. 50–60% could be detected in comparison to activity in phosphate buffer (pH 2). The three recombinant isoenzymes showed as well a higher relative activity in maleate buffer at pH 3 than the native isoenzyme mixture, but lower relative activity in Tris or Bis-Tris buffer at pH 6 or 6.5.

All studied laccases were stable from 4 to 45°C during incubation for 3h and remained stable from 4 to 30°C after incubation for 16 h. After 16 h incubation at 45 °C activity decreased to approx. 50%, and after 3 h incubation at 60 °C all isoenzymes were completely inactive. Stability of PcL35 at 65 °C was further analysed and it could be seen that activity increased after incubation for 2 min at 65 °C (due to a maturation process[24]), but decreased drastically within further incubation; after 30 min the laccase was completely inactive.

Furthermore the methanol stability of laccases was investigated as this alcohol is often required to dissolve substrates in the aqueous reaction system. As expected, activity decreased with increasing methanol concentrations using native laccase from *P. cinnabarinus* and recombinant PcL35, which was more pronounced for the latter (Fig. S1 in Supplementary data). An explanation might be the different glycosylation pattern of native and recombinant enzymes. In comparison, activity of the native isoenzyme mixture of *T. versicolor* increased with increasing methanol concentration up to 20–30% (v/v), at concentrations above 30% the activity also started to decrease until 80% of methanol, where laccases are completely inactive. The same effect was observed for the commercial available laccase from *Myceliophthora thermophila.*

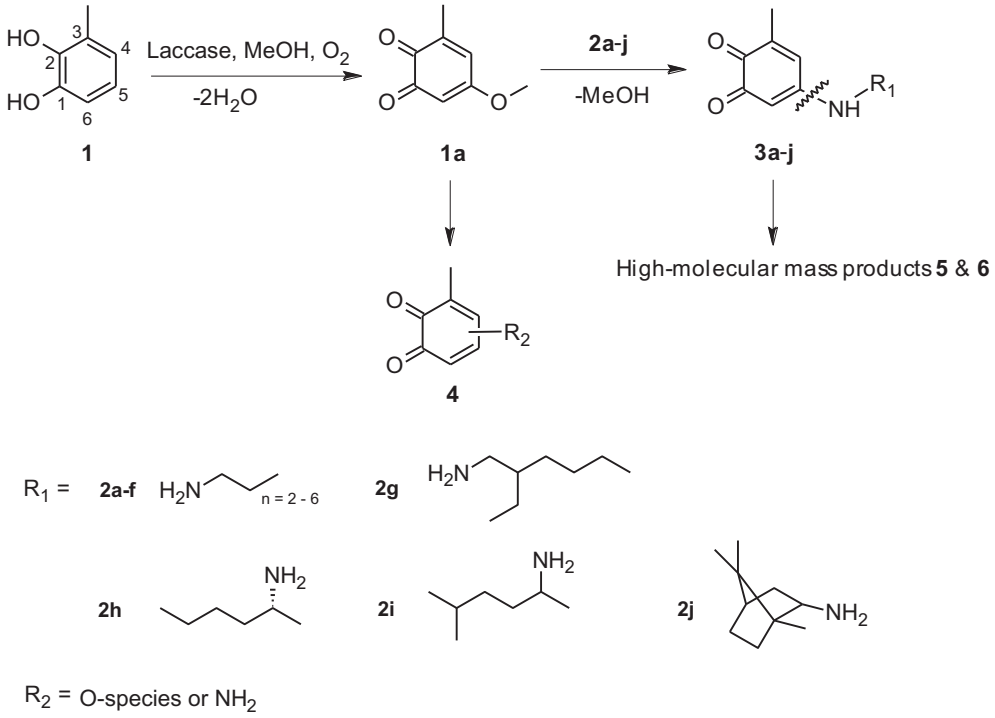
The two recombinant laccases from *T. versicolor* showed the same behaviour as PcL35 and displayed strong negative correlation of activity with increasing methanol concentrations until 50%, where both laccases were completely inactive.

2.2. Synthesis of secondary amine structures via C–N coupling

Coupling-reactions between 3-methylcatechol (**1**) and the primary amines (**2a**–**j**) were studied using native and recombinant laccases from *P. cinnabarinus* SBUG-M 1044 and *T. versicolor* SBUG-M 1050 and only marginal differences between the enzymes were observed. Formation of the secondary amines (**3a**–**j**) (Scheme 1) was determined via HPLC analyses, additionally successful amination could also be monitored qualitatively by formation of yellow and later red reaction mixtures.

addition reactions of water or CH_3OH from stock solutions and/or reaction milieu to activated aromatics. Unfortunately, a definite evidence for the occurrence of the non-substituted *o*-methylbenzoquinone could not be achieved in HPLC analysis as it was described for other *para*-dihydroxylated enzyme substrates.[25] Nevertheless, in time-course analysis an evidence for a displacement reaction, where the methoxy group was replaced by the amine group, exists. Similar nucleophile displacement reactions on readily eliminable groups (e.g., methoxy substituents) of activated hydroquinones were previously described by Schäfer and Aguado[26] for reactions focused on a sodium iodate-mediated synthesis of substituted 2,5-diamino-1,4-benzoquinones.

During the formation of secondary amines **3a**–**j**, the generation of the magenta-coloured by-product **4** was monitored as well. Compound **4** was unavailable for further heteromolecular coupling with the used primary amines **2a**–**j** and instable during preparation



Scheme 1. Reaction pathway for the laccase-mediated heteromolecular coupling of 3-methylcatechol with primary amines **2a**–**j** aiming at the synthesis of secondary amines **3a**–**j**.

In the HPLC analysis, the decrease of the concentration of **1** was detected due to simultaneous appearance of a yellow coloured product **1a**, which was subsequently converted into the heteromolecular coupling products **3a**–**j** and partially to the by-product **4** (Fig. S2 in Supplementary data). Efforts in the structural characterization of compound **1a** via NMR-spectroscopy were not successful, because during sample preparation by dehydration via lyophilisation, **1a** decomposed into several other products. However, LC–MS-analysis in a mixture of 50% methanol and 50% acetic acid in water (0.1%), revealed a defined single peak with a molecular mass of 153.1 g moL^{-1} $[(M+H)^+]$. The corresponding mass for a sodium addition (175.1 g moL^{-1}, $[M+Na]^+$) could be observed as well (Table S1 in Supplementary data). Therefore, we postulate for the structure of **1a** the activated 3-methylbenzoquinone substituted by electrophile addition with a methoxy group. Since reactants were prepared as methanolic stock solutions, we propose that this low-molecular mass substituent was preserved from methanol, which was coupled non-enzymatically to the *ortho*-methylbenzoquinone as already described for 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide by Manda et al.,[25] who also determined for NMR analysis as it was already described for **1a**. The by-product resulted in identical UV-absorption spectra as the secondary amines **3a**–**j**, suggesting a 3-methylbenzoquinone's substitution at the cyclic ring system and the formation of the *ortho*-quinoid structure same position determined for the synthesized secondary amine products, but with a low-molecular mass substituent causing significantly more hydrophilic characteristics (Table S1 in Supplementary data). LC–MS-analysis of a fraction solely containing compound **4**, obtained from solid phase extraction, resulted in a peak possessing a molar mass of 138.1 g moL^{-1} $[(M+H)^+]$ and a molecular mass for the corresponding sodium adduct (160.1 g moL^{-1}, $[M+Na]^+$) (Table S1 in Supplementary data), which indicates a substitution of the *ortho*-methylbenzoquinone with a low-molecular mass substituent also originating from reaction milieu. Referring to the estimated molar mass of product **4** (137 g moL^{-1}) in comparison to the activated substrate molecule (122 g moL^{-1}), the mass difference between both leads to the assumption of an addition of either one NH_2-group or a certain kind of oxygen species. The structure of compound **4** could not be terminatory identified due to it's instability within further preparation

