Enzymatic cyclizations using laccases: Multiple bond formation between dihydroxybenzoic acid derivatives and aromatic amines†

Veronika Hahn,*[a] Timo Davids,[a] Michael Lalk,[b] Frieder Schauer[a] and Annett Mikolasch[a]

Received 28th September 2009, Accepted 3rd February 2010
First published as an Advance Article on the web 8th March 2010
DOI: 10.1039/b920081a

Oxidative C–N bond formation followed by cyclization of dihydroxybenzoic acid derivatives with aromatic and heteroaromatic amines was catalyzed in the presence of oxygen by laccases [E.C. 1.10.3.2] from the white rot fungi *Pycnoporus cinnabarinus* and *Myceliophthora thermophila*. The laccase-catalyzed formation of cycloheptenes, cyclooctenes, diazaspiro cyclohexenes, and phenazines was investigated for the first time with regard to the ring size and substituents of the aromatic amines as well as to the substitution patterns of the substrates. Differences to C–N bond formation without cyclization are discussed.

Introduction

Enzyme-catalyzed oxidations using atmospheric oxygen as a non-toxic oxidant are of considerable general interest. Laccases (E.C. 1.10.3.2, benzenediol:dioxygen oxidoreductase) contain four copper atoms in the catalytic centre which are responsible for the electron transport during substrate oxidation.[1] The spectrum of laccase substrates is broad, ranging from monophenols[2] to anilines[3] to *ortho*- and *para*-dihydroxylated substrates.[4] The oxidation of suitable substrates results in radicals, which can undergo various reactions such as the coupling of two different reaction partners. In the case of phenolic compounds bond formations like C–C,[4b,5] C–O,[6] and C=C[7] or in case of reactions with amines C–N[4c-e,8] or C=N[4c,7,8] may be formed.

Laccase-catalyzed bond formation can be used in green chemistry for the synthesis of many different substances, *e.g.* the formation of benzofurans and benzofuran-1-ones,[9] naphthoquinones,[10] aminobenzoquinones,[4c-e,11] substituted imidazoles,[12] 3-substituted 1,2,4-triazolo[4,3-*b*][4.1.2]benzothiadiazine-8-ones,[13] and phenoxazinone derivatives,[14] as well as the dimerization of salicylic esters[2c] and penicillins.[15]

Thus the exploitation of laccases in organic synthesis is a promising field of application in addition to their use in biobleaching of pulp,[16] in textile dye degradation,[17] as biosensors,[18] and in the food industry.[19] The mild and environmentally friendly reaction conditions, which do not require high temperatures or pressures, are crucial factors for the choice of laccase as biocatalyst, for example in fine-chemical synthesis.[20]

Although the list of laccase-catalyzed reactions is long, the process of amine derivatization has so far been limited to the use of substituted hydroquinones as coupling partners for various aliphatic[21] or especially aromatic amines.[4c–e,11] As a result, most of the laccase-mediated procedures described in the literature result in the formation of mono- or diaminated quinones[4c,e] or quinonimines.[4c,22]

To broaden the range of application of laccases in fine-chemical synthesis, we exploited the laccases of *Pycnoporus cinnabarinus* and *Myceliophthora thermophila* for the synthesis of cycloheptenes, cyclooctenes, diazaspiro cyclohexenes, and phenazines. In the presence of oxygen and laccase, 2,5-dihydroxybenzoic acid derivatives and aromatic amines gave rise to cyclic products with different substitution patterns.

Results

General observations

The amines (**2a–g**) used in this study consist of five- or six-membered rings containing in the aromatic ring either only carbon atoms or, alternatively, one or two nitrogen atoms. Furthermore all amines are substituted with at least one amino group and an additional carboxamide (**2a–d**), amino (**2e,f**), or carboxyl group (**2g**). The different amines were incubated with 2,5-dihydroxybenzoic acid derivatives (**1a–d**) and the laccase from *Pycnoporus cinnabarinus*. The predominant reaction was oxidative C–N bond formation followed by cyclization, resulting in heterocyclic heptenes (**3a–l**), which in a few cases can be converted into diazaspiro products (**5a,b**). Moreover, cyclooctenes (**4a–f**) or phenazines (**6a–e**) were formed during laccase-catalyzed reaction.

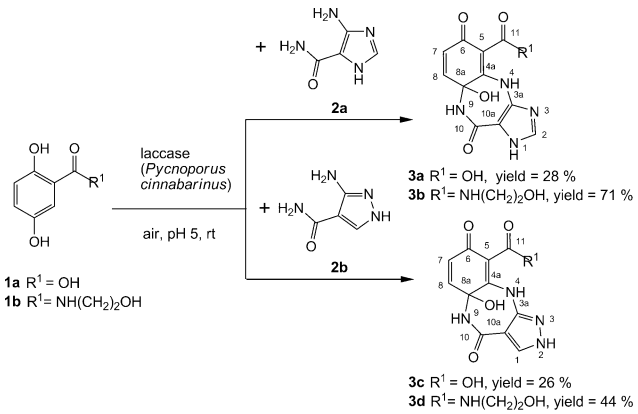
The type of cyclization products recovered was dependent on the ring size, the amination grade of the amine, as well as on the structure of the 2,5-dihydroxybenzoic acid derivative used. The reaction course and product formation were followed using HPLC equipped with a diode array detector. The products were isolated by solid-phase extraction, followed by lyophilization and structural characterization by MS and NMR.

[a]*Institut für Mikrobiologie, Ernst-Moritz-Arndt-Universität Greifswald, Friedrich-Ludwig-Jahn Straße 15, 17487, Greifswald, Germany. E-mail: veronikahahn@gmx.at; Fax: +49 3834 864202; Tel: +49 3834 864229*
[b]*Institut für Pharmazie, Ernst-Moritz-Arndt-Universität, Friedrich-Ludwig-Jahn Straße 17, 17487, Greifswald, Germany*

† Electronic supplementary information (ESI) available: Experimental methods; ^{1}H and ^{13}C NMR spectra; HSQC, HMBC spectra and ^{1}H–^{13}C NMR correlations; retention time, UV-vis, and MS data. See DOI: 10.1039/b920081a

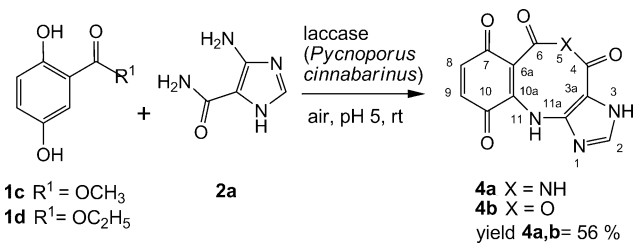
Reaction of five-membered-ring amines with 2,5-dihydroxybenzoic acid derivatives

In the reactions of 2,5-dihydroxybenzoic acid (**1a**) and 2,5-dihydroxy-*N*-(2-hydroxyethyl)benzamide (**1b**) with 4-aminoimidazole-5-carboxamide (**2a**) and 3-aminopyrazole-4-carboxamide (**2b**), the cycloheptenes **3a–d** were readily produced by laccase-mediated bond formation (Scheme 1). All reactions were performed with 0.5 U laccase from *Pycnoporus cinnabarinus* as catalyst in sodium acetate buffer pH 5.0 at room temperature. The yields of the substituted cycloheptenes **3a–d** ranged from 25 to 70%.



Scheme 1 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives **1a,b** and five-membered-ring amines **2a,b** for the synthesis of cycloheptenes **3a–d**. Yields refer to isolated yields.

In the laccase-catalyzed reactions of 2,5-dihydroxybenzoic acid methyl ester (**1c**) and of 2,5-dihydroxybenzoic acid ethyl ester (**1d**) with **2a**, the cyclooctenes **4a,b** were isolated as product mixtures (Scheme 2). The presence of two products was established by mass spectroscopy (**4a** LC/MS *m/z* AP-ESI pos. mode $[M + H]^+$ 259.0513 (calculated 259.0462); **4b** LC/MS *m/z* AP-ESI pos. mode $[M + H]^+$ 260.0368 (calculated 260.0302)). However, these products had identical chromatographic behaviour and identical NMR spectra.



