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Synthesis of 3-(3,4-dihydroxyphenyl)-propionic acid derivatives by N-coupling of amines using laccase

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Abstract—Derivatization of the natural compound 3-(3,4-dihydroxyphenyl)-propionic acid (dihydrocaffeic acid) can be achieved by laccase-catalyzed N-coupling of aromatic and aliphatic amines. Incubation of 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid with laccase in aqueous medium and in the presence of oxygen yielded 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid as the main product (>80%). Reaction with hexylamine resulted in 3-(6-hexylamino-3,4-dihydroxyphenyl)-propionic acid as the only product (60%). © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) has been detected in various plant species, some bacteria (e.g. *Azospirillum lipoferum*), various insects, and many white rot fungi which produce it as an extracellular enzyme.[1] These multicopper oxidases catalyze the oxidation of various aromatic compounds while concomitantly reducing molecular oxygen to water.[2] The ensuing oxidative coupling reaction is important in the synthesis of numerous naturally occurring complexes such as lignins, tannins, melanins, alkaloids, and humic substances. This same reaction is involved in transformation, oligomerization, and polymerization processes which can be useful for medical and industrial applications (e.g. waste detoxification, textile dye transformation, biosensor and diagnostic applications, or food treatment[3]).

Only a few applications have been described that use laccase to catalyze organic syntheses. These include the oxidation of aromatic alcohols to aldehydes,[4,5] the synthesis of substituted imidazoles,[6] the dimerization of penicillin or cephalosporin monomers or oxidation of a cephalosporin,[7,8] the laccase catalyzed synthesis of 3-substituted 1,2,4-triazolo(4,3-*b*)(4,1,2)benzothiadiazine-8-ones[9] as well as the oxidative coupling of mithramycin and hydroquinone.[10]

The aim of the present study was to investigate whether aromatic compounds, substituted by *ortho* hydroxyl groups, can be transformed into derivatives by heteromolecular coupling catalyzed by laccase. As a model we used the antiviral natural compound 3-(3,4-dihydroxyphenyl)-propionic acid whose derivatives may be interesting for pharmaceutical purposes.

2. Results and discussion

2.1. Transformation of 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid by crude preparation of laccase

Incubation experiments with crude preparation of laccase (580 nmol mL^{-1} min^{-1}) supplemented with 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid (substrates **1** and **2**, Fig. 1) yielded only the single

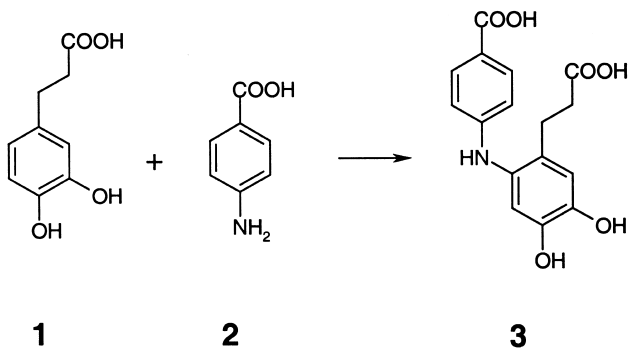

Figure 1. 3-(3,4-Dihydroxyphenyl)-propionic acid (substrate **1**), 4-aminobenzoic acid (substrate **2**) and the product 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid (**3**) formed due to a laccase catalyzed reaction.

Keywords: 3-(3,4-dihydroxyphenyl)-propionics; laccase; biotransformation.

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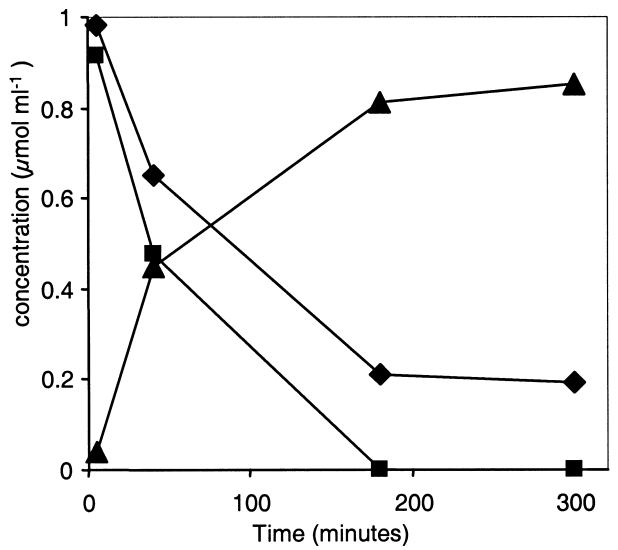