steps, however a displacement reaction between this *ortho*-methylbenzoquinone's low-molecular mass substituent and the amine group of coupling agents had never took place as it was examined for compound **1a**. Therefore, generation of product **4** was negatively affecting the yields of the desired secondary amines.

Regardless to the by-product formation, maximum concentrations of secondary amines were observed after 1–2 h, depending on the used laccase preparation, the pH of sodium acetate buffer and the concentrations of reactants. However, prolonged incubation led to the formation of the high-molecular mass coupling products **5** and **6** (Fig. S2 in Supplementary data), exclusively occurring in heteromolecular transformation reactions. Attempts to isolate these high-molecular mass products as stable pure compounds or in a mixture were not successful due to products high reactivity and therefore structural data cannot be provided within this study.

Generally, formation of secondary amines could be increased with higher amounts of aliphatic coupling agents **2a**–**j** and best results were observed in reactions with a ratio of **1** and primary amines of 1–5 mM (Table 2, Fig. 1). For example, the product formation of **3f** could be increased by 30%, of **3g** by 18%, of **3h** by 8% and **3j** by 26% by using 5 mM instead of 1 mM amino donor. Further increases were observed when using directly 5 mM of the amino donor instead of the methanolic stock solutions, in case of formation of **3g** a 12% enhancement and for **3i** a 16% enhancement could be monitored (Table 2). The direct use of amino partners in 10 mM and 40 mM concentration ranges resulted in an additional increase of product generation, as determined for **3h** (+9%) and **3i** (+34%). Complementary to those findings, the use of the amino donor in excess resulted in a suppressed formation of the by-product **4**.

The use of larger amounts of enzyme activities gave only marginally increased product formation by 5%. Nevertheless, laccase units above 4 U mL^{-1} caused a decreased formation of secondary amines due to increasing generation of by-product **4** and high-molecular by-products **5** and **6** (data not shown). Interestingly, when using low activities of recombinant PcL35 (0.2 U mL^{-1}), a simultaneous appearance of a further product (**7**) was detected. Based on its UV–vis-absorption characteristics (λ_{max} 262 nm), it appears similar to the UV–vis-absorption behaviour of **1** (λ_{max} 274 nm) rather than to the secondary amine structure (λ_{max} 220, 298, 490 nm). However, with further incubation product **7** was converted into the desired secondary amine. When increasing PcL35 activity up to 0.4 U mL^{-1} again only the targeted products **3a**–**j** were detected.

According to literature known pH optima and optima based on this study, all biocatalyses were performed in corresponding buffer

Table 2
Influence of amine donor **2a**–**j** concentrations (methanolic stock solutions, v/v), enzyme units used and type of laccase preparation on the conversion of the laccase substrate, formation of secondary amines **3a**–**j** in 5 mL scale

Amino donor	Ratio of **1** to amino donor [mM]	Enzyme (U mL^{-1})[a]	Laccase preparation	Conversion of **1** [%]		Formation of monoaminated product[b] [%]
				40 min	120 min	
2a	1:5	2	Native PcL	74	100	10
2b	1:5	1	Native PcL	60	93.5	16
2c	1:5	1	PcL35	20	72.2	0.5
	1:5	1.5		37	76.3	1
	1:5	2		44	81.5	1
	1:5	0.5	Native PcL	56	72.6	2
2d	1:5	0.5	TvL5	41	90.3	16
	1:5	0.5	TvL10	39	79.7	12
	1:5	0.5	Native PcL	33	81.4	14
2e	1:5	2	Native PcL	57	100	58
2f	1:5	0.015	TvL5	2	18.6	0
	1:5	0.03	TvL5	7	24.5	0
	1:5	0.05	TvL5	20	32.4	0
	1:5	0.15	TvL5	46	96.5	19
	1:1	1	Native PcL	94	100	15
	1:5	1	Native PcL	88	100	46
2g	1:1	1	Native PcL	94	100	12
	1:5	1		100	100	30
	1:5[c]	1		89	100	42
	1:10[c]	1		93	100	35
	1:40[c]	1		92	100	29
	1:40[c]	2		89	100	30
2h	1:1	1	Native PcL	84	100	16
	1:5	1		98	100	23
	1:5[c]	1		84	100	19
	1:5[c]	2		92	100	18
	1:5[c]	4		100	100	25
	1:10[c]	1		93	100	29
2i	1:1	1	Native PcL	98	100	37
	1:5	1		100	100	30
	1:5[c]	1		67	100	47
	1:10[c]	1		88	100	81
2j	1:1	1	Native PcL	99	100	18
	1:5	1		100	100	45
	1:5[c]	1		95	100	45

[a] Based on ABTS assay.
[b] Evaluated yields of product formation [%] compared to a 100% conversion into desired compounds **3a**–**j**, estimated in HPLC analysis of 5 mL scale in reaction after 2 h.
[c] In selected reactions instead of applying methanolic stock solutions the direct use of amine donors was tested.

