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Regioselective Synthesis of 3-Hydroxyorthanilic Acid and Its Biotransformation into a Novel Phenoxazinone Dye by Use of Laccase

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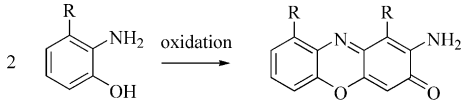
A natural phenoxazinone derivative, cinnabarinic acid (**2**, CA, R = CO_2H), could be obtained in vitro with the aid of purified laccase from the fungi *Pycnoporus cinnabarinus* by oxidative dimerization of 3-hydroxyanthranilic acid (**1**, 3-HAA, R = CO_2H). With the aim of gaining access to a new class of water-soluble chromophores and potential bioactive molecules, the regioselective sulfonation of 2-hydroxyaniline was investigated. Straightforward insertion of a sulfonate group into a carbon–metal bond provided an efficient and selective process for the synthesis of 3-hydroxyorthanilic acid (**3**, 3-HOA, R = SO_3H). This sulfonated compound was then subjected to laccase oxidation in order to mimic the cinnabarinic acid synthesis. A practical HPLC method to study the oxidized products was developed, and the major product of biotransformation – namely 2-amino-3-oxo-3*H*-phenoxazin-1,9-disulfonic acid (**4**, LAO, R = SO_3H) – was isolated and identified as the sulfonate analogue of cinnabarinic acid.

Introduction

Cinnabarinic acid (**2**) is a red phenoxazinone compound responsible for the colour of the *Pycnoporus* species.[1–3] White rot fungi produce cinnabarinic acid from 3-hydroxyanthranilic acid (**1**, 3-HAA) through oxidative dimerization catalysed by laccase (Scheme 1). Furthermore, phenoxazinones have also been identified in various other biological systems, including pigments and antibiotics. Actinomycin, for instance, is an antibiotic formed by phenoxazinone synthase, an enzyme from *Streptomyces* species.[1] Other phenoxazines of industrial interest include the Meldola Blue and Nile Red dyes.[4]



1: R = CO_2H (3-HAA) **2**: R = CO_2H (CA)

3: R = SO_3H (3-HOA) **4**: R = SO_3H (LAO)

Scheme 1. Biomimetic synthesis of phenoxazinone dyes.

The search for water-soluble long-wavelength fluorochromophores for the detection of biological and organic molecules has recently attracted great interest. Indeed, fluorescence-based techniques are sensitive, simple and selective. In particular, phenoxazinone chromophores represent potentially good leads for the development of fluorescent probes for the detection of hydroxyl radicals.[5] These radicals are largely responsible for DNA damage by endogenous effects but also by several indirect effects.[6] However, the poor solubilities of the currently available phenoxazinone derivatives represent a drawback for biological applications. One way to make these molecules soluble in aqueous media is to add a highly polar substituent such as a sulfonate group.

To the best of our knowledge, selective sulfonation at a specific position in phenoxazinones has never been reported before. From the point of view of direct sulfonation of the phenoxazinone core, the extent and selectivity of the reaction may be hard to control. Similar reactions for the synthesis of sophisticated analogues of 3*H*-phenoxazin-3-one by halogenation failed.[7–9]

Furthermore, the usual chemical synthesis of phenoxazine derivatives involves the condensation of nitroso compounds at elevated temperatures.[10–12] Such processes are highly toxic and energy-consuming. Moreover, the cyclization of sulfonated nitrosoanilines as building blocks is not very effective.[12] On the other hand, it is possible to introduce a sulfonic group regioselectively onto a phenoxazine precursor[13] that could undergo further oxidative coupling in the presence of the appropriate catalyst.[2,3]

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Nowadays, the use of enzymes integrated with traditional synthetic methods in order to develop more environmentally friendly processes is a popular field of study.[14,15] The aim of this particular study was to investigate the biomimetic synthesis of a sulfonated phenoxazinone derivative based on the use of laccase. A regioselective sulfonation method to synthesize 3-hydroxyorthanilic acid (3-HOA), the sulfonic analogue of 3-hydroxyanthranilic acid, was developed, and the laccase-catalysed oxidation of this compound was studied further.

Results and Discussion

Synthesis of 3-Hydroxyorthanilic Acid (3-HOA)

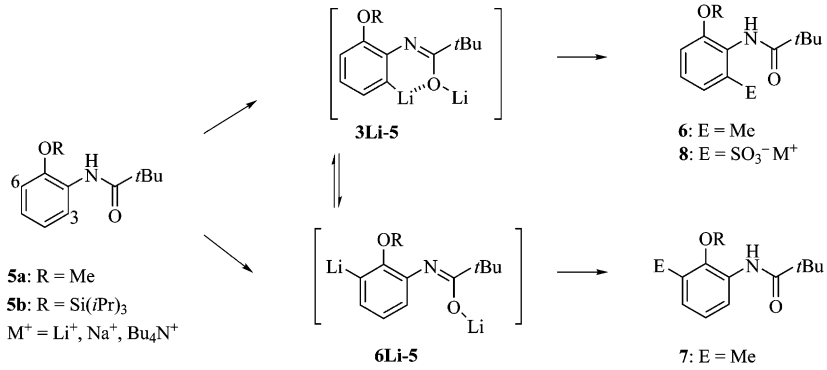
The sulfonation reaction is a rather old chemical process. Sulfonic acids clearly impact on industrial chemistry in various subfields of organic synthesis. They are used as protecting groups, as ligand modifiers[16,17] and as bioisosteres in medicinal chemistry.[18–20]

Direct sulfonation of aromatic compounds is still the most common approach used when the aryl core is rather complex. The regiochemistry is then controlled by the ring substituents. Another efficient method for polysubstituted aromatic compounds proceeds through the production of a sulfonated intermediate from a commercial sulfonic acid. This intermediate is then broken down in a selective way. Using such reactions, 3-HOA (**3**) has previously been reported as a by-product from the reduction of an azoic dye.[21]

However, sulfonation regiochemistry can be also controlled either by modulating the reactivity of sulfur trioxide by means of dioxane,[22] or by using a silane substituent already present on the ring to direct the insertion reaction.[23] The *ipso* substitution of arylsilanes by various electrophiles has recently led to numerous interesting transformations, but this method first requires the introduction of a silane group at the correct position through an *ortho*-directed metallation reaction. Therefore, the direct insertion of oxidized sulfur into the carbon–metal bond, as reported before with unsubstituted *N*-protected aniline,[13] would be the most straightforward strategy for producing 3-HOA.