Figure 2. Kinetics of substrate depletion, 3-(3,4-dihydroxyphenyl)-propionic acid ■ (substrate **1**), and 4-aminobenzoic acid ◆ (substrate **2**), and the formation of the product 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid ▲ (**3**) during the laccase catalyzed coupling reaction.

cross-coupling product **3**. Over the time course of incubation, a color change in the initially clear reaction mixture was noted. After 3 h, the reaction fluid turned pale yellow and subsequently dark red.

High-performance liquid chromatography (HPLC) analysis of the reaction mixture revealed the simultaneous decrease of the two substrates and the increase of one product (Fig. 2). Sufficient amounts of the product **3** (>80% yield) for spectral characterization was formed over an incubation period of 3 h. After that time 4-aminobenzoic acid was still present in the reaction mixture, but 3-(3,4-dihydroxyphenyl)-propionic acid was no longer detectable. Laccase transformed 3-(3,4-dihydroxyphenyl)-propionic acid up to 20% by homomolecular reactions. This amount of 3-(3,4-dihydroxyphenyl)-propionic acid was not available for the cross-coupling reaction with 4-aminobenzoic acid. Similar reaction kinetics were described for a hybrid dimer formation from 3,4-dichloroaniline and syringic acid. Within 60 min the syringic acid was transformed totally, while about 50% of the aniline derivative remained in the incubation assay.[11] In that experiment homomolecular coupling of the hydroxylated coupling partner diminished the yield of the hybrid dimer more drastically than we could observe in our experiments.

Table 2. ^{13}C NMR data of the substrates 3-(3,4-dihydroxyphenyl)-propionic acid (**1**) and 4-aminobenzoic acid (**2**) and the product (**3**) formed due to the coupling by laccase

Product **3**	Substrate **1**	Substrate **2**	Proton assignment	
δ (ppm)	δ (ppm)	δ (ppm)	Product	Substrates
27.2	31.5	/	CH_2CH_2COOH	CH_2CH_2COOH
36.3	37.1	/	CH_2CH_2COOH	CH_2CH_2COOH
114.0	/	114.3	C3/5-C_6H_4	C3/5-C_6H_4
117.0	116.3	/	C2–C_6H_2	C2–C_6H_3
117.5	116.4	/	C5–C_6H_2	C5–C_6H_3
119.4	/	119.0	C1–C_6H_4	C1–C_6H_4
/	120.5	/		C6–C_6H_3
130.1	134.7	/	C1–C_6H_2	C1–C_6H_3
131.8	/	/	C6–C_6H_2	
132.7	/	132.7	C2/6-C_6H_4	C2/6-C_6H_4
144.6	144.6, 146.2	/	C3/4-C_6H_2	C3/4-C_6H_3
153.8	/	154.6	C4–C_6H_4	C4–C_6H_4
173.6	/	170.7	COOH	COOH
174.3	176.9	/	COOH	COOH

The separation of the product was performed by HPLC. Comparison of proton–proton spin systems and couplings of the ^1H NMR-spectra and mass spectra of the compound **3** with those of the substrates **1** and **2** showed structural similarities and led to the later identification of product **3** as 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid (Fig. 1).

This identification is based on the following data: mass spectral analysis of the silylated compound **3** showed a molecular mass of 605. This mass was attributed to the coupling of 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid and the silylation of four functional groups. The fragment ion at 590 (M^+-15) indicated the loss of one methyl group. The loss of six methyl groups led to the ion peak at 515. The molecular mass ion peaks of the silylated substrates 3-(3,4-dihydroxyphenyl)-propionic acid (398) and 4-aminobenzoic acid (209) and the typical fragment ions [3-(3,4-dihydroxyphenyl)-propionic acid 383 M^+-15, 368 M^+-30; 4-aminobenzoic acid 194 M^+-15] in the mass spectral analysis of product **3** indicated the coupling of these substrates to produce 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid.