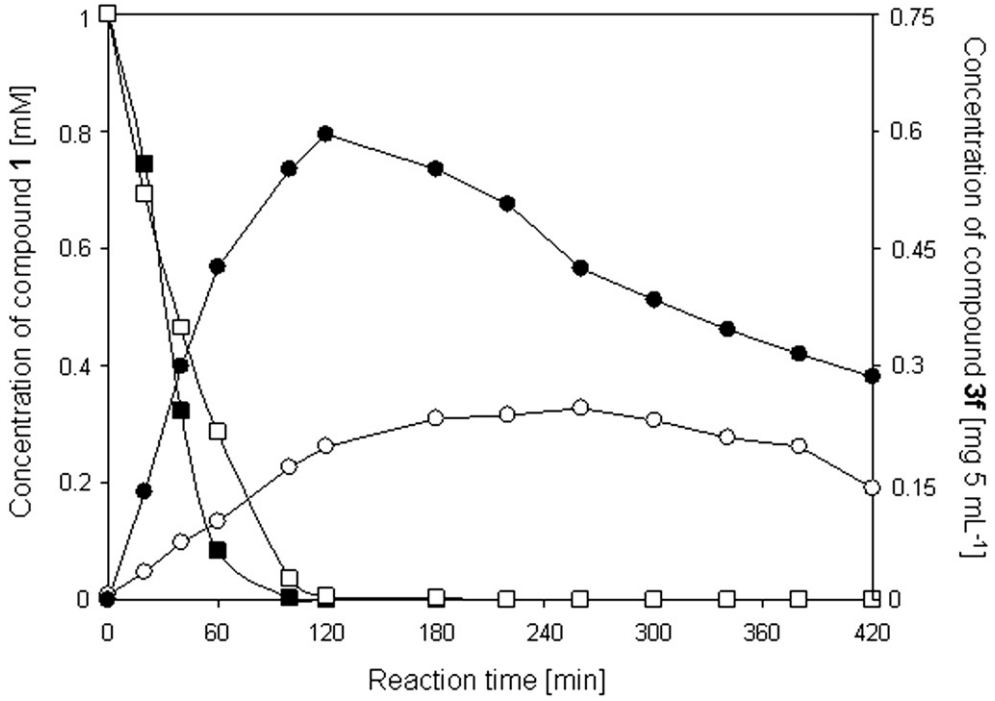


Fig. 1. Effect of concentrations of aliphatic amines on substrate consumption (1 mM amine: open square; 5 mM amine: closed square) and product formation (1 mM amine: open circle; 5 mM amine: closed circle) in a 5 mL scale, exemplary shown for the reaction between 3-methylcatechol and *n*-nonylamine mediated by 2 U mL^{-1} native *P. cinnabarinus* laccase (20 mM sodium acetate, pH 5).

systems to assure catalytic efficiency and preventing acidification by release of protons due to enzymatic substrate activation. Independent from an optimal catalytically environment of laccases, also the physicochemical nature of **1** referring to the pH of reaction milieu resulted in a significant influence on the formation of the desired secondary amine structures **3a**–**j**. According to this, in experiments using *M. thermophila* laccase in 20 mM phosphate citrate buffer at pH 7 higher amounts of by-products were observed in comparison to reactions at lower pH ranges (data not shown). Therefore, application of laccases preferring slightly acidic pH values (pH 4) reduced the formation of by-products such as **4**, since a higher amount of *ortho*-dihydroxylated enzyme substrate is available for synthesis of monoaminated target products.

Consequently, reaction progress and yields are also based on reactant properties, which are positively affected by the pH optima of the laccases from *P. cinnabarinus* and *T. versicolor*. An increased by-product formation was also observed in the synthesis of **3d** and **3f** using small amounts of recombinant isoenzymes TvL5 and TvL10 at pH 4, (Fig. 2), whereas increased enzyme activity resulted in increased product formation.

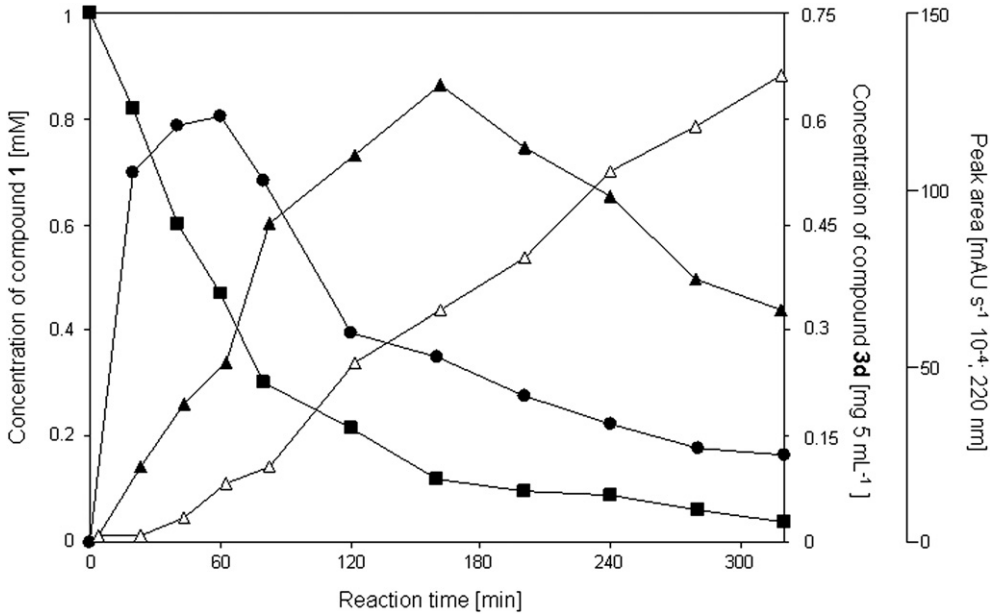


Fig. 2. Formation and decrease of desired secondary amine (filled circle) due to increasing accumulation of high-molecular weight products **5** (closed triangle) and **6** (open triangle) in comparison to substrate consumption (filled square) in a reaction of 1 mM 3-methylcatechol and 5 mM *n*-heptylamine catalyzed by recombinant TvL10 (20 mM sodium acetate, pH 4) (mAu=milliabsorption units).

In this study, we mainly focused on the optimization of product formation detectable by HPLC for the synthesis of novel secondary amines **3a**–**j**. With increased amino donor molarities (5–40 mM) and more enzyme units, the formation of **3e**–**3j** in good to high amounts (up to 81%) within only 2 h (Table 2) were possible.

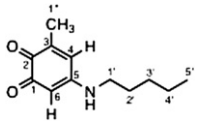
2.3. Structural characterization of secondary amines

Structural characterization of the secondary amine products is exemplary explained here for compound **3b** (Table 3). The signals appearing at 5.53 ppm and 6.74 ppm in the ^{1}H NMR spectrum of **3b** could be assigned to H-6 and H-4, respectively. Within this, the signal of H-4 exists as a broad signal according to its long-range coupling to *meta*-positioned H-6 and further methyl protons of C-1″ as also indicated by coupling constants (Table 3). The coupling of *n*-pentylamine to the C5 position is clearly confirmed by the correlation of H-1′ protons with C-5. The presence of a quinoid, hydroquinoid or quinone imine structure was proved by ^{13}C and HMBC data. As quinones revealed low-field shifted resonance signals (180 ppm up to 190 ppm), **3b** was identified as a 1,2-quinoid structure according to ^{13}C NMR signals at 175.4 ppm for C-1 and 185.7 ppm for C-2 as described by Mikolasch et al.[5b] for the amination of catechols and for the hybrid dimer quinone production from 3,4-dichloroaniline with protocatechuic acid and syringic acid.[27] Additionally, the HMBC spectra confirmed the presence of a 1,2 quinoid structure as in 3-methyl-5-(pentylamino)-1,2-benzoquinone.

lipophilic biomembranes and aqueous cytosolic areas.[28] Furthermore, it is accepted as an important parameter in QSAR-studies (*q*uantitative *s*tructure *a*ctivity *r*elationships) and in areas of designing pharmaceutical active compounds focused on their characteristics like absorption, biological availability, metabolism and toxicity.[29] As *ortho*-dihydroxylated benzenediols are commonly described as pharmaceutical valuable compounds,[30] showing activities as analgesics[31] and other interesting physiological activities, for example, as extremely important pharmacophores in numerous biologically active compounds,[32] log *P* of the synthesized alkyl aminated *ortho*-benzoquinones **3a**–**f** was of great interest (Table 4).