Our selected synthetic path thus starts from commercial *o*-aminophenol, which already has the required substituents for a dimerization process catalysed by laccase (Scheme 1). A minimal number of steps, inexpensive protecting groups and purification methods are required for further scaling up. Pivaloyl amide was chosen to protect the amine, and a few classical protecting groups (alkyl and silyl ethers) were tested for masking the phenol. Through this preliminary screening, the two combinations of methyl ether/pivalamide[24,25] (**5a**) and triisopropylsilyl ether/pivalamide (**5b**) were selected for the development of a direct sulfonation process (Scheme 2).

Metallation of **5** followed by quenching of the dianion intermediate with methyl iodide was first studied in order to optimize the experimental conditions and to control the regioselectivity (Table 1). Deprotonation of **5a** at –78 °C in THF with the *n*BuLi/TMEDA couple or with the more basic *t*BuLi failed, with the starting material being recovered (entries 1–2). A similar result was obtained with **5b** (entry 3). When a solution of **5a** and *t*BuLi was warmed up to –20 °C before addition of MeI at the same temperature, however, methylation products **6a** and **7a** (Scheme 2) were formed in 7:2 ratio and with a conversion rate of 90% (entry 4). At –10 °C, the regioselectivity in favour of the desired regioisomer **6a** was enhanced to 8:1 (entry 6). In the case of the more hindered precursor **5b**, the yield was slightly lower (entry 5) at –20 °C and the reaction could not be performed at –10 °C, due to the instability of the silyl ether. Raising the temperature of the dianion solution seems to allow the equilibration of **3Li-5** and **6Li-5** intermediates, favouring the dianion stabilized by intramolecular complexation. Since *n*BuLi is better suited than *t*BuLi to handling at room temperature,[26] the next reactions were performed with the less basic *n*BuLi. Deprotonation of **5a** at 0 °C to 25 °C and quenching at 25 °C thus furnished an 80% yield of **6a**, without detectable regioisomer **7a** (entry 7). Similarly, the yield of **6b** (entry 8) was 70%. The following conditions were therefore retained for deprotonation



Scheme 2. Direct sulfonation: quenching of the dianion intermediate. Reagents and conditions: a) 1.1 equiv. *t*BuCOCl, 3 equiv. $NaHCO_3$, H_2O/EtOAc, 25 °C 4 h; b) 1.1 equiv. MeI, 1.1 equiv. K_2CO_3, DMF, 0 °C, 1 h then 25 °C, 8 h; c) 2.4 equiv. *n*BuLi, THF, 0 °C, 10 min, then 25 °C, 3 h; d) 1.5 equiv. $C_3H_9N.SO_3$, –10 °C to 0 °C, 2 h, then 25 °C, 15 h; e) 0.9 equiv. $HSO_4{\cdot}N(Bu)_4$; f) anion-exchange resin IRA-900, MeOH/H_2O, then HCl/H_2O; g) 3 equiv. BCl_3, CH_2Cl_2, –20 °C, 1 h, then 25 °C, 8 h; h) 0.5 equiv. TFA, MeOH, 80 °C, 3 h; i) HCl (6 N), 80 °C, 20 h; j) HBr (48% in water), 120 °C, 17 h.

Table 1. Experimental conditions for *ortho*-metallation and quenching.[a]

Entry (series)	Base (2.4 equiv.)	T_0, T_1[c]	T_2[d]	E = Me[e]				E = $SO_3^-M^+$[f]	
				5	**6**	**7**	Others[g]	**5**	**8**
1 (a)	*n*BuLi/TMEDA	–78, –78	–78	100%	–	–	–		
2 (a)	*t*BuLi	–78, –78	–78	100%	–	–	–		
3 (b)	*t*BuLi	–78, –78	–78	80%	–	–	20%		
4 (a)	*t*BuLi	–78, –20	–20	10%	70%	20%	–		
5 (b)	*t*BuLi	–78, –20	–20	20%	50%[b]	–	30%		
6 (a)	*t*BuLi	–78, –10	–10	10%	80%[b]	10%	–		
7 (a)	*n*BuLi	0, 25	25	20%	80%[b]	–	–		
8 (b)	*n*BuLi	0, 25	–20	20%	70%[b]	–	10%		
9 (a)	*n*BuLi	0, 25	–78				–	85%	15%[b]
10 (a)	*n*BuLi	0, 25	–20				–	30%	70%[b]
11 (a)	*n*BuLi	0, 25	25				–	40%	60%[b]
12 (b)	*n*BuLi	0, 25	–20				10%	40%	50%[b]

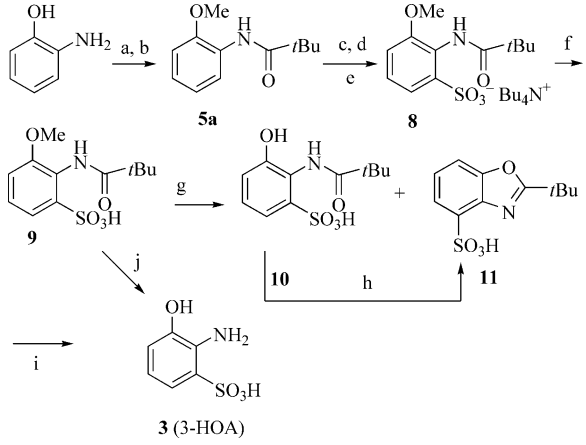
[a] NMR yields in $CDCl_3$: ratios of conversion were determined by comparison of residual proton signals for H-3 (δ = 8.42 and 8.44 ppm respectively for **5a** and **5b**) with H-4 (δ = 6.83 ppm) and CH_3 (δ = 2.20 ppm) for **6a**; with H-3 (δ = 8.22 ppm) and CH_3 (δ = 2.30 ppm) for **7**; with H-4 (δ = 6.81 ppm) and CH_3 (δ = 2.17 ppm) for **6b**. [b] Products isolated and structures confirmed by MS, ^{1}H NMR and ^{13}C NMR spectroscopy. [c] The base was allowed to react with **5a**–**b** at temperature T_0 for 15 min, then for 3 h at temperature T_1 (°C). [d] The dianion was allowed to react with the electrophile for 2 h at temperature T_2 (°C). [e] Quenching was achieved by addition of 1 equiv. of MeI to the dianion solution. [f] Quenching was achieved by addition of the dianion solution to a suspension of sulfur electrophile (SO_3·C_3H_9N); the reaction time of 2 h was extended by 15 h at 25 °C. [g] Products arising from partial deprotection of the silyl ether; TMEDA = tetramethylethylenediamine.