Scheme 2 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives **1c,d** and the five-membered-ring amine **2a** for the synthesis of cyclooctenes **4a,b**. Yields refer to isolated yields.

The reactions of 2,5-dihydroxybenzoic acid methyl ester (**1c**) and of 2,5-dihydroxybenzoic acid ethyl ester (**1d**) with **2b** also result in a product mixture consisting of four cyclooctenes (Fig. 1). Additionally to the quinonoid cyclooctenes **4c,d**, the corresponding hydroquinonoid products **4e,f** were analyzed by NMR and MS.

In addition to the cycloheptenes **3a–d**, the cyclooctenes **4a–f** were formed as by-products in small amounts. For structural

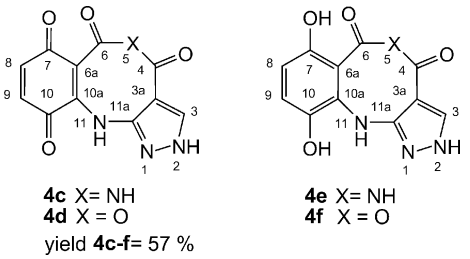


Fig. 1 Cyclooctenes **4c–f**. Yields refer to isolated yields.

characterization of the by-products, we isolated the cyclooctene mixture resulting from **1a** and **2b** and detected the expected products **4c–f**.

In order to study the influence of different laccases on the product pattern or the product yields, the laccase of *Myceliophthora thermophila* in citrate phosphate buffer (CPB) pH 7.0 was used in addition to the laccase of *Pycnoporus cinnabarinus* in sodium acetate buffer (SAB) pH 5.0. Independent of the source of laccase, the same cycloheptenes or cyclooctenes were formed, though the amounts of various products differed over a wide range. In course of the reactions of **1a–d** with **2a,b** the synthesis of the respective major products cycloheptenes or cyclooctenes proceeded very rapidly over the first 20 min and in general reached maximum yield after 2 h (as example, the formation of **3a** and **4a,b** are shown in Fig. 2). The highest product yields were achieved with *P. cinnabarinus* laccase rather than with the enzyme from *M. thermophila*. Reactions using laccase of *P. cinnabarinus* resulted in an 89% increase of product amount for **3a** and a 35% increase for **4a,b** compared with the *M. thermophila* laccase. Interestingly, the *P. cinnabarinus* enzyme leads to a more efficient formation of the cycloheptene **3a** than to the production of cyclooctenes **4a,b**.

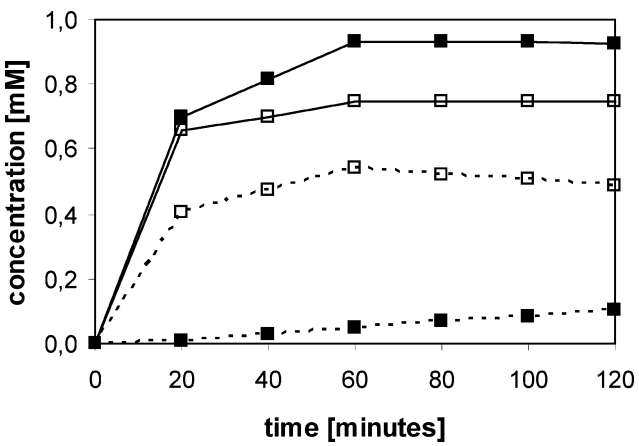


Fig. 2 Reaction course of product formation for **3a** (■) and **4a,b** (□) at equimolar concentrations (1mM) of reactants 2,5-dihydroxybenzoic acid (**1a**) or 2,5-dihydroxybenzoic acid methyl ester (**1c**) and 4-aminoimidazole-5-carboxamide (**2a**) using *Pycnoporus cinnabarinus* (solid lines) laccase in SAB pH 5.0 and *Myceliophthora thermophila* (dashed lines) laccase in CPB pH 7.0.

Moreover, using *P. cinnabarinus* laccase the substrates **1a,c** were consumed within 60 min and the concentration of amine **2a** decreased 89% for the reaction with **1a** and 93% for **1c**.

Homomolecular products of **1a,c** were either not detected or present only in trace amounts, indicating a nearly complete conversion of reactants to the products **3a** and **4a,b**. We attribute the less-than-theoretical yield to the isolation procedure, which was not optimized. To address the question of the influence of the amount of reaction partners on the product pattern or the product yield, some of the reactions were run using different concentrations of the reactants (1 : 1, 1 : 2, 2 : 1 and 2 : 2 mM). In the reaction of **1a** with **2a**, the yield of **3a** was only influenced to a minor degree by the amount of reactants, independent of the laccase used (Fig. 3).

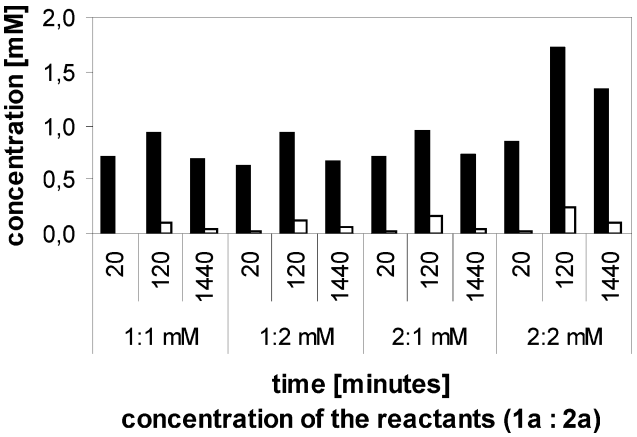
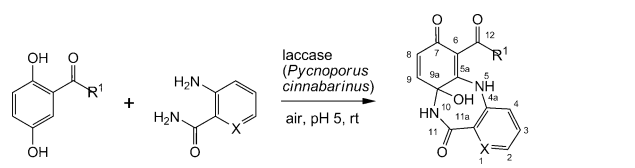


Fig. 3 Reaction courses of product formation for **3a** at different concentrations of reactants 2,5-dihydroxybenzoic acid (**1a**) and 4-aminoimidazole-5-carboxamide (**2a**) using the laccase from *Pycnoporus cinnabarinus* (black columns) and *Myceliophthora thermophila* (white columns).

Increasing the concentration of **1a** or **2a** did not result in an increase either of product yield or of transformation rate. The only positive effect was that with 2 : 2 mM reactants; approximately double the amount (but the same yield) of products was obtained with either laccase. The other reactions of **1** with **2** were also less influenced by the amount of reactants, independent of the used laccase (data not shown).

Reaction of six-membered-ring amines with 2,5-dihydroxybenzoic acid derivatives

The reactions performed with six-membered-ring amines 2-aminobenzoic acid amide (**2c**) and 3-aminopyridine-2-carboxylic acid amide (**2d**) also resulted in the formation of cycloheptenes **3e–h** as the main products (Scheme 3). The product yields depend on both the 2,5-dihydroxybenzoic acid derivative and the amino partner used. The product yield decreased in the following order of substrate **1b** > **1c** > **1d** and amino partner **2c** > **2d**. In the reaction of **1c** or **d** with **2d**, the cycloheptene **3i,j** could not be isolated and was only detected with HPLC/UV-vis and MS.



1b R^1= $NH(CH_2)_2OH$
1c R^1= OCH_3
1d R^1= OC_2H_5

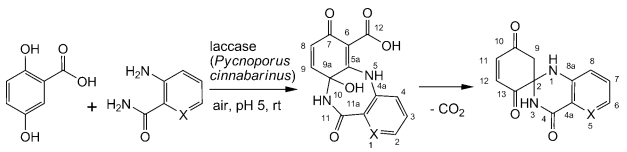
2c X = CH
2d X = N

3e R^1 = $NH(CH_2)_2OH$, X = CH, yield = 38 %
3f R^1 = OCH_3, X = CH, yield = 30 %
3g R^1= OC_2H_5, X = CH, yield = 11 %
3h R^1= $NH(CH_2)_2OH$, X = N, yield = 16 %

Scheme 3 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives **1b–d** and six-membered-ring amines **2c,d** for the synthesis of cycloheptenes **3e–h**. Yields refer to isolated yields.