The log *P* of the secondary amines with increasing chain length resulted in an increased hydrophobicity of the laccase-mediated reaction products, and therefore each product should elute in-between the hydrophilic and hydrophobic reference substances. Accordingly, the use of three different hydrophobic references was essential. A comparison of the log *P* data derived from chromatographic analysis with theoretical log *P* values, obtained from ACD/ChemSketch, suggested 2-(4-hydroxyphenyl) ethylalcohol as hydrophilic standard while 3-methylcatechol (log *P*=0.75) was used as second reference. The newly synthesized secondary amines with C_4 and C_5 alkyl substituents (**3a** and **3b**) were determined to be more hydrophilic (log *P*=−0.75 to 0.41). In contrast, amination of **1a** with alkyl substituents with longer alkyl chain length (**3c**–**f**) led to rather high log *P* values up to 3.64. Generally, an additional methylene group enhanced log *P* about 0.89±0.21.

Table 3
^{1}H and ^{13}C NMR assignments and HMBC/^{1}H,^{1}H COSY correlations for **3b**[a]

Structure of **3b**	Pos.	^{13}C	^{1}H	^{1}H,^{13}C correlations (HMBC)	^{1}H,^{1}H COSY
	4	133.5	6.74, br, 1H	C-2 (185.7), C-6 (94.6), C-1″ (15.6)	H-3′/H-4′ (1.40)
	6	94.6	5.53, d, 1H (2.5)	C-1 (175.4),[b] C-2 (185.7), C-4 (133.5)	H-4 (6.74)
	1′	45.1	3.33, t, 2H (7.2)	C-5 (160.7), C-2′ (29.0), C-3′ (30.3)	H-2′ (1.71)
	2′	29.0	1.71, m, 2H	C-1′ (45.1), C-3′ (30.3), C-4′ (23.5)	H-1′ (3.33), H-3′/H-4′ (1.40)
	3′	30.3	1.40, m, 4H	C-1′ (45.1),[b] C-3′ (30.3), C-4′ (23.5),	H-2′ (1.70), H-5′ (0.96)
	4′	23.5		C-5′ (14.4)[b]	
	5′	14.4	0.96, t, 3H (7.0)	C-3′ (30.3), C-4′ (23.5)	H-3′/H-4′ (1.40)
	1″	15.6	1.98, d, 3H (1.3)	C-4 (133.5), C-2 (185.7), C-3 (142.7)	H-4 (6.74)

[a] Chemical shifts are expressed in δ (ppm) referred to TMS (calibration was done on solvents signals: δ MeOH-d_4=3.31 (^{1}H), 49.0 (^{13}C)). *J* values are in hertz in brackets.
[b] Correlation with low intensity.

Table 4
log *P* values determined in comparison to the theoretical log *P* values for the secondary amines **3a**–**f**

Compound		log *P*[a]		Theoretical log *P*	log *P* difference (−CH_2)	
		3,5-Dihydroxybenzoic acid	2-(4-Hydroxyphenyl) ethyl alcohol	ACD/ChemSketch	HPLC	Theoretical
1	3-Methylcatechol	−0.007	0.75	1.34		
3a	3-Methyl-5-(butylamino)-1,2-benzoquinone	−2.15	−0.75	1.34	1.16	0.53
3b	3-Methyl-5-(pentylamino)-1,2-benzoquinone	−0.58	0.41	1.88	0.88	0.53
3c	3-Methyl-5-(hexylamino)-1,2-benzoquinone	0.77	1.29	2.41	0.73	0.53
3d	3-Methyl-5-(heptylamino)-1,2-benzoquinone	1.77	2.02	2.94	0.59	0.53
3e	3-Methyl-5-(octylamino)-1,2-benzoquinone	n.d.	2.61	3.47	1.03	0.53
3f	3-Methyl-5-(nonylamino)-1,2-benzoquinone	n.d.	3.64	4.00		

n.d.: not determined.
[a] log *P* according to Donavan and Pescatore.[33]

2.4. Evaluation of log *P* and product stability

Since log *P* is used for quantitative description of the hydrophobicity of compounds, it can be used as a key criterion of pharmacokinetic properties by means of dispersive behaviour in

Stability studies for **3a**–**j** showed that they remained stable as dry chemicals at room temperature and 4 °C. Furthermore, stability in organic solvents controlled via HPLC analysis confirmed stability in methanol and acetonitrile, while they decomposed and lost their typical reddish colour in DMSO or THF.

3. Conclusion

In conclusion, we demonstrate the use of native and several recombinant laccase isoenzymes for the synthesis of a variety of secondary amines, which are potential biologically active compounds and thus of great interest for pharmaceutical industry. Product formation could be increased by applying excessively concentration of the amino donors set either as a methanolic stock solution, but ideally used directly in ranges from 5 to 10 mM. However, preventing formation of a two-phase system due to hydrophaty of alkylamines, enzyme substrate molecule **1** was always introduced as a methanolic stock solution in a 1 mM concentration (v/v). Based upon reaction kinetics, maximal yields of secondary amines were recovered after **2h** using 1 U mL^{-1} of especially native laccase preparations. Referring to this, increase of enzyme activities were found to repress concentration levels of aimed synthesis products due to a simultaneous elevation of low-molecular mass by-product **4**. Since the herein studied laccases are stable in methanol to a certain extent and no alternative chemical methods for the product synthesis exist, the introduced laccase-catalyzed coupling reaction, progressed at enzymes optimal pH ranges of pH 4 and 5, respectively, presents the general applicability of recombinant and native enzymes in organic synthesis and may serve as a tool in cases where chemical methods are exercisable.

4. Experimental section

4.1. General

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany) unless stated otherwise. Restriction enzymes, ligase, polymerases, cDNA preparation kits, reagents for DNA isolation and purification were obtained from New England BioLabs GmbH (Frankfurt am Main, Germany), Fermentas (St. Leon-Roth, Germany) and Qiagen (Hilden, Germany), all primers, cloning and expression vectors, as well as *Pichia* strains from Invitrogen (Darmstadt, Germany). Sequencing was performed at GATC (Konstanz, Germany).