of **5**: treatment at low temperature (≤0 °C) with *n*BuLi (2.4 equiv.) in THF, then further reaction at 25 °C for 3 h.

Insertion of oxidized sulfur into a carbon–metal bond is usually achieved with gaseous sulfur dioxide or trioxide. While such reagents are troublesome to use, solid sulfur trioxide complexes[27] are much easier to handle and in general give good yields. Synthesis of the sulfonated intermediate **8** (M^+ = Li^+) was performed by addition of the dianion solution to SO_3·trimethylamine complex as the electrophile, firstly at low temperature (Table 1). Quenching of **3Li-5a** at –78 °C was unsuccessful (entry 9), while quenching between –20 °C and +25 °C furnished a 60–70% yield of sulfonate **8a** (entries 10–11) but only a 50% yield of sulfonate **8b** (entry 12), without detectable contamination by the 6-regioisomers.

Hence, precursor **5a** was selected for large-scale synthesis (Scheme 3). The dianion was produced under the optimized conditions, this solution then was added to SO_3·Me_3N at –10 °C, and the reaction mixture was kept at low temperature for two hours before being allowed to run overnight at room temperature. Isolation of the sulfonic acid was achieved by forming its tetrabutylammonium salt **8a** (M^+ = Bu_4N^+), a method inspired by the production of monobactam antibiotics.[28] The following steps of Scheme 3 involved the so-called "cosmetic reactions".

Methyl ether cleavage in **8a** with BBr_3[29] or BCl_3 in dichloromethane gave the expected product in high yield. However, subsequent hydrolysis of the pivalamide, either in strongly acidic or in basic media, was sluggish and the isolation of the final product by chromatography on reversed phase rather difficult, due to the presence of unidentified by-products. This prompted us to investigate the use of a less expensive process to isolate the free sulfonic acid **9** before performing the deprotections (Scheme 3). An anion-



Scheme 3. Optimised synthesis of 3-hydroxyorthanilic acid.

exchange resin bearing quaternary ammonium groups was chosen to quench the aromatic sulfonate. Deprotonated **9** could be trapped on the column cationic residues and purified by removing all other organic compounds. Free sulfonic acid **9** was then eluted in quantitative yield. Sulfonic acids are often poorly soluble in organic solvents; nevertheless the methyl ether cleavage of **9** with BCl_3 could be worked up in dichloromethane. Purification by filtration through RP18 silica gel yielded the desired compound. Interestingly, it was present as a 7:3 mixture of noncyclized (**10**) and cyclized (**11**) product. The presence of the sulfonic acid in the *ortho* position seems to activate the amide function towards intramolecular nucleophilic attack from the phenol and the loss of a water molecule. Total cyclization into 2-*tert*-butylbenzoxazole-4-sulfonic acid (**11**) was achieved by gentle heating in the presence of a catalytic amount of trifluoroacetic acid. Compared with the chal-

lenging synthesis of benzoxazole-4-carboxylic derivatives,[19,30] this sulfonic analogue offers a promising means of access to a new class of valuable intermediates for fine chemicals.

The last step of our synthesis consisted of a clean acidic hydrolysis of the imidate bond of **11** with HCl (6 N). Alternatively, simultaneous deprotection of both functions could be achieved from precursor **9** by heating in aqueous HBr[31] solution. The final target 3-HOA was obtained with an overall yield of 50%. The purity of compound **3** as determined by HPLC was about 95%.

The structures of the sulfonated products **8**–**11** and **3** were unambiguously determined by NMR spectroscopy. The question of whether the sulfonate group had been inserted into the position *ortho* to the amino or to the hydroxy group was best answered by HMQC and HMBC spectra performed on **3** (3-HOA). The signals of the aromatic protons H-4, H-5 and H-6 were assigned to their relative carbons by HMQC. A J^3 correlation between the carbon at position C-2 (substituted with NH_2) and the two proton signals appearing as doublets in positions C-4 and C-6 was observed.

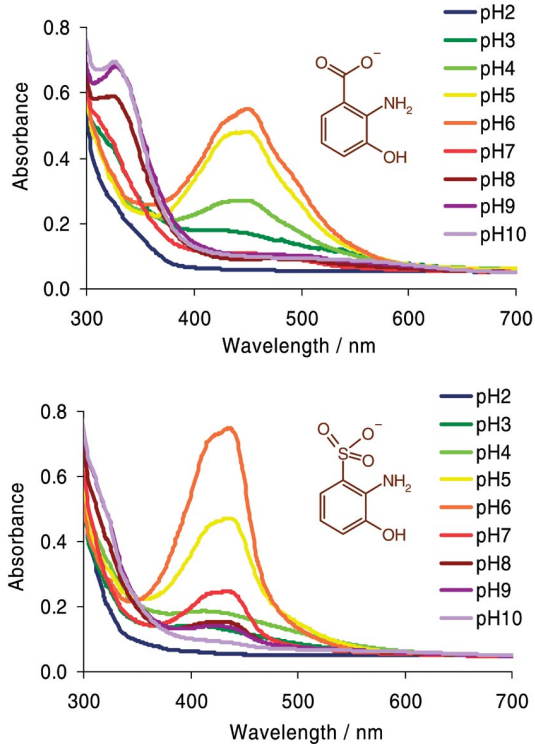


Figure 1. UV/Vis spectra of 3-HAA and 3-HOA oxidation media. Reaction media contained substrate (1 mM), laccase (100 U L^{-1}) and phosphate (100 mM) at pH values ranging from 2 to 10. After 24 h incubation at 25 °C, the spectra of 10-fold diluted solutions were recorded.