Table 1. ^1H NMR data of the substrates 3-(3,4-dihydroxyphenyl)-propionic acid (**1**) and 4-aminobenzoic acid (**2**) and the product (**3**) formed due to the coupling by laccase

Product **3**		Substrate **1**		Substrate **2**		Proton assignment
δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	
2.68	^3J=7.3	2.50	^3J=7.8	/		t, 2H, CH_2, CH_2CH_2COOH
3.01		2.75		/		t, 2H, CH_2, CH_2CH_2COOH
5.99						s, 1H, CH, C5–C_6H_2
6.57						s, 1H, CH, C2–C_6H_2
6.93	^3J=8.3	/		6.62	^3J=8.8	m(d), 2H, CH, C2/6-C_6H_4
/		6.51	^3J=8.1 ^4J=2.2	/		dd, 1H, CH, C6–C_6H_3
/		6.64	^4J=2.2	/		d(s), 1H CH, C2–C_6H_3
/		6.65	^3J=8.1	/		d, 1H CH, C5–C_6H_3
8.06	^3J=8.3	/		7.75	^3J=8.8	m(d), 2H, CH, C3/5-C_6H_4

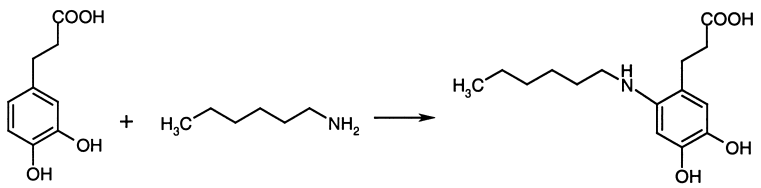

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Figure 3. 3-(3,4-Dihydroxyphenyl)-propionic acid (substrate **1**), 1-hexylamine (substrate **4**) and the product 3-(6-hexylamino-3,4-dihydroxyphenyl)-propionic acid (**5**) formed due to a laccase catalyzed reaction.

The investigation of the product **3** by high-resolution mass analyses revealed a molecular weight of 316.0600 (317.0899 was calculated for the molecular formula of $C_{16}H_{14}NO_6$). This finding indicates the missing of one proton in product **3**, pointing to a radical structure for this substance.

The coupling of the substrates 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid to the 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid is confirmed by the presence of all carbons of the 4-aminobenzoic acid and of the 3-(3,4-dihydroxyphenyl)-propionic acid in the ^{13}C NMR spectrum of the product (Table 2).

Moreover, all protons of the two substrates, besides the proton at C-6 of the 3-(3,4-dihydroxyphenyl)-propionic acid, were detectable in the ^{1}H NMR spectrum of the product (Table 1, amino and hydroxyl protons were not analyzed with the method used).

The multiplicity of the signals of the C-2 and C-5 proton of 3-(3,4-dihydroxyphenyl)-propionic acid changed to singlets in the ^{1}H NMR spectrum of the product, indicating a further substituent at the C-6 position, because no *meta*-coupled singlets could be observed. The chemical shift to lower field of the C-5 proton demonstrated the presence of an electron-withdrawing group. The signals of the substituted 4-amino-benzoic acid were comparable with the signals of the substrate 4-aminobenzoic acid. This fact together with the changes of the signals of 3-(3,4-dihydroxyphenyl)-propionic acid mentioned above suggests that the structure of the coupling product is 3-[6-(4-carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid. No benzoquinone-imino structures were observed as described for the hybrid dimer formation from 3,4-dichloraniline with protocatechuic acid and syringic acid.[11,12]

2.2. Transformation of 3-(3,4-dihydroxyphenyl)-propionic acid and 1-hexylamine by crude preparation of laccase

In the reaction mixture containing laccase (580 nmol mL^{-1} min^{-1}), 3-(3,4-dihydroxyphenyl)-propionic acid **1**, and 1-hexylamine **4** one cross-coupling product **5** (Fig. 3) was produced. The color of the reaction mixture changed from initially colorless to dark red at the end of incubation.