4.2. Isolation of native laccases

Laccases from the white-rot fungus *P. cinnabarinus* SBUG-M 1044 were produced with 3,4-dimethoxybenzyl alcohol (10 mM) as an enzyme inducer, isolated and purified as recently described by Kordon et al.[34] Under these conditions *P. cinnabarinus* produced laccase as a single extracellular enzyme with an activity of 0.5 U mL^{-1}. Laccases from *T. versicolor* SBUG-M 1050 were obtained as described earlier by Jonas et al.[35] after 9 d of cultivation in nitrogen-rich medium with a maximal recovered activity of 0.5 U mL^{-1} in cell-free culture supernatants.

4.2.1. Cloning of recombinant laccases. During isolation of laccases from their native white-rot fungi, whole cells from culture supernatant were separated by filtration through a glass fibre filter in a Buchner funnel and used for total RNA isolation by using TRI REAGENT™. Afterwards polyA RNA was enriched with Oligotex® and cDNA preparation was performed using the Protoscript® First Strand cDNA Synthesis Kit. The second DNA strand was then synthesized via PCR and the following primer, based on literature known laccase sequences, in all possible combinations of forward (FW) and reverse (RV) primers.

Primers used for isolation of *P. cinnabarinus* isoenzymes:

FW_AF170093: ATGTCGAGGTTCCAGTCCCTC
RV_AF170093: TCAGAGGTCGCTGGGGTCAA
FW_AF025481: ATGTCCAGATTCCAATCTCTCC
RV_AF025481: TCAGAGATCGCTGGGGTCAA
FW_AF123571: ATGATCAGCATGGGCTTCCG
RV_AF123571: CTAATGGTCAGACTCCGGGA

Primers used for isolation of *T. versicolor* isoenzymes:

FW_Y1801: ATGTCGAGGTTTCACTCTCTTCTCGCTTTCGTCG
RV_Y1801: TTACTGGTCGCTCGGGTCGCGCGCG
FW_AY693776: FTV2: ATGGGTCTGCAGCGATTCAGCTTCTTCGTCACCC
RV_AY693776: TCACTGGTTAGCCTCGCTCAGCCCG
FW_AB212734: ATGGGCAAGTTTCACTCTTTTGTGAACG
RV_AB212734: TCAGAGGTCGGACGAGTCCAAAGCACCG
FW_AB212733: ATGGGCAGGGTCTCATCTCTCTGCGCGC
RV_AB212733: TTAGAGGTCGGATGAGTCAAGAGCGTTGTACG
FW_AM422387: ATGGGCAGGTTCTCATCTCTCTGCGCGC
FW_AF414109: ATGTCGAGGTTTCACTCTCTTTTCGCTTTCGTCG

The PCR product was analysed on a 1% agarose gel and a 1.5 kb fragment was isolated and purified via QIAquick Gel Extraktion Kit. The pure fragment was ligated into pCR®II_TOPO® vector, which was used for transformation of *E. coli* DH5alpha. Transformands were plated on agar plates containing X-gal for a blue-white screening. White colonies were used for a colony PCR to identify clones bearing laccase encoding fragments. Appropriate colonies were used for plasmid isolation and sequencing. Laccase encoding fragments were used for cloning into the vectors pET22b(+) for expression in *E. coli* and pGAPZalpha_B for expression in yeast, where the native signal peptides were removed for expression in *E. coli* and replaced by the alpha-mating factor signal peptide of *S. cerevisiae* for expression in yeast. Therefore laccase encoding genes were amplified using standard PCR protocols and the following primers for the expression in *E. coli* without their native signal peptides:

TvL5 forward: GGCGTATACATATGGGTATCGGTCCTGTCGCCG
TvL5 reverse: GTGGTGCTCGAGTGCGGCCGCCTGGTAGCTCGGGTCGC GCGCG
TvL10 forward: GGCGTATACATATGGCCATCGGGCCGGTGGCG
TvL10 reverse: GTGGTGCTCGAGTGCGGCCGCCTGGTTAGCCTCGCTC AGCCCG
TvL13 forward: GGCGTATACATATGGCGATTGGGCCCGTCACCG
TvL13 reverse: GTGGTGCTCGAGTGCGGCCGCGAGGTCGGACGAGTCC AAAGCACCG
TvL20 forward: GGCGTATACATATGGCTATCGGGCCTGTGACCGACC
TvL20 reverse: GTGGTGCTCGAGTGCGGCCGCGAGGTCGGATGAGTC AAGAGCG
PcL35 forward: GGCGTATACATATGGCCATAGGGCCTGTGGCGG
PcL35 reverse: GTGGTGCTCGAGTGCGGCCGCGAGGTCGCTGGGGT CAAGTGC

All PCR products were cloned into pET22b(+) using restriction enzymes *Nde*I and *Not*I. For cloning of laccase isoenzymes into pGAPZalpha_B for expression in *P. pastoris* without their native signal peptides and secretion via alpha factor of *S. cerevisiae* all laccase encoding genes were amplified using standard PCR protocols and the following primers:

TvL5 forward: GCTGCAGGAATTCTGGGTATCGGTCCTGTCGCCG
TvL5 reverse: GAAAGCTGGCGGCCGCCTGGTAGCTCGGGTCGCG
TvL10 forward: GCTGCAGGAATTCTGGCCATCGGGCCGGTGGCG
TvL10 reverse: CTAGAAAGCTGGCGGCCGCCTGGTTAGCCTCGCTC AGCC
TvL13 forward: CTCGAGCCGCGCGGCGGCCGCTGGCGATTGGGCCC GTCACCGAC
TvL13 reverse: CGCTTGTTCTAGAAAGAGGTCGGACGAGTCC

TvL20 forward: GCTGCAGGAATTCTGGCTATCGGGCCTGTGACCG
TvL20 reverse: CTAGAAAGCTGGCGGCCGCGAGGTCGGATGAGTCA AGAGC
PcL35 forward: GCTGCAGGAATTCTGGCCATAGGGCCTGTGGCGG
PcL35 reverse: GAAAGCTGGCGGCCGCGAGGTCGCTGGGGTC

PCR products TvL5, TvL10, TvL20 and PcL35 were cloned into pGAPZalpha_B using restriction enzymes *Eco*RI and *Not*I, TvL13 was cloned using *Xba*I and *Not*I. Later, also constructs containing the native signal peptide for a secretion while expressed in yeast, were produced by cloning into pGAPZ_B. The laccase encoding genes were amplified using standard PCR protocols and the following primers:

TvL5 forward: CGACGAGGAATTCAAGCATGGCATCGAGGTTTCACT CTCTTCTC
TvL5 reverse: CCGCTGGCGGCCGCTCTGGTAGCTCGGGTCGCG
TvL10 forward: CGACGAGGAATTCAAGCATGGGTCTGCAGCG
TvL10 reverse: CCGCTGGCGGCCGCTCTGGTTAGCCTCGCTCAGCC
TvL13 forward: GCGCGTACCTCGAGGATGGGCAAGTTTCAC
TvL13 reverse: CCGCTGGCGGCCGCTGGACGAGTCC
TvL20 forward: CGACGAGGAATTCAAGCATGGGCAGGTTCTC
TvL20 reverse: CCGCTGGCGGCCGCTGAGGTCGGATGAGTCAAGAGC
PcL35 forward: CGACGAGGAATTCAAGCATGGCATCGAGGTTCCAGT CCCTC
PcL35 reverse: CCGCTGGCGGCCGCTGAGGTCGCTGGGGTC

PCR products TvL5, TvL10, TvL20 and PcL35 were cloned into pGAPZ_B using restriction enzymes *Eco*RI and *Not*I, TvL13 was cloned using *Xho*I and *Not*I.