Biotransformation of 3-HAA and 3-HOA by Use of Fungal Laccase – HPLC Analysis

3-HAA and 3-HOA (1 mM) were subjected to laccase (100 U L^{-1}) oxidation in phosphate solution (0.1 M) over 24 h at 25 °C, at pH values ranging from 2 to 10 (Scheme 1). The biotransformations were followed by UV spectroscopy (Figure 1). With both substrates, appearance of colour was rapidly observed at pH 3–5. Indeed, the commercial laccase Oxizym LA displays its maximum activity towards a reference compound – namely 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) – at pH 3 (data not shown). Media containing 3-HAA became progressively orange/brown between pH 3 and 7, while media containing 3-HOA became bright yellow under the same conditions. After 24 h incubation, maximum colouration was obtained at pH 6–7. It can be noted that at lower pH, the colour of the media did not evolve significantly after 15 h. It seems that the replacement of the carboxylic acid function by the sulfonic acid one did not drastically affect the substrate reactivity. Media containing 3-HAA showed a light coloration upon storage at pH 8 to 10, even in the absence of laccase. This phenomenon is probably due to substrate autooxidation.[32] Such a reaction was less visible in the case of 3-HOA. The shape of the UV/Vis spectrum of 3-HAA oxidation medium around pH 6–7 strongly resembled that of pure cinnabarinic acid (CA),[2] while spectra recorded at lower pH differed significantly. Similarly, with 3-HOA, UV/Vis spectra shape of the media seemed to indicate that several by-products are present at lower pH. This suggests that more neutral conditions favour the appearance of the highly coloured phenoxazinone product; this was checked more accurately through HPLC analyses.

The HPLC method used for 3-HAA oxidation products analysis was inspired by Iwahashi et al.[32] The biotransformation media showed the appearance of cinnabarinic acid (CA) and several by-products, amounts of which increased when reactions were performed at low pH. The identification of CA as the oxidation product through different processes had already been studied.[33–35] We confirmed that the main product of 3-HAA oxidation by laccase is CA by comparison of the UV/Vis scan with data reported by Eggert et al.[2] and by mass spectrometry analysis (APCI, positive mode), which showed the expected peak [M + H] at 301 *m/z*, in accordance with previous literature results.[2,3,32]

Analysis of 3-HOA biotransformation media required an appropriate method to be set up. Separation of organic sulfonic acids by HPLC is not a trivial issue.[36,37] Our optimal analytical conditions allowed the elution of the substrate after a few minutes, while the oxidized products were detected over the 40 min run. All the oxidized products were detected at 220/254 nm, and the orange-red coloured compounds were also monitored at 440/460 nm (Table 2, entries 1–5).

HPLC analyses showed the appearance of at least three orange-coloured compounds in the crude media at retention times (t_R) of 2.1, 23 and 26 min. As observed in the case of 3-HAA, the numbers and amounts of by-products were higher at lower pH. At pH 6 no residual substrate could be detected, and the compound eluting at 23.3 min was produced with a conversion ratio of about 85%.

Table 2. HPLC studies of biotransformation reactions.[a]

Entry	pH	Buffer	Retention time t_R [min][b] and ratio of peak area (%)[c] 3-HOA $t_R = 1.9$	By-products $2 < t_R < 24$	LAO $t_R = 23$–24	By-products $t_R > 25$
1[d]	3	yes	2%	25%	–	50%
2[d]	4	yes	2%	30%	7%	40%
3[d]	5	yes	–	15%	75%	10%
4[d]	6	yes	–	6%	85%	10%
5[d]	7	yes	–	25%	40%	20%
6[e]	4	no	10%	55%	10%	20%
7[e]	5	no	–	15%	48%	20%
8[e]	6	no	–	2%	70%	25%
9[f]	6	no	–	13%	65%	20%
10[g]	6	no	–	3%	85%	9%
11[h]	6	no	–	–	90%	7%

[a] The elution system for the analysis of 3-HOA and its oxidation products consisted of: A (H_2O/MSA, 100:0.1, v/v), B (H_2O/CH_3CN/MSA, 75:25:0.01, v/v/v), C (H_2O). After a 5 min isocratic step at 100% A, a linear gradient to 100% C over 5 min was applied, followed by a second gradient to 100% B over 15 min. A 10 min isocratic step at 100% B was applied to ensure the elution of all oxidation products, online UV scans were performed, samples were taken after 24 h. [b] Retention times obtained as triplicates. [c] %Area of peaks detected at 235 nm. [d] Sample diluted at 0.5 mM with MSA/H_2O. [e] Sample at 5 mM, directly taken from crude reaction mixture. [f] Sample of 5 mM concentration from crude medium of large-scale biotransformation. [g] Sample at 5 mM of fraction collected after chromatography on RP18. [h] Sample at 5 mM of fraction collected after preparative HPLC.

Use of preparative HPLC was envisaged for purifying this main oxidation product. The presence of phosphate salts from the buffer caused problems when concentration of the crude reaction mixtures was necessary, so the laccase oxidation reaction of 3-HOA was therefore assayed in absence of buffer, the pH being adjusted to pH 4, 5 and 6 by addition of NaOH/HCl. The colouration of the unbuffered media followed almost the same kinetics as that of the buffered ones, and no significant pH modification was observed during the biotransformations. HPLC analyses confirmed that the same products were obtained in almost similar amounts (Table 2, entries 6–8). The UV/Vis spectrum of the major red compound at room temp. and 23.3 min was similar to that of a 2-aminophenoxazinone.[2,3] Its mass spectrum indicated the presence of a dimeric structure.

Identification of the Main Product Obtained Through 3-HOA Oxidation by Laccase

A large-scale biotransformation was conducted at a higher concentration of substrate (5 mM) and enzyme (200 U L^{-1}) at pH 6 in phosphate-free medium. The reaction was stopped after 24 h by decreasing the pH with MSA in order to inactivate the laccase.