The product yields were dependent on the laccase used. The yield of **3e** in the reaction of **1b** with **2c** performed with *P. cinnabarinus* laccase was 46% higher than with *M. thermophila* laccase. In the reaction of **1c** with **2c** only 16% of **3f** was formed with *M. thermophila* laccase compared with *P. cinnabarinus* laccase.

In the reaction of **1a** with **2c**, the cycloheptene product **3k** decreased within 24 h and a new product **5a** was formed (Scheme 4). The conversion of the cycloheptene was also observed during stability measurements of the isolated product, indicating that transformation of **3k** to **5a** is also possible during NMR analysis. A similar reaction was observed for **1a** and **2d**, and this was confirmed by HPLC/UV-vis and MS analyses. The diazaspiro structures of products **5a,b** may be attributed to the loss of the carboxyl group.



1a

2c X = CH
2d X = N

3k X = CH
3l X = N

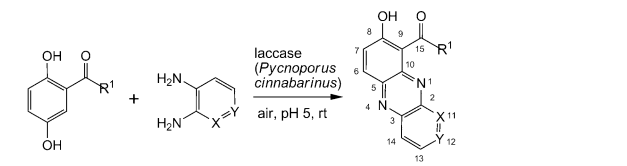
5a X = CH, yield = 27 %
5b X = N, yield = 26 %

Scheme 4 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid **1a** and six-membered-ring amines **2c,d** for the synthesis of diazaspiro cyclohexenes **5a,b**. Yields refer to isolated yields.

In some reactions of **2c** and **2d** cyclooctenes were detected by HPLC/UV-vis as unstable by-products in very low concentrations (data not shown).

All amines **2a–d** have a carboxamide group in the *ortho*-position to the amino group. To determine if reactants with a second amino group or a carboxyl group in the *ortho*-position are also able to react with 2,5-dihydroxybenzoic acid derivatives **1a–d** to form cyclic products, we used diaminopyridines **2e,f** and 2-aminobenzoic acid **2g** as reactants in laccase-catalyzed reactions.

The two neighboring amino groups on the heteroaromatic ring in **2e,f** resulted in the formation of phenazines **6a–e** as cyclization products (Scheme 5).



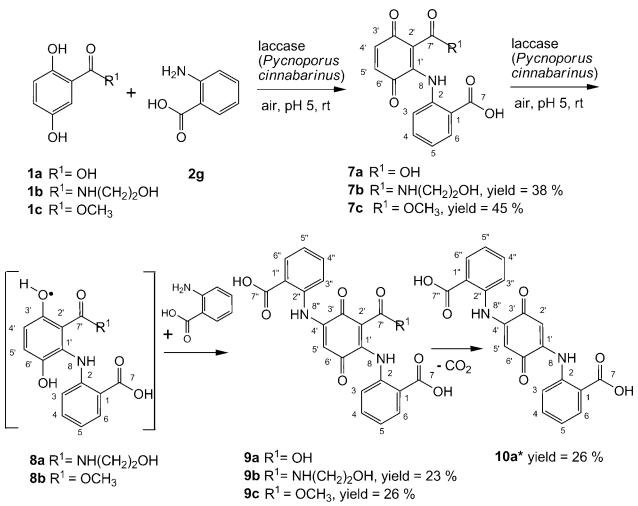
1a R^1= OH
1b R^1= $NH(CH_2)_2OH$
1c R^1= OCH_3
1d R^1= OC_2H_5

2e X = N, Y = CH
2f X = CH, Y = N

6a R^1 = $NH(CH_2)_2OH$, X = N, Y = CH, yield = 48 %
6b R^1 = OCH_3, X = N, Y = CH, yield = 25 %
6c R^1= OC_2H_5, X = N, Y = CH, yield = 40 %
6d R^1= OH, X = CH, Y = N, yield = 20 %
6e R^1= OCH_3, X = CH, Y = N, yield = 27 %

Scheme 5 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives **1a–d** and six-membered-ring diamines **2e,f** for the synthesis of phenazines **6a–e**. Yields refer to isolated yields.

Though all of these laccase-catalyzed reactions with reactants **2a–f** yielded cyclic products, we were unable to detect cyclization between 2-aminobenzoic acid **2g** and 2,5-dihydroxybenzoic acid derivatives **1a–c**. In contrast to cyclization, we obtained in reactions of **2g** mono- (**7a–c**) and diaminated (**9a–c**) products, as well as reactive radical intermediates (**8a,b**) (Scheme 6). The monoaminated products and the reactive radical intermediates were isolated as mixtures of the quinonoid form and the hydroquinonoid radical, which have not so far been described elsewhere. The reactive radical intermediates were detected in NMR spectra as by-products, which were present in low amounts. From the reaction mixture of **1a** with **2g** the monoaminated product **7a** and the simple diaminated product **9a** were only detected as intermediates (**7a** by HPLC/UV-vis and MS, **9a** as a by-product of **10a** in MS and NMR analyses). The final product of the reaction of **1a** with **2g** is the decarboxylated diaminated product **10a**.



Scheme 6 Laccase-catalyzed reaction of 2,5-dihydroxybenzoic acid derivatives **1a–d** and 2-aminobenzoic acid **2g**. Yields refer to isolated yields. * **10a** can only be obtained from **1a**.

Detailed structural characterization of 3a, 4a,b, and 5a

The different types of laccase-synthesized products have different characteristic UV-vis spectra. The cycloheptenes were mostly coloured yellow to brown and the cyclooctenes red violet to black. Product **3a** showed two absorption maxima under 240 nm and one around 380 nm. MS measurement with AP-ESI in both positive and negative modes showed the molecular mass of **3a** to be 276. This mass can be attributed to an amination of **2a** on the quinonoid form of **1a** and a cyclization *via* the carboxylamide group of **2a** on the carbonyl group (C-8a) of the quinone, resulting in a seven-membered non-aromatic ring. ^{1}H NMR spectral data of **3a** showed characteristic signals for both reactants (Table 1). Multiplicity of H-7 and H-8 suggests the first amination step takes place at C-4a. The chemical shift of the proton at N-4 was strongly shifted to lower field due to formation of a hydrogen bond with the amidic oxygen. The HMBC correlations (Fig. 4A) of the proton H-4 unambiguously fixed the first amination at C-4a. Signals for the phenolic hydroxy group at 7.46 ppm and for the amidic amine group at 9.19 ppm were observed. Both signals showed HMBC correlations to C-4a (162.7 ppm), C-8 (142.7 ppm), and C-8a (73.8 ppm), supporting the concept of a cyclization step of the amidic amine group at C-8a and the removal of the *para*-quinonoid character. Additionally, ^{13}C NMR showed only one typical signal for quinones in the region of 180 ppm, indicating only one quinonoid carbonyl group for **3a**. The HMBC spectrum also showed correlations between the proton H-7 and C-8a and the proton H-8 and the quinonoid carbonyl carbon and the C-4a, unambiguously showing **3a** to be the aminated and cyclized product 8a-hydroxy-6,10-dioxo-1,4,6,8a,9,10-hexahydro-1,3,4,9-tetraazabenzo[*f*]azulene-5-carboxylic acid.