The decrease of the substrate 3-(3,4-dihydroxyphenyl)-propionic acid and the increase of one product were analyzed by HPLC (hexylamine is not detectable by the analytical method used). Sufficient amounts of the product **5** (60% yield) for spectral characterization was formed over an incubation period of 6 h. The homomolecular reactions of 3-(3,4-dihydroxyphenyl)-propionic acid were almost completely suppressed by the transformation with hexylamine.

Preparative HPLC led to the isolation of product **5**. The structure has been determined by ^{1}H NMR and mass spectroscopy. The mass *m/z* 496 (sylilated compound, $M^{+}-1$), the sum of the mass of the sylilated substrate **1** (398) and of the substrate **3** (101) minus two protons, was attributed to the cross-coupling of the two substrates. The fragmentation of $M^{+}-15$ ($M^{+}-CH_3$) in the mass spectrum of product **5**, was attributed to the loss of a methyl group. Further cleavage of methylene and methyl groups resulted in the fragment ions *m/z* 468 ($M^{+}-29$, CH_2CH_3), 440 ($M^{+}-57$, $CH_2CH_2CH_2CH_3$), 425 ($M^{+}-72$,

Table 3. ^{1}H NMR data of the substrates 3-(3,4-dihydroxyphenyl)-propionic acid (**1**) and 1-hexylamine (**4**) and the product (**5**) formed due to the coupling by laccase

Product **5**		Substrate **1**		Substrate **4**		Proton assignment
δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	δ (ppm)	J (Hz)	
0.92	^{3}J=6.8	/		0.91	^{3}J=6.7	t, 3H, CH_3
1.35		/		1.32		m, 6H, 3×CH_2, $CH_3(CH_2)_3CH_2CH_2NH$
1.64		/		1.45		m, 2H, CH_2, $CH_3(CH_2)_3CH_2CH_2NH$
2.44	^{3}J=7.7	2.50	^{3}J=7.8	/		t, 2H, CH_2, CH_2CH_2COOH
2.81	^{3}J=7.0	/	^{3}J=7.8	2.61	^{3}J=6.9	t, 2H, CH_2, $CH_3(CH_2)_3CH_2CH_2NH$
2.90	^{3}J=7.7	2.75		/		t, 2H, CH_2, CH_2CH_2COOH
5.58		/		/		s, 1H, CH, C5–C_6H_2
6.28		/		/		s, 1H, CH, C2–C_6H_2
/		6.51	^{3}J=8.1, ^{4}J=2.2	/		dd, 1H, CH, C6–C_6H_3
/		6.64	^{4}J=2.2	/		d(s), 1H, CH, C2–C_6H_3
/		6.65	^{3}J=8.1	/		d, 1H, CH, C5–C_6H_3

$CH_2CH_2CH_2CH_3/CH_3$), 365 (M^+-132, $CH_2CH_2CH_2$-$CH_3/5\times CH_3$). The loss of the hexylamino substituent and six methyl groups led to the fragment ion 307 (M^+-190, $HN(CH_2)_5CH_3/6\times CH_3$). High-resolution mass analyses of product **5** revealed a molecular weight of 280.1543 (281.1622 was calculated for the molecular formula of $C_{15}H_{23}NO_4$) This finding indicates the missing of one proton in product **5** comparable with **3**, pointing to radical structure for the substances.

The structure 3-(6-hexylamino-3,4-dihydroxyphenyl)-propionic acid is fully compatible with the NMR data of the product **5**. The ^1H NMR spectrum showed signals for two aromatic methines and for 17 protons of the aliphatic chains (Table 3). All these aliphatic signals were also found in the spectra of the substrates. Comparison of the ^1H NMR spectrum of the product with those of the substrates showed only changes of the aromatic signals. The multiplicity of the signals of the C-2 and C-5 proton of 3-(3,4-dihydroxyphenyl)-propionic acid changed to singlets in the ^1H NMR spectrum of the product, indicating a further substituent at C-6 to give 3-(6-hexylamino-3,4-dihydroxyphenyl)-propionic acid. Like in case of product **3** no *meta*-coupled singlets could be observed in the ^1H NMR spectrum of **5**.