The ratio of substrate conversion was determined by analytical HPLC of the crude mixture (Figure 2). Detection at 220/254 nm clearly showed the complete consumption of 3-HOA. The major red compound, named LAO (which stands for laccase acid orange), was obtained efficiently and the proportion of by-products was rather low (Table 2, entry 9). Freeze drying gave a residual red powder, HPLC analysis of which indicated a purity of about 65% in LAO. A portion of this residue was then subjected to chromatography on reversed-phase silica RP-18 with water as solvent. The major red fraction was collected again and analysed by HPLC. LAO was isolated with a purity of about 80% (Table 2, entry 10). However, another unidentified oxidized product with a red colouration was still present (t_R = 26 min). Finally, preparative HPLC followed by filtration through a short pad of reversed-phase for removal of the residual MSA allowed the recovery of LAO with a purity of about 90% (Table 2, entry 11). The ESI mass spectrum of purified LAO showed a peak at 371 *m/z* with 100% intensity corresponding to the structure **4** (Scheme 1) minus one atom of hydrogen. Adducts of sodium and potassium could also be detected in lower proportions. ^{1}H NMR in DMSO showed the two hydrogen atoms of the amino group

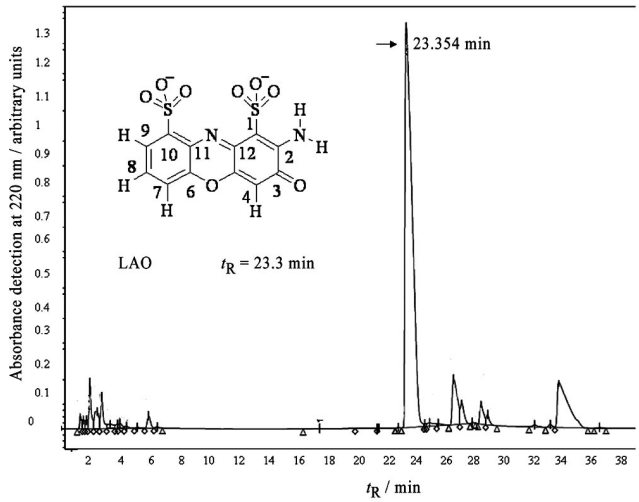


Figure 2. HPLC chromatogram of bioconversion reaction with commercial laccase Bioscreen (*Trametes versicolor*) at 25 °C with substrate 3-HOA (5 mM). pH 6 (adjusted with HCl/NaOH), sample taken after 24 h of reaction. LAO, major red compound of interest. Elution conditions from Table 2.

Table 3. NMR spectroscopic data (δ values, ppm) for 2-amino-3-oxo-3*H*-phenoxazin-1,9-disulfonic acid (**4**, LAO).

H atom	δ[a]	δ[b]	C atom	δ[c]
–	–	–	1	123.2
2	–	9.87 (br s), 9.45 (br s)	2	139
–	–	–	3	176.3
4	6.56 (s)	6.65 (s)	4	105
–	–	–	5	150.1
–	–	–	6	141.6
7	7.76 (d), J = 8 Hz	7.74 (d), J = 7.5 Hz	7	117
8	7.43 (t), J = 8 Hz	7.5 (dd), J = 7.5 Hz; 6.9 Hz	8	127
9	7.57 (d), J = 8 Hz	7.58 (d), J = 6.9 Hz	9	123.8
–	–	–	10	136.8
–	–	–	11	118.3
–	–	–	12	153.7

[a] 500 MHz, D_2O. [b] 300 MHz, DMSO/CD_3OD (95:5, v/v). [c] 75 MHz DMSO/CD_3OD (95:5, v/v), D1 = 5 s; for atoms numbering, see Figure 2.

as nonequivalents at 9.9–9.4 ppm. ^{1}H NMR in D_2O gave the proton signals corresponding to the expected core for the sulfonic analogue of cinnabarinic acid. ^{13}C NMR clearly showed the twelve carbon atoms of phenoxazinone **4** (Table 3). 2D correlation experiments (HMQC and HMBC) provided unambiguous structural assignment of the novel dye LAO. The J^3 coupling observed by HMBC established a correlation between the proton H-8 and the two carbons C-10 and C-6. Similar correlations could be detected for the protons H-7 and H-9 and the carbons C-9/C-11 and C-7/C-11, respectively. Finally, a correlation between the proton H-4 and the two carbons C-12 and C-2 allowed the complete characterization of LAO.

Conclusions

We have disclosed the first efficient synthesis of 3-hydroxyorthanilic acid (3-HOA), in five steps from 2-aminophenol, with an overall yield of 50%. This compound had previously been reported only as a by-product, and had not been fully characterized. Our process, making use of the selective *ortho*-metallation reaction to instal the sulfonate group, was feasible on multi-gram scales. Moreover, the study of O/N deprotection conditions for intermediate **9** led to the discovery of a novel benzoxazole derivative **11**, which will be further exploited for the preparation of tetrasubstituted benzenesulfonic acids. Regarding 3-HOA as a good potential substrate for laccase, similarly to 3-HAA, we were able to prepare a new red dye of phenoxazinone type (LAO) resulting from an oxidative dimerization of the precursor. This biotransformation mimics the well known synthesis of cinnabarinic acid (CA). Our study has established the best experimental conditions (pH, concentrations, ...) for producing high yields of LAO, with a minimum amount of side-products, as well as analytical and preparative conditions for HPLC analysis and purification of sulfonated products.

The mechanism of laccase oxidation is still under investigation. For instance, the increased reactivity of aminophenol derivatives at acidic pH values suggests that their protonation state could modify the relative oxidation potentials of aromatic amine and hydroxy functions.[38] The primary oxidation product would be a protonated arylimino radical rather than the protonated aryloxy radical usually obtained in neutral solutions.

The new phenoxazinone derivative (LAO) has interesting colour properties: it has a high molar extinction coefficient at 450 nm and is totally water-soluble, as required for biological detection systems.