The products **4a,b** could only be isolated as a mixture, and this mixture has three absorption maxima under 400 nm and a weak maximum around 500 nm. MS with AP-ESI in positive mode showed two different mass peaks. The molecular mass of 259 was attributed to an amination of **2a** on the quinonoid form of **1c** and a cyclization *via* the amino group of the carboxylamide group of **2a** with the side chain of **1c** resulting in **4a**. The molecular mass of 260 was also attributed to an amination of **2a** on the quinonoid form of **1c** and a cyclization *via* the carboxylamide group of **2a** on the side chain of **1c**, resulting in an ester bond formation of **4b**. Each of these products contains an eight-membered non-aromatic ring due to the cyclization. The postulated product structures were confirmed by NMR. ^{1}H NMR spectral data of **4** showed characteristic signals for both reactants (Table 1). Multiplicity of H-8 and H-9 suggests that the first amination step takes place at C-10a. The HMBC spectrum showed correlations between the protons H-8 and H-9 and the quinone carbonyl carbons in the typical range of 180 ppm, unambiguously showing **4a,b** to have quinonoid structures substituted at C-10a. Based on the NMR data we are not able to distinguish between **4a** and **4b**. ^{13}C NMR data of C-4 (163.8 ppm) and C-6 (153.1 ppm) are both in the ranges of anhydride and of imide carbon atoms.[23] All results together – MS and NMR – led to the identification of **4a,b** as 3,11-dihydro-1,3,5,11-tetraazabenzo[*a*]cyclopenta[*d*]cyclooctene-4,6,7,10-tetraone and 3,11-dihydro-5-oxa-1,3,11-triazabenzo[*a*]-cyclopenta[*d*]cyclooctene-4,6,7,10-tetraone respectively.

Product **5a** showed one absorption maximum at 213 nm and a weak maximum under 400 nm. MS measurements with AP-ESI in both positive and negative modes showed the molecular mass of **5a** to be 242, indicating the coupling of both reactants **1a** and **2c** and a decarboxylation. ^{1}H NMR spectral data of **5a** showed the presence of two aliphatic protons (Table 1). ^{13}C NMR, HSQC, and HMBC indicated that these aliphatic protons are the C-9 methylene protons. This methylene group could be formed by decarboxylation coupled with dearomatization. Additionally, ^{13}C NMR showed two typical signals for the aliphatic carbonyl carbons C-10 (194.0 ppm) and C-13 (191.6 ppm). ^{1}H–^{13}C correlations (Fig. 4C) of H-11 and H-12 unambiguously showed the decarboxylation and dearomatization of **1a**. The multiplicities and ^{1}H–^{13}C correlations of H-11 and H-12 suggest that the amination has taken place at C-2. The HMBC correlations of the protons H-1 and H-3 showed multiple cross-signals with C-2, C-4a and C-9, demonstrating the two N–C bond formations at C-2. The analyses confirmed the diazaspiro character of the product **5a** as 1′*H*,2*H*,3′*H*,5*H*-spiro[cyclohex-3-ene-1,2′-quinazoline]-2,4′,5-trione.

Table 1 ^{1}H and ^{13}C assignments and HMBC correlations for **3a**, **4a,b**, and **5a**[a]

	^{13}C	^{1}H	^{1}H–^{13}C correlations
3a			
C-2	137.3	7.88, s, 1H	C-3a (139.5), C-10 (158.3)[b], C-10a (114.9)
C-7	124.7	6.36, d, 1H, $J = 10.1$	C-4a (162.7)[c], C-5 (93.2), C-6 (185.5)[c], C-8a (73.8), C-11 (170.6)[b]
C-8	142.7	6.79, d, 1H, $J = 10.1$	C-4a (162.7), C-5 (93.2)[b], C-6 (185.5), C-7 (124.7)[c], C-8a (73.8)[c]
NH (N-1)		13.43, s, 1H	—
NH (N-4)		13.78, s, 1H	C-4a (162.7)[b], C-5 (93.2), C-8a (73.8), C-10a (114.9)
NH (N-9)		9.19, s, 1H	C-4a (162.7), C-8 (142.7), C-8a (73.8), C-10 (158.3), C-10a (114.9)
OH (at C-8a)		7.46, s, 1H	C-4a (162.7), C-8 (142.7), C-8a (73.8)
OH (at C-11)		15.08, s, 1H	C-5 (93.2), C-11 (170.6)
4a,b			
C-2	126.4	8.26, s, 1H	C-3a (124.5), C-11a (139.8)
C-8	142.6	6.77, d, 1H, $J = 10.3$	C-6 (153.1)[b], C-6a (100.2), C-9 (134.2)[b], C-10 (184.8)
C-9	134.2	6.84, d, 1H, $J = 10.3$	C-7 (180.8), C-8 (142.6)[b], C-10a (148.8)
NH		7.31, s, 1H	C-3a (124.5)[b]
NH		7.84, s, 1H	—
5a			
C-5	126.8	7.59, m, 1H, $J = 7.7, 1.4$	C-4 (164.0), C-4a (114.4)[c], C-7 (133.3), C-8a (144.7)
C-6	117.9	6.70, m, 1H, $J = 7.8, 7.2, 0.9$	C-4 (164.0)[c], C-4a (114.4), C-5 (126.8)[b], C-7 (133.3)[b], C-8 (114.0), C8a (144.7)[b]
C-7	133.3	7.23, m, 1H, $J = 7.9, 7.3, 1.6$	C-5 (126.8), C-6 (117.9)[c], C-8a (144.7), C-8 (114.0)[b]
C-8	114.0	6.66, d, 1H, $J = 7.8$	C-4 (164.0))[b], C-4a (114.4), C-5 (126.8)[c], C-6 (117.9), C-8a (144.7)[c]
C-9	48.5	3.08, d, 1H, $J = 17.1, 0.9$	C-2 (70.7), C-10 (194.0), C-11 (141.4)[b], C-13 (191.6)
		3.36, d, 1H, $J = 17.1$	C-2 (70.7), C-8a (144.7)[b], C-10 (194.0), C-13 (191.6)[b]
C-11	141.4	6.85, d, 1H, $J = 10.3, 0.9$	C-6 (117.9)[c], C-9 (48.5)[b], C-13 (191.6), C-10 (194.0)[c]
C-12	137.7	6.88, d, 1H, $J = 10.3$	C-2 (70.7), C-10 (194.0), C-11 (141.4)[c], C-13 (191.6)[c]
NH (N-1)		7.64, s(b), 1H	C-2 (70.7), C-4a (114.4), C-9 (48.5)[b]
NH (N-3)		8.14, s(b), 1H	C-2 (70.7), C-4a (114.4), C-4 (164.0), C-9 (48.5)[b], C-5 (126.8)[c]

[a] Chemical shifts are expressed in δ (ppm) calibrated on the resonances of the residual nondeuterated solvent DMSO. J values are in Hz [b] Signals with low intensity. [c] Signals with very low intensity.