Both 4-aminobenzoic acid and *n*-hexylamine reacted with 3-(3,4-dihydroxyphenyl)-propionic acid in the same way to products that kept their 3,4-dihydroxy substructure and carried an additional amino substituent. Both structure and high-resolution mass analyses imply that the products are formed by a $R-NH_2$ attack of a cation radical of 3-(3,4-dihydroxyphenyl)-propionic acid. No hints for an involvement of *ortho*-quinones in the reaction were found.

3. Experimental

3.1. 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).

3.2. Culture media and conditions

3.2.1. 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 maintained at 4°C. Broth cultures were prepared by inoculating a nitrogen-rich medium containing 5 g glucose, 1 g KH_2PO_4, 0.5 g L-asparagine, 0.5 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 L^{-1}) with two or 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 Ref. 13). Incubation was performed under static conditions 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 17,000 rpm. For the production of the ligninolytic enzyme laccase, 75 mL medium inoculated with 7.5 mL of the homogenized pre-culture was incubated in 300 mL Erlenmeyer flasks for 24 h with 3,4-dimethoxybenzyl alcohol (10 mM), a known inducer of laccase. Cultures were agitated in a water bath (GFL model 1092, Burgwedel, Germany) at 30°C and 160 rpm.

3.2.2. Transformation of 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid or 1-hexylamine using a crude preparation of laccase. A crude preparation of laccase was prepared as follows: *P. 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 Q-Sepharose for 1 h and the adsorbed enzymes were extracted from the Q-Sepharose with 20 mM histidine buffer (pH 6.3). This enzyme extract was then concentrated by ultrafiltration (Centriprep, 10 kDa, Amicon GmbH, Witten, Germany) and desalted using a Sephadex G-25 Superfine column (Pharmacia, Freiburg, Germany).

The crude preparation of laccase (activity 2320 nmol mL^{-1} min^{-1}) was diluted 1:4 with 20 mM sodium acetate buffer, pH 5. The substrates [3-(3,4-dihydroxyphenyl)-propionic acid (1 mM) and 1-hexylamine (6 mM) or 4-aminobenzoic acid (1 mM)] were added to 2 mL of this solution, and the reaction mixture was incubated for 3 h at 30°C (**1** and **2**) or 6 h (**1** and **4**) at room temperature with agitation at 100 rpm.

Laccase activity was determined spectrophotometrically at 420 nm with ABTS (2,2-azino-bi-(3-ethyl-benzthiiazolin-sulfonate)) as substrate[14] using the method described by Jonas et al.[15]

3.3. Analytical HPLC

For routine analysis, the reaction mixtures were analyzed using an HPLC system (Hewlett–Packard GmbH, Bad Homburg, Germany) consisting of an HP 1050 Series Pump, and HP 1040 M Series I Diode-Array-Detector, and an HP HPLC Chem Station. An endcapped, 5 μm, LiChroCart 125-4 RP 18 column (Merck, Darmstadt, Germany) was used at a flow rate of 1 mL min^{-1}. A solvent system consisting of methanol (eluent A) and 20 mM phosphate buffer pH 4.8 (eluent B), starting from an initial ratio of 20% A and 80% B and reaching 100% methanol within 14 min, was used.

3.4. Isolation

3.4.1. General procedure for the isolation of transformation products. An RP18 silicagel column (polypropylene 3 mL, 200 mg adsorbent material, Baker, Gross-Gerau, Germany) was charged with the reaction mixture.

The homomolecular products of 3-(3,4-dihydroxyphenyl)-propionic acid were eluted with a solution consisting of 10% methanol and 90% 20 mM sodium acetate buffer (pH 5). The cross-coupling products **3** and **5** were eluted with acetonitrile.