Experimental Section

General Methods: ^{1}H and ^{13}C NMR spectra were recorded with a Bruker Avance 500 or a Bruker Avance 300 spectrometer. Spectra were obtained in [D_6]DMSO, $CDCl_3$, D_2O or CD_3OD. Chemical shifts are reported in ppm relative to the solvent signals.[39] ^{13}C NMR were obtained with broadband proton decoupling (D1 = 5 s). Low-resolution mass spectra were acquired with a Thermo Finnigan LCQ spectrometer, either in positive mode APCI, or negative mode ESI or EI methods (70 eV). High-Resolution Mass Spectrometry (HRMS) analysis by the ESI method was performed at the University of Mons Hainaut (Belgium). Melting points were recorded with an Electrothermal apparatus calibrated with benzoic acid. Infrared spectra were recorded with a Shimadzu FT-IR 84005 spectrometer with KBr plates. The HPLC system consisted of Waters pumps and a Waters 996 photodiode array detector (for analytic separations) or a Waters 486 absorbance detector (for semipreparative HPLC) (Waters, Milford, Massachusetts, USA). Analytical separations were performed with Waters XterraMSC18 column (4.6 × 100 mm, 5 μm) (Waters, Milford, Massachusetts, USA) fitted with a conventional pre-column. Detection was performed at 220/254 nm with control at 440/460 nm, and on-line UV/Vis absorbance scans were performed. Semipreparative separation was performed with a Waters Xterra-PrepMSC18 OBD column (30 × 100 mm, 5 μm) (Waters, Milford, Massachusetts, USA) fitted with a conventional pre-column. Detection was performed at 300 nm. All the solvents were of HPLC grade. Flow rate was 1 mL min^{-1} for analytical separation and 15 mL min^{-1} for semipreparative separation.

Organic Synthesis: For standard working practice, see recent publication.[40] Reactions with organolithium reagents were carried out under argon in flame-dried glassware. Anhydrous tetrahydrofuran and dichloromethane were purchased from Fluka, and chemical reagents and reactants were from Aldrich and Acros. Thin layer chromatography was carried out on aluminium-backed silica gel plate F254 (Merck) and visualized by use of UV irradiation or iodine vapour. Column chromatography was carried out either with flash silica gel (60–200 μm) or with silica gel 60-RP18 (40–60 μm) as the stationary phase with elution by the flash chromatography technique.

Biotransformation: Laccase from *Trametes versicolor* was purchased as a brownish powder from Bioscreen e. K. (Uebach-Palenberg, Germany) under the commercial trademark Oxizym LA (batch no. LA 2000/001). Laccase activity was determined by oxidation of ABTS [2′,2-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid)] (4 mM, Sigma–Aldrich) into a stable cationic radical ABTS$^{\cdot+}$ (absorption coefficient, εM = 34220 M^{-1} cm^{-1}) in tartaric buffer (100 mM) at pH 4.5 and at 25 °C. One unit was defined as the amount of enzyme to oxidize 1 μmol of ABTS per minute. Analytical biotransformations were performed at 25 °C in solutions containing 3-HAA or 3-HOA (1 mM), phosphate buffer (0.1 M) and laccase (100 U L^{-1}), unless otherwise mentioned. All reactions were monitored by spectrophotometry between 300 and 800 nm with a Beckman DU-800 spectrophotometer (Analis, Namur, Belgium) connected to a high-performance temperature controller.

***N*-(2-Hydroxyphenyl)-2,2-dimethylpropionamide:** $NaHCO_3$ (110.1 mmol, 9.2 g) and pivaloyl chloride (40.37 mmol, 4.9 mL) were added to a solution of 2-aminophenol (36.7 mmol, 4 g) in ethyl acetate/water (250/300 mL). The mixture was stirred for 4 h at room temperature. The layers were separated and the organic layer was washed with aqueous HCl (1 N). The organic layer was dried with Na_2SO_4 and the solvents were evaporated in vacuo. The residue was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate, 7:3) to give the title compound as a white solid.[41] Yield 91% (6.5 g). $C_{11}H_{15}NO_2$ (193.11). ^{1}H NMR (500 MHz, DMSO): δ = 9.79 (s, 1 H, OH), 8.54 (s, 1 H, NH), 7.76 (dd, $J_{H,H}$ = 7.8, 1.9 Hz, 1 H, Ar–H), 6.93 (td, $J_{H,H}$ = 7.8, 1.9 Hz, 1 H, Ar–H), 6.86 (dd, $J_{H,H}$ = 6.8, 1.9 Hz, 1 H, Ar–H), 6.77 (td, $J_{H,H}$ = 7.8, 1.9 Hz, 1 H, Ar–H), 1.23 (s, 9 H, 3 × CH_3) ppm. ^{13}C

NMR (75 MHz, DMSO): δ = 176.1 (CO amide), 147.4 (C-1), 126.2 (C-2), 124.29 (C-5), 121.5 (C-4), 118.8 (C-3), 115.2 (C-6), 38.7 [$C(CH_3)_3$], 27 ($3\times CH_3$) ppm. EI-MS: *m*/*z* (%) = 193.3 (55) $[M]^+$, 136 (7), 109 (15), 85 (45), 57.1 (40).

***N*-(2-Methoxyphenyl)-2,2-dimethylpropionamide (5a):** K_2CO_3 (3.4 mmol, 472 mg) and methyl iodide (3.4 mmol, 0.21 mL) were added to a solution of *N*-(2-hydroxyphenyl)-2,2-dimethylpropionamide (3.1 mmol, 600 mg) in DMF (2 mL). The solution was stirred for 24 h at 20 °C. The reaction mixture was diluted with water (15 mL) and extracted with ethyl acetate (3×15 mL). The organic layer was dried with $MgSO_4$ and evaporated in vacuo. The residue was purified by column chromatography on flash silica gel (cyclohexane/ethyl acetate, 9:1, 5:5, and 0:10) to give the title compound.[42] Yield 94%. $C_{12}H_{17}NO_2$ (207.27). ^{1}H NMR 300 MHz ($CDCl_3$): δ = 8.42 (d, $J_{H,H}$ = 8 Hz, 1 H, Ar–H), 8.13 (s, NH), 7.03 (t, $J_{H,H}$ = 7.8 Hz, 1 H, Ar–H), 6.95 (t, $J_{H,H}$ = 6.8 Hz, 1 H, Ar–H), 6.87 (d, $J_{H,H}$ = 7.8 Hz, 1 H, Ar–H), 3.89 (s, 3 H, OCH_3), 1.31 (s, 9 H, $3\times CH_3$) ppm. ^{13}C NMR (75 MHz, $CDCl_3$): δ = 176.4 (CO amide), 147.9 (C-1), 127.8 (C-2), 123.3 (C-5), 121.07 (C-4), 119.4 (C-3), 109.7 (C-6), 55.7 (O–CH_3), 39.9 [$C(CH_3)_3$], 27.5 ($3\times CH_3$) ppm. EI-MS: *m*/*z* (%) = 207.1 (100) $[M]^+$, 176 (10), 164 (7), 150 (15), 123 (45), 107.9 (40), 57.1 (48).