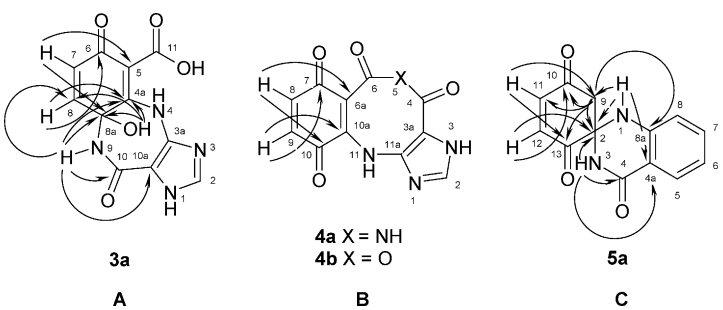
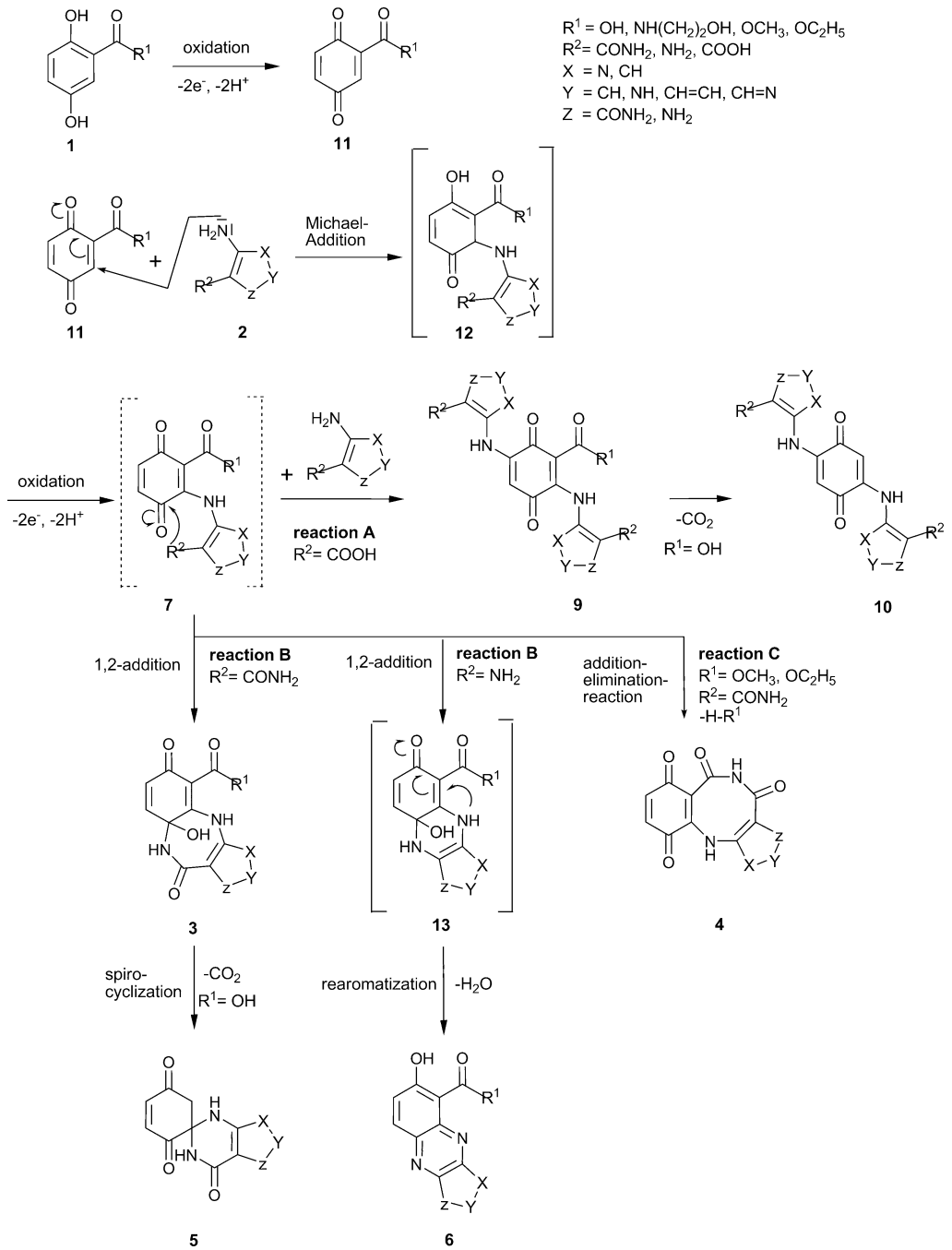


Fig. 4 Product numbering and important HMBC correlations (H → C) of (A) **3a**, (B) **4a,b**, (C) **5a**.

Discussion

The structures of the products synthesized with the aid of laccases were defined by MS, NMR and UV-vis spectral analyses. The course of reaction (whether cyclization or not) was dependent on the number of amino groups of the amino partners **2a–g**. The path of cyclization of aminated products is determined by the ring size and substituents of the aromatic or heteroaromatic amino partner, by the substitution of the used laccase substrate as well as the reaction conditions such as the particular laccase used and buffer pH.

The synthesis of cyclic products can be described as a regioselective domino reaction (Scheme 7). The first step is the laccase-catalyzed oxidation of the respective 2,5-dihydroxybenzoic acid derivative with oxygen, resulting in quinonoid derivatives that then undergo an amination by intermolecular Michael addition (1,4-addition). The amination is affected by the amino group linked directly to the aromatic ring, as in **2a–g**. The position of amination is *ortho* to the COR-group of **1a–d** as described for other aminations.[4c,11a,c,g] After a second oxidation the monoaminated products **7**, which were isolated for $R^2 = COOH$ but only postulated for $R^2 = CONH_2$ or NH_2, are formed. Products **7** with $R^2 = COOH$ undergo a second amination (reaction A, Scheme 7) *para* to the first one, resulting in the formation of products **9**, which, in case of $R^1 = OH$, were decarboxylated to **10**. The analyzed hydroquinonoid radicals **8** as intermediates are described for the first time for laccase-mediated amination, though the formation of radicals in the course of laccase-catalyzed substrate oxidation has long been known.[1b,7,24] The radicals described in our study could be formed during the



Scheme 7 Possible reaction mechanism.

second laccase-catalyzed oxidation before binding the second amine molecule. The chemical synthesis of trimers like **9** has been described in the past.[4c,25]

Starting from **7**, different reaction courses for cyclization (reactions B and C, Scheme 7) are possible depending on the ring size (X, Y, Z) and amino substituent R^2 of the amino partner **2** as well as on the substituent R^1 of the laccase substrate **1**. In case of R^1 = OH or $NH(CH_2)_2OH$ and $R^2 = CONH_2$ the reaction step is an intramolecular 1,2-addition of the R^2-nitrogen to the carbonyl carbon C-6′ of **7**, forming stable cycloheptenes **3**. The cycloheptene formation is dominant in the reactions of five-membered **2** as well as for six-membered **2** with $R^2 = CONH_2$, and this is independent of the laccase substrate used. In the case of R^1 = OH the cycloheptenes **3** are not final products of the reaction. Compound **3** undergoes a decarboxylation coupled with a migration of amide nitrogen from C-9a to C-5a, resulting in **5**. The formation of diazaspiro products was described for chemical spirocyclization *e.g.* by Kuckländer *et al.*[26]

In contrast to these products **3** and **5**, the intramolecular 1,2-addition is only the first reaction step in the case of **7** with $R^2 = NH_2$, **13** being hypothetical reactive intermediates. These reactive species can undergo rapid dehydratization coupled with rearomatization resulting in the isolated phenazines **6**. The chemical reaction of 2,3-dibromo-1,4-naphthoquinone with

1,2-diaminobenzole[27] proceeded in a similar way to our laccase-catalyzed reactions with **2e** and **2f**. The authors also noted that the monoaminated product formed reacted further by attacking the carbonyl group of the quinone to yield an angular 5-bromo-6-hydroxy-benzo[*a*]phenazine. A similar product was described in the reaction of *ortho*-phenylenediamine with 2-hydroxy-1,4-naphthoquinone. The formation of an *ortho*-quinone was postulated as an intermediate which further reacts with the diamine resulting in benzo[*a*]phenazin-5-ol.[28] The chemical synthesis of phenazines by amination and substitution of one hydroxyl group has been described by Fandy *et al.*[29]

Furthermore, **7** gives mainly **4** in the case of $R^1 = OCH_3$ or OC_2H_5 and $R^2 = CONH_2$ and X = N, Y = CH, Z = NH. The ester groups of **1** undergo an addition–elimination reaction by the R^2 amino group and a loss of R^1 (reaction C, Scheme 7). The more reactive ester groups of **1c,d** can react with the amino group of $R^2 = CONH_2$ to produce a less reactive imide group, whereas the less reactive substituted amide group of **1b** is more stable. The hydroxy proton of the carboxyl group of **1a** is acidic, so that both are involved in an acid–base-reaction than in amide formation, and this may explain why we could not detect products **4** from **1a**. Surprisingly, we also found acid anhydrides **4b,d,f** in MS analyses in addition to the **4a,c,e** products. Additionally, it is not possible to distinguish between anhydride and imide structures by NMR. Because of this, we can only postulate the structures from the reaction mechanisms. The reaction of the more reactive ester groups of **1c,d** can only react with the amino group of $R^2 = CONH_2$ to an imide group by loss of alcohol. The reaction of these ester groups with the amino group to acid anhydrides has not been described so far, so we propose that acid anhydrides as well as the hydroquinones from the quinones[11b] are generated during MS analyses.