4.3. Recombinant expression of laccases in yeast

After cloning different laccase encoding gene fragments into the shuttle vector pGAPZalpha_B, the plasmid was used for transformation of *P. pastoris* X33 according to the manufacturer. Cells were plated out on YPDS agar plates containing 100 μg mL^{-1} Zeocin. Colonies grown after approx. 3 d were inoculated on YPDS plates containing 500, 1000 and 2000 μg mL^{-1} Zeocin to select multi-copy recombinants. These clones were used for recombinant constitutive expression according to the manufactures protocol. Laccases were secreted into the culture supernatant by the alpha secretion factor from the expression vector.

4.4. Activity measurements of laccases

Generally laccase activity was determined by monitoring the oxidation of 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid)diammonium (ABTS) spectrophotometrically at 420 nm (ε=3.6×10^4 M^{-1} cm^{-1}) in sodium acetate buffer (20 mM pH 5) at 30 °C as described earlier.[35] One unit (1 U) is defined as the amount of enzyme, which converts 1 μmol substrate per minute under assay conditions.

For estimation of pH optima, malate, sodium acetate, sodium phosphate, Bis-Tris or Tris buffers (all 20 mM) were used. To determine the stability and activity in methanol, the enzyme activity was monitored by adding different methanol concentrations to the sodium acetate buffer (20 mM, pH 5) followed by activity measurements using the ABTS assay.

4.5. Laccase-mediated coupling of 3-methylcatechol with aliphatic amines

Examinations on the laccase-mediated coupling of 3-methylcatechol with various linear primary amines ranging from C_4 (*n*-butylamine) to C_9 (*n*-nonylamine), the branch-chained amines **2g**–**i** and cyclic (*R*)-(+)-bornylamine **2j** were performed in sealed 6 mL brown glass flasks in a final reaction volume of 5 mL at room temperature with agitation at 200 rpm in presence of atmospheric oxygen. For enrichment of the expected heteromolecular products, reactions were scaled up in 100 mL Erlenmeyer-flasks, which were protected against light, containing 50 mL of reaction mixture using the same conditions as already described. Educts were prepared as 50 mM methanolic stock solutions and biocatalysis conducted either in equimolar concentrations of the aromatic or aliphatic amines (1 mM) or with an excess of the aliphatic coupling agents (5, 10 or 40 mM). Reactions were generally performed in sodium acetate buffer (20 mM) at pH 4 or 5[34,35] depending on the pH optima of the native and recombinant *P. cinnabarinus* and *T. versicolor* laccases.

4.6. Analytical HPLC

For kinetic studies, reaction mixtures were sampled in regular intervals over 24 h and analysed via HPLC. Grading of analytes was accomplished with a LiChroCart 125-4 RP-18 endcapped column of 5-μm particle size. The mobile phase consisted of an aqueous component A (0.1% phosphoric acid) and a methanol component B at an initial ratio of 10% B to 90% A, simultaneously raised up to 100% B within 14 min. The flow rate was always adjusted to 1 mL min^{-1} with an injection volume of 40 μL.

4.7. Determination of log *P*

Lyophilized secondary amines **3a**–**f** were analysed for their *n*-octanol/water partition coefficient using a modified log *P* method as described by Donovan and Pescatore[33] with instruments and analytical parameters as already described. Chromatographic separation was conducted on a 20 mm×4.0 mm short HPLC-column of 5 μm (250 Å) particle size with an octdecyl-poly(vinylalcohol) ODP 50 matrix. Samples were eluted with an aqueous phase consisting of phosphoric acid (0.1%) and methanol. Measurements were conducted at an initial ratio of 10% methanol to 90% phosphoric acid, whereby methanol concentration raised up to 100% in 9.4 min with 6 min equilibration time. The flow rate was adjusted to 1 mL min^{-1} and injection volumes set to 5 μL. To get evidence of change in log *P* by modifying the activated *ortho*-benzenediol with linking to primary amines **2a**–**f** consisting of increasing chain length, 3-methylcatechol as well as the synthesized dimeric compounds **3a**–**f** were individually combined in standard mixtures containing hydrophobic (toluene, ibuprofen, triphenylamine) and also hydrophilic (3,5-dihydroxybenzoic acid or 2-(4-hydroxyphenyl)ethyl alcohol) reference substances. Standard mixtures were prepared by adding 20 mg of the hydrophilic substance to 2 mL of hydrophobic reference and filled up to a final volume of 200 mL with methanol. For log *P* determination, 0.5 mg of the product was diluted in 1 mL of standard mixtures and subsequently measured. For arylalkyl products **3a**,**b** ranging from C_4 to C_5 side chains, toluene was set as hydrophobic standard. Ibuprofen was used for products **3c**,**d** carrying C_6 and C_7-substitutents, whereas triphenylamine was applied for **3e**,**f** with addition of *n*-octylamine or *n*-nonylamine. Examinations were conducted in standard mixtures containing either 3,5-dihydroxybenzoic acid or 2-(4-hydroxyphenyl)ethyl alcohol as hydrophilic reference. With known log *P* values (Table S2 in Supplementary data) of reference substances, *n*-octanol/water partition coefficients of the secondary amines were calculated. Products **3a**–**f** were analysed in triplicate. Results obtained from log *P* measurements were validated with a software tool of ACD/ChemSketch, whereby theoretical log *P* values of the products could be estimated.

4.8. Isolation of heteromolecular coupling products

The secondary amines were purified by solid reverse phase extraction with a silicagel column (Strata C18-E 60 mL Giga Tubes, 10 g absorbent material), progressively charged with 50 mL of the reaction mixture. Homomolecular by-products were eluted with 50 mL of a solution composed of 50% methanol and 50% double destilled water. The desired alkylamine substituted heteromolecular coupling products were obtained with mixtures consistent of 60% methanol and 40% acetic acid (0.1% v/v). After elution, the fraction containing the desired coupling product was diluted to a final methanol concentration of 5% with bidestilled water and frozen at −20 °C for 24 h, followed by freezing at −80 °C for further 4 h. Via lyophilisation, products were obtained as dry chemicals and further available for aimed characterization studies.