2-(2,2-Dimethylpropionylamino)-3-methoxybenzenesulfonic Acid (9): *n*-Butyllithium in cyclohexane (2.5 M, 40.5 mmol, 16.2 mL) was added dropwise under argon at –10 °C to a solution of **5a** (19.3 mmol, 4 g) in anhydrous THF (40 mL). The solution was kept for 15 min at low temperature (between –10 °C and 0 °C), then warmed to 25 °C and kept for 3 h at room temperature. The dianion solution was added dropwise at – 10 °C to a stirred suspension of crystalline sulfur trioxide/trimethylamine complex (powder dried prior to use under vacuum for 1 h, 28.95 mmol, 4.028 g) in anhydrous THF (10 mL). The reaction mixture was allowed to react for 2 h at low temperature (between –10 °C and 0 °C; white-orange suspension) and was then warmed up to room temperature for 20 h (orange-brown solution). Aqueous NaOH (10% w/w, 150 mL) was then added and the mixture was extracted with diethyl ether (3×100 mL) to remove residual starting material. Tetrabutylammonium hydrogen sulfate (17.37 mmol, 5.89 g) was added to the aqueous solution, and the mixture was extracted with ethyl acetate (3×100 mL). The combined organic phases were dried with $MgSO_4$ and evaporated (black oil). This residue was purified on anion-exchange resin IRA-900 (chloride form) with MeOH/H_2O (1:1, v/v) as neutral eluent and MeOH/1 N HCl (1:1, v/v) as acidic eluent. The IRA mass needed was 100 g. First the resin was placed in H_2O (200 mL) and allowed to swell for 24 h, and then the resin was placed in a column (diameter 2 cm) and washed with HCl (1 N, 150 mL), followed by 150 mL of MeOH/HCl 1 N (1:1, v/v). The crude salt **8**, dissolved in the minimum possible amount of MeOH, was added dropwise onto the resin. The resin was allowed to stand undisturbed for 30 min after complete percolation of the mixture into the resin. Afterward the resin was washed with neutral eluent (500 mL) to recover the tetrabutylammonium chloride salt. The resin was then washed with of acidic eluent (500 mL) and the solution was concentrated under vacuo to yield the title compound as a brown gummy solid. Yield 70% (3.9 g). $C_{12}H_{17}NO_5S$ (287). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.48 (dd, $J_{H,H}$ = 7.8, 1.5 Hz, 1 H, Ar–H), 7.30 (t, $J_{H,H}$ = 8.4 Hz, 1 H, Ar–H), 7.17 (dd, $J_{H,H}$ = 7.8, 1.5 Hz, 1 H, Ar–H), 3.83 (s, 3 H, OCH_3), 1.33 (s, 9 H, $3\times CH_3$) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 179.5 (CO amide), 156.4 (C-3), 141.8 (C-1), 127.9 (C-5), 124.1 (C-2), 119.7 (C-6), 115.5 (C-4), 56.5 (OCH_3), 39.9 [$C(CH_3)_3$], 27.6 ($3\times CH_3$) ppm. HRMS-ESI negative mode: *m*/*z* $[M - H]^-$ calcd. for $C_{12}H_{16}NO_5S$: 286.0749; found 286.0758. ESI-MS: *m*/*z* (%) = 286.1 (100) $[M - H]^-$, 270.9 (25), 214 (70), 206 (40).

2-(2,2-Dimethylpropionylamino)-3-hydroxybenzenesulfonic Acid (10): BCl_3 (1 M, 10.78 mmol, 10.8 mL) was added dropwise to a solution of **9** (13.5 mmol, 1.02 g) in CH_2Cl_2 (15 mL), kept at 0 °C. The mixture was left 1 h at 0 °C and then overnight at 25 °C. The reaction mixture was cooled to 0 °C, and H_2O (20 mL) was added dropwise to quench the residual BCl_3. After evaporation, the residue was dissolved in MeOH (6 mL) and added to RP18 (0.1 g) and concentrated under vacuum. The residue was filtered through a 0.5 μm filter with MeOH/H_2O (1:1 v/v, 60 mL), and the solution was concentrated to yield the title compound as a brown solid. Yield 91% (0.87 g). NOTE: The compound exists partially in the form of benzoxazole **11** in a ratio of 70:30 in favour of product **10**. $C_{11}H_{15}NO_5S$ (273.3055). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.52 (d, $J_{H,H}$ = 6.8 Hz, 1 H, Ar–H), 7.16 (t, $J_{H,H}$ = 7.8 Hz, 1 H, Ar–H), 7.05 (d, $J_{H,H}$ = 6.8 Hz, 1 H, Ar–H), 3.68 (s, 0.5 H), 1.36 (s, 9 H, $3\times CH_3$) ppm.