Whereas the five-membered amino partners **2a,b** react to **3** and/or **4** depending on the substituent R^1 of **1**, the six-membered amino partners **2c,d** prefer to form **3**, and react further to give **5** only in the case of $R^1 = OH$.

Furthermore, cycloheptene and cyclooctene formation was more effective with *P. cinnabarinus* laccase than with *M. thermophila* laccase, contrary to the di- and trimer formation in reactions of 1,4-hydroquinones with amino acids described previously by Hahn *et al.*[4e] Although it is known that the pH value of the buffer may be important especially for the quinone formation, it is more important for high product yield to use the laccase in a reaction medium in which the highest possible activity of the enzyme can be achieved.[4c]

Furthermore, despite the utilization of an oxidative enzyme and oxygen hydroquinonoide, products **4** were formed. Although aminohydroquinones are very susceptible to oxidation[26] these products were part of a mixture of four different products. This is one example of the unusual reaction course and reaction products described in this study. In particular, the formation of the cyclooctenes and cycloheptenes as well as the diazaspiro products is unique for enzyme- and non-enzyme-catalyzed reactions of amines containing a neighboring amino and carboxylamide group with 2,5-dihydroxybenzoic acid derivatives.

The cyclization products described here could be the basis not only for the production of fine chemicals but also for the synthesis of new pharmaceuticals. Cycloheptenes can be regarded as diazepines, and a famous representative of this group is diazepam, which is used as an anti-convulsant and anti-anxiety agent.[30] Additionally, diazepines have antitumor,[31] antimicrobial and anthelmintic activities,[32] as well as anti-HIV activity.[33] Furthermore, cyclooctenes are regarded as diazocines or oxazocines and possess (for example) anti-inflammatory properties.[34] In this context the novel synthetized compounds may enlarge the spectrum of medically important substances. Further studies are ongoing to test the synthesized products for their biological activity.

Conclusion

In this study we have described a new method for the synthesis of cycloheptenes, cyclooctenes, diazaspiro cyclohexenes, and phenazines catalyzed by laccase as a one-pot reaction sequence. The potential of laccases in organic chemistry is thus broadened towards the formation of cyclic compounds, and constitutes an additional tool for organic chemists, for example in fine chemical synthesis. Furthermore, the synthesis of substances that are not accessible by standard procedures, and the use of environmentally friendly reaction conditions, enable laccase-catalyzed processes to fill the gaps in classical organic chemistry.

Experimental

Enzymes

The used laccase was obtained from *Pycnoporus cinnabarinus* SBUG-M 1044. The 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), from where it can be obtained.

Cultivation of *Pycnoporus cinnabarinus* SBUG-M 1044 and crude preparation of laccase was carried out as we reported previously.[35] This enzyme preparation contains only 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.[35,36]

Laccase from *Myceliophthora thermophila* (expressed in genetically modified *Aspergillus* sp.) was obtained from Novozymes (Bagsvaerd, Denmark). It was used as received (activity 1000 U g^{-1}; substrate: syringaldazine) in citrate phosphate buffer (CPB, 18 mM citrate, 165 mM phosphate) at its pH optimum of pH 7.0.[36b,37]

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[38] using the method described by Jonas *et al.*[35] 1 U is defined as 1 μmol mL^{-1} min^{-1}.

Experimental procedures

Analytical procedure. For analytical experiments amines (1 mM, 2 mM) and the respective dihydroxylated compound (1 mM, 2 mM) were incubated with laccase (activity 0.5 U). Reaction mixtures were incubated with agitation at 200 rpm at room temperature in the dark. The reaction mixtures were analyzed by HPLC. The separation of the substances was

achieved by RP18 column at a flow rate of 1 mL min^{-1}. 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.

Product isolation. All reaction mixtures for product isolation were performed with laccase of *Pycnoporus cinnabarinus* (final activity 0.5 U) in SAB. Isolation steps were performed by solid-phase extraction with a RP18 silica gel column (60 mL, 10 g adsorbent material, Phenomenex, Strata, Germany). Product **3a** was isolated from the reaction mixture (340 mL) after an incubation period of 2 h (**1a**:**2a**, 2 : 2 mM assay). After charging the column with 10 mL reaction mixture, 100 mL of methanol–distilled water (10 : 90 v/v) was used to remove undesired impurities. Elution of the yellow–green fraction was performed with 30 mL of methanol–distilled water (40 : 60 v/v). Product **4a,b** was isolated from reaction mixture (460 mL) after an incubation period of 24 h (**1c**:**2a**, 2 : 2 mM assay). After charging the column with 20 mL reaction mixture and washing steps with 80 mL of methanol–distilled water (5 : 95 v/v) and 10 mL of methanol–distilled water (50 : 50 v/v), the product was eluted with 10 mL of methanol–distilled water (50 : 50 v/v). **5a** was a product resulting from conversion of **3k**. Product **3k** was isolated from reaction mixture (400 mL) after an incubation period of 20 min (**1a**:**2c**, 1 : 1 mM assay). After charging the column with 40 mL reaction mixture and washing steps with 50 mL of distilled water and 20 mL of methanol–distilled water (10 : 90 v/v), the product was eluted with 30 mL of methanol–distilled water (30 : 70 v/v).

For mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy, the isolated products were dried by lyophilization.

The products were characterized by MS using electrospray ionization under atmospheric conditions (API-ES) (drying and nebulizing gas: nitrogen) on a Bruker-Daltoniks micrOTOF instrument (Bremen, Germany; software: HyStar). The NMR spectra were recorded on a Bruker Avance 600 instrument (Rheinstetten, Germany) at 600 MHz. The solvent used was DMSO-d_6. Chemical shifts are expressed in δ (ppm) calibrated on the resonances of the residual nondeuterated solvent. *J* values are given in Hz.

8a-Hydroxy-6,10-dioxo-1,4,6,8a,9,10-hexahydro-1,3,4,9-tetraazabenzo[*f*']azulene-5-carboxylic acid (3a)

Synthesis and isolation as described above. Yellow–green solid. Yield 27.82% (51.9 mg): mp (decomposition) 168–170 °C. ^{1}H NMR: δ 6.36 (d, J = 10.1, 1H, H-7), 6.79 (d, J = 10.1, 1H, H-8), 7.46 (s, 1H, OH, on C-8a), 7.88 (s, 1H, H-2), 9.19 (s, 1H, NH, H-9), 13.43 (s, 1H, NH, H-1), 13.78 (s, 1H, NH, H-4), 15.08 (s, 1H, OH, on C-11). ^{13}C NMR: δ 73.8 (C-8a), 93.2 (C-5), 114.9 (C-10a), 124.7 (C-7), 137.3 (C-2), 139.5 (C-3a), 142.7 (C-8), 158.3 (C-10), 162.7 (C-4a), 170.6 (C-11), 185.5 (C-6). HMBC correlations see Table 1. R_f (HPLC) 6.1 min, UV-vis (MeOH) λ_{max} 204, 234, 387 nm. MS *m/z* AP-ESI: pos. ion mode $[M + H]^+$ 277.0610 (calculated 277.0567), $[2M + H]^+$ 553.1167 (calculated 553.1062); AP-ESI: neg. ion mode $[M - H]^-$ 275.0414 (calculated 275.0422), $[2M - H]^-$ 551.0892 (calculated 551.0917), $[3M - H]^-$ 827.1346 (calculated 827.1411).