4.9. Structural characterization by LC–MS, HRMS and NMR studies

The heteromolecular coupling products were characterized by liquid chromatography coupled with mass spectroscopy. For sample ionization, the API-ES method was used in positive ion $[M+H]^+$ mode. All measurements were performed on a 1200 Series 6120 Quadrupole mass spectrometer (Agilent Technologies, Böblingen, Germany). Grading of analytes was accomplished on a ZORBAX SB-C18 column (2.1×50 mm) possessing a 1.8 μm pore size (Agilent Technologies, Böblingen, Germany) with a mobile phase consistent of acetonitrile and 0.1% ammonium formate. HRMS measurements were conducted on an ESI-TOF/MS (Agilent Technologies, Böblingen, Germany) using positive ion $[M+H]^+$ mode within a direct injection of methanolic samples lacking a previous chromatographic column separation. HRMS measurements were accomplished using a MeOH/0.1% acetic acid mobile phase in a 9:1 ratio. For complete structure determination of the isolated products, HMBC-, HSQC-, and HH-COSY were recorded on a Bruker spectrometer (Advance 600, 600 MHz, Karlsruhe, Germany) in deuterated methanol (MeOH-d_4).

*4.9.1. 3-Methyl-5-(butylamino)-1,2-benzoquinone (**3a**).* Light red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.53 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.34 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.69 (m, 2H, H-2′); 1.45 (m, 2H, H-3′), 0.99 (t, $^3J_{3',4'}$=7.4 Hz, 3H, H-4′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.6 (C-2), 175.6 (C-1), 160.4 (C-5), 142.4 (C-3), 133.4 (C-4), 94.4 (C-6), 44.2 (C-1′), 31.0 (C-2′), 21.0 (C-3′), 15.1 (C-1″), 13.8 (C-4′). HPLC R_f 8.8 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC–MS R_f 4.2 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 193; found *m*/*z* 194.1. HRMS (ESI) calcd for $C_{11}H_{15}NO_2$ 194.11756; found 194.11758.

*4.9.2. 3-Methyl-5-(pentylamino)-1,2-benzoquinone (**3b**).* Reddish-crimson solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (br, 1H, H-4), 5.54 (d, $^4J_{4,6}$=2.5 Hz, 1H, H-6), 3.33 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.3 Hz, 3H, H-1″), 1.71 (m, 2H, H-2′), 1.40 (m, 4H, H-3′, H-4′), 0.96 (t, $^3J_{4',5'}$=7.0 Hz, 3H, H-5′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.7 (C-2), 175.4 (C-1), 160.7 (C-5), 142.7 (C-3), 133.5 (C-4), 94.6 (C-6), 45.1 (C-1′), 30.3 (C-3′), 29.0 (C-2′), 23.5 (C-4′), 15.6 (C-1″), 14.4 (C-5′). ^{1}H,^{1}H COSY and ^{1}H,^{13}C correlations (HMBC): see Table 3 and Supplementary data. HPLC R_f 10.2 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC–MS R_f 4.9 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 207; found *m*/*z* 208.1. HRMS (ESI) calcd for $C_{12}H_{17}NO_2$ 208.13321; found 208.1332.

*4.9.3. 3-Methyl-5-(hexylamino)-1,2-benzoquinone (**3c**).* Red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.53 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.33 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.70 (m, 2H, H-2′), 1.43 (m, 2H, H-3′), 1.39–1.33 (m, 4H, H-3′, H-4′, H-5′), 0.93 (t, $^3J_{5',6'}$=7.0 Hz, 3H, H-6′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.8 (C-2), 175.8 (C-1), 160.5 (C-5), 142.7 (C-3), 133.7 (C-4), 94.7 (C-6), 45.1 (C-1′), 32.6 (C-4′), 29.3 (C-2′), 27.8 (C-3′), 23.6 (C-5′), 15.4 (C-1″), 14.3 (C-6′). HMBC correlations see Supplementary data. HPLC R_f 11.6 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC–MS R_f 5.6 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 221; found *m*/*z* 222.1. HRMS (ESI) calcd for $C_{13}H_{19}NO_2$ 222.14886; found 222.14856.

*4.9.4. 3-Methyl-5-(heptylamino)-1,2-benzoquinone (**3d**).* Dark-red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.53 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.33 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.70 (m, 2H, H-2′), 1.45–1.29 (m, 8H, H-3′, H-4′, H-5′, H-6′), 0.91 (t, $^3J_{6',7'}$=6.9 Hz, 3H, H-7′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.6 (C-2), 160.5 (C-5), 142.6 (C-3), 133.6 (C-4), 94.5 (C-6), 44.8 (C-1′), 32.7 (C-5′), 29.8 (C-4′), 29.0 (C-2′), 27.9 (C-3′), 23.5 (C-6′), 15.3 (C-1″), 14.3 (C-7′). HMBC correlations see Supplementary data. HPLC R_f 12.7 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC–MS R_f 6.2 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 235; found *m*/*z* 236.1. HRMS (ESI) calcd for $C_{14}H_{21}NO_2$ 236.16451; found 236.16459.

*4.9.5. 3-Methyl-5-(octylamino)-1,2-benzoquinone (**3e**).* Dark-red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (br, 1H, H-4), 5.53 (d, $^4J_{4,6}$=2.5 Hz, 1H, H-6), 3.33 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.4 Hz, 3H, H-1″), 1.70 (m, 2H, H-2′), 1.45–1.29 (m, 10H, H-3′, H-4′, H-5′, H-6′, H-7′), 0.90 (t, $^3J_{7',8'}$=7.1 Hz, 3H, H-8′). HPLC R_f 13.6 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC–MS R_f 6.7 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 249; found *m*/*z* 250.1. HRMS (ESI) calcd for $C_{15}H_{23}NO_2$ 250.18016; found 250.17915.

*4.9.6. 3-Methyl-5-(nonylamino)-1,2-benzoquinone (**3f**).* Red-brown solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.75 (br, 1H, H-4); 5.53 (d, $^4J_{4,6}$=2.5 Hz, 1H, H-6), 3.33 (t, $^3J_{1',2'}$=7.2 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.4 Hz, 3H, H-1″), 1.70 (m, 2H, H-2′), 1.45–1.25 (m, 12H, H-3′, H-4′, H-5′, H-6′, H-7′, H-8′), 0.90 (t, $^3J_{8',9'}$=7.1 Hz, 3H, H-9′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.5 (C-2), 160.4 (C-5), 142.3 (C-3), 133.2 (C-4), 94.2 (C-6), 44.6 (C-1′), 32.7 (C-7′), 30.2 (C-4′), 29.7, 21.5 (C-5′, C-6′), 28.9 (C-2′), 27.8 (C-3′), 23.4 (C-8′), 15.0 (C-1″), 14.3 (C-9′). HMBC correlations see Supplementary data. HPLC R_f 14.3 min. UV–vis (MeOH) λ_{max} 220, 298, 490 nm. LC-MS R_f 7.3 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 263 ; found *m*/*z* 264.1. HRMS (ESI) calcd for $C_{16}H_{25}NO_2$ 264.19581; found 264.19619.