2-*tert*-Butylbenzoxazole-4-sulfonic Acid (11): TFA (1.8 mmol, 0.2 mL) was added to a solution of a **10**+**11** mixture as a dry powder (3.6 mmol, 1 g) in MeOH (10 mL). The mixture was stirred for 3 h at 80 °C. The reaction mixture was then cooled to 25 °C and evaporated under vacuo to yield the title compound as a pale yellow solid; yield 99% (0.92 g). $C_{11}H_{13}NO_4S$ (255). M.p. 293–295 °C. ^{1}H NMR (300 MHz, CD_3OD): δ = 7.86 (d, $J_{H,H}$ = 7.5 Hz, 1 H, Ar–H), 7.77 (d, $J_{H,H}$ = 7.5 Hz, 1 H, Ar–H), 7.48 (t, $J_{H,H}$ = 7.8 Hz, 1 H, Ar–H), 1.56 (s, 9 H, $3\times CH_3$) ppm. ^{13}C NMR (125 MHz, $CD_3OD/CDCl_3$): δ = 176.8 (C-2), 151.1 (C-1), 134.3 (C-4), 125.3 (C-6), 124.7 (C-3), 113.3 (C-5, C-7), 35.8 [$C(CH_3)_3$], 20.5 ($3\times CH_3$) ppm. IR (KBr): $\tilde{\nu}$ = 3448, 2977, 2360, 1566, 1473, 1427, 1253, 1191, 1141, 1045, 891, 802, 682 cm^{-1}. HRMS-ESI negative mode: *m*/*z* calcd: 278.0463 for $C_{11}H_{12}NO_4NaS$, found 278.0466. ESI-MS: *m*/*z* (%) = 254.2 [M – H] (100), 190.2 (15).

2-Amino-3-hydroxybenzenesulfonic Acid (3, 3-HOA). Method i: Compound **11** (3.1 mmol, 0.8 g) was added to a solution of HCl (6 N, 21 mL). The mixture was heated at 80 °C for 24 h. Then the solution was cooled down and the solvents were evaporated. The residue was dissolved in MeOH (20 mL), and activated charcoal was added (50 mg). The mixture was stirred at 40 °C for 30 min. The solution was then filtered through a short pad of celite and the pad was washed with hot methanol (30 mL). The filtered solution was concentrated and dried under vacuo to yield the title compound as a light brown solid. Yield 90% (0.53 g). The purity of the compound can be monitored by HPLC (t_R = 1.8 min). $C_6H_7NO_4S$ (189.1). M.p. 210–215 °C (slow dec.). ^{1}H NMR (300 MHz, CD_3OD): δ = 7.41 (dd, $J_{H,H}$ = 7.8, 1.35 Hz,1 H, Ar–H), 7.34 (t, $J_{H,H}$ = 8.1 Hz, 1 H, Ar–H), 7.07 (dd, $J_{H,H}$ = 7.8, 1.35 Hz, 1 H, Ar–H) ppm. ^{13}C NMR (125 MHz, CD_3OD): δ = 152.6 (C-3), 140.6 (C-1), 130.6 (C-5), 119.4 (C-6), 118.8 (C-4), 115.8 (C-2) ppm. IR (KBr): $\tilde{\nu}$ = 3487, 3436, 3220, 3066, 2931, 2692, 1635, 1490, 1469, 1365, 1307, 1191, 1064, 1037, 902, 798, 675 cm^{-1}. HRMS-ESI negative mode: *m*/*z* calcd. 188.0018 for $C_6H_6NO_4S$; found 188.0018. ESI-MS: *m*/*z* (%) = 188.07 [M – H] (100), 376.8 [2 M – 2 H] (75), 187.1 (6), 108 (5), 79.8 (15).

2-Amino-3-hydroxybenzenesulfonic Acid (3, 3-HOA). Method j: Compound **9** (1.04 mmol, 0.3 g) was added to a solution of HBr (48%, 6 mL). The mixture was heated at 120 °C for 13 h. Afterwards the reaction mixture was cooled down. Part of the product was recovered as a solid by precipitation in situ. The filtered solution was concentrated, and the residue was dissolved in MeOH (20 mL). Activated charcoal was added (50 mg), and the mixture was agitated at 40 °C for 30 min. The solution was then filtered

through a short pad of celite, and the celite was washed with hot methanol (30 mL). The filtered solution was concentrated and dried under vacuum to yield the title compound as a brown solid. Yield 95% [0.17 g including 20% as hydrobromide salt (0.05 g)] from precipitation in situ.

2-Amino-3-oxo-3*H*-phenoxazin-1,9-disulfonic Acid (4, LAO): A solution of laccase (200 U L^{-1}) and 3-HOA (5.29 mmol, 1.02 g) in water (final volume of 100 mL) adjusted to pH 6 with HCl and NaOH was prepared. The stirred mixture was kept for 24 h at 25 °C. The reaction was stopped by decreasing the pH with MSA (pH ≈ 2). The crude mixture was freeze-dried to give a red powder (1 g). Part of this powder (400 mg) was purified by chromatography on reversed phase (water as eluent) to yield an almost pure fraction of LAO (270 mg). Part of this fraction (70 mg) was subjected to preparative HPLC to yield the pure title compound as a deep red solid (30 mg). Yield of about 70% determined by HPLC. $C_{12}H_8N_2O_8S_2$ (372.3305). ^{1}H NMR and ^{13}C NMR: see Table 3. M.p. >300 °C. IR (KBr): $\tilde{\nu}$ = 3433, 2364, 1635, 1593, 1473, 1415, 1218, 1053 cm^{-1}. UV: λ_{max} at 435 and 339 nm (0.1 M phosphate buffer, pH 7). 435 nm (ε = 13981 L mol^{-1} cm^{-1}). UV: λ_{max} at 460 and 330 nm (0.1 M phosphate buffer, pH 2). 460 nm (ε = 5426 L mol^{-1} cm^{-1}). HRMS-ESI negative mode: *m/z* calcd. 370.9644 for $C_{12}H_7N_2O_8S_2$; found 370.9641. ESI-MS: *m/z* (%) = 371.16 [M – H] (100); 393.1 [M – H + Na] (40); 291.2 (20).

Supporting Information (see also the footnote on the first page of this article): ^{1}H and ^{13}C NMR spectra of compounds **9** (Figures S2, S3), **11** (Figures S4, S5), **3** (Figures S6, S7), **4** (Figures S8, S9) are provided as additional material. UV/Vis spectra of compound **4** at pH 7, pH 2 (Figures S10) are also furnished.

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