Product mixture: 3,11-Dihydro-1,3,5,11-tetraazabenzo-[*a*]cyclopenta[*d*]cyclooctene-4,6,7,10-tetraone (4a), 3,11-dihydro-5-oxa-1,3,11-triazabenzo[*a*]cyclopenta[*d*]cyclooctene-4,6,7,10-tetraone (4b)

Synthesis and isolation as described above. Dark violet solid. Yield 47.70% (90.6 mg): mp (decomposition) 149–153 °C. ^{1}H NMR: δ 6.78 (d, J = 9.7, 1H, H-8), 6.85 (d, J = 9.7, 1H, H-9), 7.32 (s, 1H, NH), 7.83 (s, 1H, NH), 8.26 (s, 1H, H-2). ^{13}C NMR: δ 100.2 (C-6a), 125.0 (C-3a), 126.3 (C-2), 134.1 (C-9), 140.0 (C-11a), 142.5 (C-8), 149.1 (C-10a), 153.8 (C-6), 164.2 (C-4), 181.0 (C-7), 185.1 (C-10). HMBC correlations see Table 1. R_f (HPLC) 6.1 min, UV-vis (MeOH) λ_{max} 246, 265, 323, 503 nm. (**4a**) MS *m/z* AP-ESI: pos. mode $[M + H]^+$ 259.0513 (calculated 259.0462). (**4b**) MS *m/z* AP-ESI: pos. mode $[M + H]^+$ 260.0368 (calculated 260.0302).

9a-Hydroxy-7,11-dioxo-7,9a,10,11-tetrahydro-5*H*-dibenzo[*b*,*e*][1,4]diazepine-6-carboxylic acid (3k)

Synthesis and isolation as described above. Yellow solid. Yield 26.99% (31.3 mg): mp (decomposition) 130–134 °C, mp 156–160 °C. R_f (HPLC) 7.6 min, UV-vis (MeOH) λ_{max} 213, 366 nm. MS *m/z* AP-ESI: neg. mode $[M - H]^-$ 285.0499 (calculated 285.0517).

1'*H*,2*H*,3'*H*,5*H*-Spiro[cyclohex-3-ene-1,2'-quinazoline]-2,4',5-trione (5a)

^{1}H NMR: δ 3.08 (d, J = 17.1, J = 0.9, 1H, H-9), 3.36 (d, 1H, H-9), 6.66 (d, J = 7.8, 1H, H-8), 6.70 (m, J = 7.8, J = 7.2, J = 0.9, 1H, H-6), 6.85 (m, J = 10.3, J = 0.9, J = 0.7, 1H, H-11), 6.88 (d, J = 10.3, 1H, H-12), 7.23 (m, J = 7.9, J = 7.3, J = 1.6, 1H, H-7), 7.59 (m, J = 7.7, J = 1.4, 1H, H-5) 7.64 (s (br), 1H, NH, H-1), 8.14 (s (br), 1H, NH, H-3). ^{13}C NMR: δ 48.5 (C-9), 70.7 (C-2), 114.0 (C-8), 114.4 (C-4a), 117.9 (C-6), 126.8 (C-5), 133.3 (C-7), 137.7 (C-12), 141.4 (C-11), 144.7 (C-8a), 164.0 (C-4), 191.6 (C-13), 194.0 (C-10). HMBC correlations see Table 1. R_f (HPLC) 5.9 min, UV-vis (MeOH) λ_{max} 213, 366 nm. MS *m/z* AP-ESI: neg. mode $[M - H]^-$ 241.0614 (calculated 241.0619). AP-ESI: pos. mode $[M + H]^+$ 243.0734 (calculated 243.0764).

Acknowledgements

Financial support by means of a scholarship from the government of Mecklenburg-Vorpommern is gratefully acknowledged. We thank R. Jack (Institute of Immunology, University of Greifswald) for help in preparing the manuscript.

Notes and references

1 (*a*) C. F. Thurston, *Microbiology*, 1994, **140**, 19; (*b*) E. I. Solomon, P. Chen, M. Metz, S. K. Lee and A. E. Palmer, *Angew. Chem., Int. Ed.*, 2001, **40**, 4570; (*c*) K. Piontek, M. Antorini and T. Choinowski, *J. Biol. Chem.*, 2002, **277**, 37663; (*d*) H. Claus, *Arch. Microbiol.*, 2003, **179**, 145; (*e*) E. I. Solomon, A. J. Augustine and J. Yoon, *Dalton Trans.*, 2008, 3921.

2 (*a*) J.-M. Bollag, K. L. Shuttleworth and D. H. Anderson, *Appl. Environ. Microbiol.*, 1988, **54**, 3086; (*b*) J.-M. Bollag, H.-L. Chu, M. A. Rao and L. Gianfreda, *J. Environ. Qual.*, 2003, **32**, 63; (*c*) S. Ciecholewski, E. Hammer, K. Manda, G. Bose, V. T. H. Nguyen, P. Langer and F. Schauer, *Tetrahedron*, 2005, **61**, 4615; (*d*) L. Gianfreda, G. Iamarino, R. Scelza and M. A. Rao, *Biocatal. Biotransform.*, 2006,