The further purification of the products **3** and **5** from the acetonitrile fraction was performed on a HPLC module

system (Merck, Darmstadt, Germany) equipped with a Model L 6200A Intelligent Pump, a Rheodyne 7161 injection valve with a 20 μL loop, a Model L-4250 absorbance detector operating at 220 nm. High purity of the product was achieved on an endcapped, 5 μm, LiChroCart 125-4 RP 18 column (Merck,) at a flow rate of 1 mL min^{-1}. For sufficient separation, the solvent system consisting of methanol (eluent A) and 20 mM ammonium acetate buffer pH 4.8 (eluent B) in a ratio of 20% A and 80% B changed to 100% A within 14 min.

3.5. Derivatization

100 μL Silyl-991 [BSTFA (*N*,*O*-bis-trimethylsilyl-trifluoracetamide)–TMCS (trimethylchlorsilane) 99:1, Macherey-Nagel, Düren] was added to about 1 mg of product **3**. After incubating the reaction mixture at 60°C for 3 h, Silyl-991 was steamed off by nitrogen. The dried product was dissolved in methanol and analyzed by gas chromatography/mass spectrometry.

100 μL of MSTFA (*N*-methyl-*N*-trimethylsilyltrifluoracetamide) was added to about 5 mg of product **5** in 400 μL methanol. The reaction mixture was heated to 60°C for 3 h and then analyzed directly by gas chromatography/mass spectrometry.

3.6. Characterization

Analysis of the silylated substrates and of silylated purified products by gas chromatography/mass spectrometry was carried out on a gas chromatograph GC 8000 linked to a mass selective detector MD 800 (Fisons Instruments, Mainz, Germany) operating at 70 eV and fitted with a 30-m BPX5 ms column (0.21 mm; 0.25 μm film, SGE, Weiterstadt, Germany).

The products were investigated by high-resolution mass analyses on a Q-TOF mass spectrometer (Q-Star Pulsar, Applied Biosystems, Forster City, USA) with a nanospray source (Protana Odense, Denmark).

The ^{1}H NMR spectra were recorded on a Bruker ARX 300 instrument (Karlsruhe, Germany) at 300 MHz in MeOH-d_4. Tetramethylsilane was used as an internal standard.

3.6.1. 3-[6-(4-Carboxyphenyl)amino-3,4-dihydroxyphenyl]-propionic acid (3). This compound was recovered as a transformation product of 3-(3,4-dihydroxyphenyl)-propionic acid and 4-aminobenzoic acid. Isolation from the reaction mixture was achieved by use of a RP18-silicagel column. Purification was carried out as described before: yield 0.26 mg mL^{-1} (>80%). 300 MHz ^{1}H NMR (MeOH-d_4) see Table 1, ^{13}C NMR (MeOH-d_4) see Table 2; MS (GC/MS silylated): M^+=605, 590 (M^+-15, CH_3), 515 (M^+-90, $6\times CH_3$), 398 ($C_{18}H_{34}O_4Si_4$), 383 ($C_{18}H_{34}O_4Si_4-15$, CH_3), 368 ($C_{18}H_{34}O_4Si_4-30$, $2\times CH_3$), 209 ($C_{10}H_{15}O_4NSi$), 194 ($C_{10}H_{15}O_4NSi-15$, CH_3), high-resolution mass analysis 316.0600.

3.6.2. 3-(6-Hexylamino-3,4-dihydroxyphenyl)-propionic acid (5). This compound was recovered as a transformation product of 3-(3,4-dihydroxyphenyl)-propionic acid and hexylamine. Isolation from the reaction mixture was achieved by use of a RP18-silicagel column. Purification was carried out as described before: yield 0.17 mg mL^{-1} (60%). 300 MHz ^{1}H NMR (MeOH-d_4) see Table 3; MS (GC/MS silylated): M^+-1=496 (M^+-H), 482 (M^+-15, CH_3), 468 (M^+-29, CH_2CH_3), 440 (M^+-57, CH_2CH_2-CH_3), 425 (M^+-72, $CH_2CH_2CH_3/CH_3$), 365 (M^+-132, $CH_2CH_2CH_3/5\times CH_3$), 307 ($M^+-190$, $HN(CH_2)_5CH_3/6\times CH_3$), high-resolution mass analysis 280.1543.

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