*4.9.7. 3-Methyl-5-(2-ethylhexylamino)-1,2-benzoquinone (**3g**).* Red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.78 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.55 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.25 (d, $^3J_{1',2'}$=6.7 Hz, 2H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.71 (m, 1H, H-2′), 1.44 (m, 2H, H-7′), 1.40–1.33 (m, 6H, H-3′, H-4′, H-5′), 0.95 (t, $^3J_{7',8'}$=7.5 Hz, 3H, H-8′), 0.93 (t, $^3J_{5',6'}$=7.0 Hz, 3H, H-6′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.4 (C-2), 175.2 (C-1), 160.6 (C-5), 142.5 (C-3), 133.2 (C-4), 94.6 (C-6), 48.3 (C-1′), 39.9 (C-2′), 31.8 (C-3′), 29.6 (C-4′), 25.0 (C-7′), 23.8 (C-5′), 15.2 (C-1″), 14.2 (C-6′), 10.8 (C-8′). HMBC correlations see Supplementary data. HPLC R_f 11.4 min. UV–vis (MeOH) λ_{max} 219, 299, 486 nm. LC–MS R_f 6.4 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated *m*/*z* 249; found *m*/*z* 250.1. HRMS (ESI) cald for $C_{15}H_{23}NO_2$ 250.18016; found 250.18086.

*4.9.8. 3-Methyl-5-(1-methylpentylamino)-1,2-benzoquinone (**3h**).* Red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.56 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.72 (m, 1H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.63 (m, 2H, H-2′), 1.37 (m, 4H, H-3′, H-4′), 1.28 (d, $^3J_{1',6'}$=6.5 Hz, 3H, H-6′), 0.93 (t,

$^3J_{4',5'}$=7.0 Hz, 3H, H-5′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)= 185.7 (C-2), 175.6 (C-1), 160.0 (C-5), 142.7 (C-3), 133.3 (C-4), 94.4 (C-6), 51.2 (C-1′), 36.6 (C-2′), 29.2 (C-3′), 23.4 (C-4′), 19.9 (C-6′), 15.3 (C-1″), 14.1 (C-5′). HMBC correlations see Supplementary data. HPLC R_f 10.7 min. UV–vis (MeOH) λ_{max} 220, 298, 491 nm. LC–MS R_f 5.3 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated m/z 221; found m/z 222.1. HRMS (ESI) calcd for $C_{13}H_{19}NO_2$ 222.14886; found 222.14854.

*4.9.9. 5-(1,4-Dimethylpentylamino)-3-methyl-1,2-benzoquinone (**3i**).* Red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.74 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.56 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.69 (m, 1H, H-1′), 1.98 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.63 (m, 2H, H-2′), 1.57 (m, 2H, H-3′), 1.28 (d, $^3J_{1',6'}$=6.5 Hz, 3H, H-6′), 1.25 (m, 1H, H-4′); 0.92 (2d, $^3J_{4',5'}$=$^3J_{4',7'}$=6.6 Hz, 6H, H-5′,7′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.5 (C-2), 175.2 (C-1), 159.7 (C-5), 142.7 (C-3), 133.5 (C-4), 94.3 (C-6), 51.3 (C-1′), 34.8 (C-2′), 28.9 (C-3′), 20.0 (C-6′), 36.1 (C-4′), 22.6 (C-5′, C-7′), 15.2 (C-1″). HMBC correlations see Supplementary data. HPLC R_f 11.4 min. UV–vis (MeOH) λ_{max} 220, 298, 492 nm. LC–MS R_f 5.8 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated m/z 235; found m/z 236.1. HRMS (ESI) calcd for $C_{14}H_{21}NO_2$ 236.16451; found 236.16449.

*4.9.10. 3-Methyl-5-[(4,7,7-trimethylnorbornan-2-yl)amino]-1,2-benzoquinone (**3j**).* Light red solid. ^{1}H NMR (600 MHz, MeOH-d_4) δ (ppm)=6.94 (dq, $^4J_{4,6}$=2.7 Hz, $^4J_{4,1''}$=1.5 Hz, 1H, H-4), 5.58 (d, $^4J_{4,6}$=2.7 Hz, 1H, H-6), 3.94 (ddd, $^3J_{2',3'a}$=10.8 Hz, $^3J_{2',3'b}$=4.4 Hz, $^4J_{2',6'b}$=2.2 Hz, 1H, H-2′), 2.45 (dddd, $^2J_{3'a,3'b}$=13.4 Hz, $^3J_{2',3'a}$=10.8 Hz, $^4J_{3'a,5'a}$=4.7 Hz, $^4J_{1',3'a}$=3.2 Hz, 1H, H-3′a), 1.99 (d, $^4J_{4,1''}$=1.5 Hz, 3H, H-1″), 1.85 (m, 1H, H-5′a), 1.80–1.75 (m, 2H, H-1′, H-6′a), 1.47 (dddd, $^2J_{6'a,6'b}$=13.8 Hz, $^3J_{5'a,6'b}$=12.1 Hz, $^3J_{5'b,6'b}$=4.5 Hz, $^4J_{2',6'b}$=2.2 Hz, 1H, H-6′b), 1.37 (ddd, $^2J_{5'a,5'b}$=12.3 Hz, $^3J_{5'b,6'a}$=9.5 Hz, $^3J_{5'b,6'b}$=4.5 Hz, 1H, H-5′b), 1.16 (dd, $^2J_{3'a,3'b}$=13.4 Hz, $^3J_{2',3'b}$=4.4 Hz, 1H, H-3′b), 1.03, 0.95 (2s, 6H, H-8′, H-9′); 0.94 (s, 3H, H-10′). ^{13}C NMR (150 MHz, MeOH-d_4) δ (ppm)=185.5 (C-2), 175.5 (C-1), 161.1 (C-5), 142.3 (C-3), 133.3 (C-4), 94.9 (C-6), 60.5 (C-2′), 52.2 (C-4′), 49.9 (C-7′), 46.3 (C-1′), 37.3 (C-3′), 28.8 (C-6′), 28.8 (C-5′), 19.9, 18.8 (C-8′, C-9′), 15.4 (C-1″), 14.6 (C-10′). HMBC and HH COSY correlations see Supplementary data. HPLC R_f 12.2 min. UV–vis (MeOH) λ_{max} 220, 306, 498 nm. LC–MS R_f 6.2 min (MeOH); API-ES: pos. mode $[M+H]^+$ calculated m/z 273; found m/z 274.1. HRMS (ESI) calcd for $C_{17}H_{23}NO_2$ 274.18016; found, 274.18003.

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Supplementary data

Supplementary data associated with this article can be found in online version, at doi:10.1016/j.tet.2011.09.123.

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