24, 177; (*e*) M. A. Tadesse, A. D'Annibale, C. Galli, P. Gentili and F. Sergi, *Org. Biomol. Chem.*, 2008, **6**, 868.
3 (*a*) T. Hoff, S. Y. Liu and J.-M. Bollag, *Appl. Environ. Microbiol.*, 1985, **49**, 1040; (*b*) T. Bertrand, C. Jolivalt, P. Briozzo, E. Caminade, N. Joly, C. Madzak and C. Mougin, *Biochemistry*, 2002, **41**, 7325; (*c*) A. Kollmann, F. D. Boyer, P. H. Ducrot, L. Kerhoas, C. Jolivalt, I. Touton, J. Einhorn and C. Mougin, *Appl. Microbiol. Biotechnol.*, 2005, **68**, 251.
4 (*a*) G. Benfield, S. M. Bocks, K. Bromley and B. R. Brown, *Phytochemistry*, 1964, **3**, 79; (*b*) I. O. Anyanwutaku, R. J. Petroski and J. P. N. Rosazza, *Bioorg. Med. Chem.*, 1994, **2**, 543; (*c*) T. H. J. Niedermeyer, A. Mikolasch and M. Lalk, *J. Org. Chem.*, 2005, **70**, 2002; (*d*) A. Mikolasch, M. Wurster, M. Lalk, S. Witt, S. Seefeldt, E. Hammer, F. Schauer, W.-D. Jülich and U. Lindequist, *Chem. Pharm. Bull.*, 2008, **56**, 902; (*e*) V. Hahn, A. Mikolasch, K. Manda, D. Gördes, K. Thurow and F. Schauer, *Amino Acids*, 2009, **37**, 315.
5 J.-M. Bollag, S.-Y. Liu and R. D. Minard, *Appl. Environ. Microbiol.*, 1979, **38**, 90.
6 J.-M. Bollag and S.-Y. Liu, *Pestic. Biochem. Physiol.*, 1985, **23**, 261.
7 K. E. Simmons, R. D. Minard and J.-M. Bollag, *Environ. Sci. Technol.*, 1989, **23**, 115.
8 K. Tatsumi, S. Wada, H. Ichikawa, S. Y. Liu and J.-M. Bollag, *Water Sci. Technol.*, 1992, **26**, 2157.
9 (*a*) S. Witayakran, L. Geldbaum and A. J. Ragauskas, *Tetrahedron*, 2007, **63**, 10958; (*b*) S. Witayakran and A. J. Ragauskas, *Eur. J. Org. Chem.*, 2009, 358; (*c*) H. Leutbecher, J. Conrad, I. Klaiber and U. Beifuss, *Synlett*, 2005, 3126; (*d*) S. Hajdok, H. Leutbecher, G. Greiner, J. Conrad and U. Beifuss, *Tetrahedron Lett.*, 2007, **48**, 5073.
10 (*a*) S. Witayakran and A. J. Ragauskas, *Green Chem.*, 2007, **9**, 475; (*b*) S. Witayakran, A. Zettili and A. J. Ragauskas, *Tetrahedron Lett.*, 2007, **48**, 2983.
11 (*a*) K. Manda, E. Hammer, A. Mikolasch, T. Niedermeyer, J. Dec, A. D. Jones, A. J. Benesi, F. Schauer and J.-M. Bollag, *J. Mol. Catal. B: Enzym.*, 2005, **35**, 86; (*b*) K. Manda, E. Hammer, A. Mikolasch, D. Gördes, K. Thurow and F. Schauer, *Amino Acids*, 2006, **31**, 409; (*c*) A. Mikolasch, T. H. J. Niedermeyer, M. Lalk, S. Witt, S. Seefeldt, E. Hammer, F. Schauer, M. Gesell, S. Hessel, W.-D. Jülich and U. Lindequist, *Chem. Pharm. Bull.*, 2006, **54**, 632; (*d*) A. Mikolasch, T. H. J. Niedermeyer, M. Lalk, S. Witt, S. Seefeldt, E. Hammer, F. Schauer, M. Gesell-Salazar, S. Hessel, W.-D. Jülich and U. Lindequist, *Chem. Pharm. Bull.*, 2007, **55**, 412; (*e*) A. Mikolasch, S. Hessel, M. Gesell-Salazar, H. Neumann, K. Manda, D. Gördes, E. Schmidt, K. Thurow, E. Hammer, U. Lindequist, M. Beller and F. Schauer, *Chem. Pharm. Bull.*, 2008, **56**, 781; (*f*) A. Mikolasch, A. Matthies, M. Lalk and F. Schauer, *Appl. Microbiol. Biotechnol.*, 2008, **80**, 389; (*g*) V. Hahn, A. Mikolasch, K. Manda, D. Gördes, K. Thurow and F. Schauer, *J. Mol. Catal. B: Enzym.*, 2009, **60**, 76.
12 A. Schäfer, M. Specht, A. Hetzheim, W. Francke and F. Schauer, *Tetrahedron*, 2001, **57**, 7693.
13 U. T. Bhalerao, C. Muralikrishna and B. R. Rani, *Tetrahedron*, 1994, **50**, 4019.
14 (*a*) C. Eggert, U. Temp, J. F. D. Dean and K. E. L. Eriksson, *FEBS Lett.*, 1995, **376**, 202; (*b*) J. Osiadacz, A. J. H. Al-Adhami, D. Bajraszewska, P. Fischer and W. Peczynska-Czoch, *J. Biotechnol.*, 1999, **72**, 141; (*c*) F. Bruyneel, E. Enaud, L. Billottet, S. Vanhulle and J. Marchand-Brynaert, *Eur. J. Org. Chem.*, 2008, **1**, 70.
15 H. Agematu, T. Tsuchida, K. Kominato, N. Shibamoto, T. Yoshioka, H. Nishida, R. Okamoto, T. Shin and S. Murao, *J. Antibiot.*, 1993, **46**, 141.
16 (*a*) M. Balakshin, C.-L. Chen, J. S. Gratzl, A. G. Kirkman and H. Jakob, *J. Mol. Catal. B: Enzym.*, 2001, **16**, 205–215; (*b*) P. Widsten and A. Kandelbauer, *Enzyme Microb. Technol.*, 2008, **42**, 293.
17 (*a*) R. Campos, A. Kandelbauer, K. H. Robra, A. Cavaco-Paulo and G. M. Gubitz, *J. Biotechnol.*, 2001, **89**, 131; (*b*) D. Wesenberg, I. Kyriakides and S. N. Agathos, *Biotechnol. Adv.*, 2003, **22**, 161; (*c*) A. Zille, B. Górnacka, A. Rehorek and A. Cavaco-Paulo, *Appl. Environ. Microbiol.*, 2005, **71**, 6711.
18 (*a*) C. G. Bauer, A. Kühn, N. Gajovic, O. Skorobogatko, P.-J. Holt, N. C. Bruce, A. Makower, C. R. Lowe and F. W. Scheller, *Fresenius J. Anal. Chem.*, 1999, **364**, 179; (*b*) B. A. Kuznetsov, G. P. Shumakovich, O. V. Koroleva and A. I. Yaropolov, *Biosens. Bioelectron.*, 2001, **16**, 73; (*c*) Y. Ferry and D. Leech, *Electroanalysis*, 2005, **17**, 113; (*d*) J. Kochana, P. Nowak, A. Jarosz-Wilkolazka and M. Bieron, *Microchem. J.*, 2008, **89**, 171.
19 (*a*) P. Piacquadio, G. De Stefano and V. Sciancalepore, *Ital. J. Food Sci.*, 2002, **14**, 291; (*b*) R. C. Minussi, G. M. Pastore and N. Durán, *Trends Food Sci. Technol.*, 2002, **13**, 205; (*c*) E. Selinheimo, K. Kruus, J. Buchert, A. Hopia and K. Autio, *J. Cereal Sci.*, 2006, **43**, 152.
20 F. Xu, *Encyclopedia of bioprocess technology: Fermentation, biocatalysis, and bioseparation*, John Wiley & Sons, New York, 1999.
21 A. Mikolasch, E. Hammer, U. Jonas, K. Popowski, A. Stielow and F. Schauer, *Tetrahedron*, 2002, **58**, 7589.
22 K. Tatsumi, A. Freyer, R. D. Minard and J.-M. Bollag, *Soil Biol. Biochem.*, 1994, **26**, 735.
23 M. Hesse, H. Meier and B. Zeeh, *Spektroskopische Methoden in der Organischen Chemie*, 6th edn, Thieme Verlag, Stuttgart, 2002.
24 T. Nakamura, *Biochem. Biophys. Res. Commun.*, 1960, **2**, 111.
25 C. Astre, *Bull. Soc. Chim. Fr.*, 1897, **15**, 1025.
26 U. Kuckländer, P. Ulmer and G. Zerta, *Chem. Ber.*, 1989, **122**, 1493.
27 H. Ulrich and R. Richter, *Methoden der Organischen Chemie, Chinone, Teil 1*, 4th edn (Band VII/3a), Thieme Verlag, Stuttgart, 1977.
28 G. Kaupp and M. R. Naimi-Jamal, *Eur. J. Org. Chem.*, 2002, 1368.
29 R. F. Fandy, H. H. Abbas, A. S. Al-Hussaini and A. S. Hammam, *J. Chin. Chem. Soc.*, 2001, **48**, 795.
30 (*a*) S. J. Childress and M. I. Gluckman, *J. Pharm. Sci.*, 1964, **53**, 577; (*b*) H. P. Goodkin and J. Kapur, *Epilepsia*, 2009, **50**, 2011.
31 T. A. Andreyanova, A. S. Sokolova and T. S. Safonova, *Pharm. Chem. J.*, 1998, **32**, 352.
32 R. Kumar and Y. C. Joshi, *J. Chem. Sci.*, 2009, **121**, 497.
33 K. Görlitzer, C. Wilpert, H. Rübsamen-Waigmann, H. Suhartono, L. Wang and A. Immelmann, *Arch. Pharm.*, 1995, **328**, 247.
34 (*a*) M. R. Stillings, S. Freeman, P. L. Myers, M. J. Readhead, A. P. Welbourn, M. J. Rance and D. C. Atkinson, *J. Med. Chem.*, 1985, **28**, 225; (*b*) S. Seto and J. Asano, *Bioorg. Med. Chem.*, 2007, **15**, 5083.
35 U. Jonas, E. Hammer, F. Schauer and J.-M. Bollag, *Biodegradation*, 1997, **8**, 321.
36 (*a*) C. Eggert, U. Temp and K. E. L. Eriksson, *Appl. Environ. Microbiol.*, 1996, **62**, 1151; (*b*) B. B. Feng, M. Voehler, L. Zhou, M. Passarelli, C. M. Harris, T. M. Harris and M. P. Stone, *Biochemistry*, 1996, **35**, 7316.
37 R. M. Berka, P. Schneider, E. J. Golightly, S. H. Brown, M. Madden, K. M. Brown, T. Halkier, K. Mondorf and F. Xu, *Appl. Environ. Microbiol.*, 1997, **63**, 3151.
38 R. Bourbonnais and M. G. Paice, *FEBS Lett.*, 1990, **267**, 99.