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Synthesis of bis-lactone lignans through laccase catalysis

Sylvain Tranchimand, Thierry Tron, Christian Gaudin, Gilles Iacazio *

Laboratoire de Bioinorganique Structurale (CBRL UMR CNRS 6517), Case 432, Faculté des Sciences et Techniques, Université Paul Cézanne Aix-Marseille III, Avenue Escadrille Normandie-Niemen, 13397 Marseille Cedex 20, France

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Abstract

We report here on the use of a biphasic reaction solvent during the laccase catalysed transformation of sinapinic and ferulic acids. As compared to buffer alone, higher product selectivity, higher yields and higher product stability were obtained simply using ethyl acetate as added non-miscible co-solvent. Reactions run in biphasic medium are marginally longer but offer an easy separation protocol allowing to obtain the highest yield reported so far for the synthesis of the two bis-lactone lignans originating from sinapinic and ferulic acids.

Keywords: Laccase; Sinapinic acid; Ferulic acid; Bis-lactone lignan; Biotransformation

1. Introduction

Laccases are blue copper oxidases that catalyse a single electron oxidation of various phenolic and non-phenolic compounds with a concomitant four-electron reduction of dioxygen to water [1]. The technological potential of laccases has been recognised long ago and numerous studies devoted to their use in various bioprocesses (delignification, ethanol production, bioremediation...) have been conducted [2,3]. Laccases have been also used as catalyser for fine chemistry [4,5] and recent achievements in the field [6–10] exemplified their potential. A key point to consider when using laccases is that a radical is the primary product of the reaction. It is generally considered that once formed, the radical product is released and is subject to further transformation linked to its own reactivity in the reaction medium. The reaction selectivity is not enzymatically controlled, but rather and to some extent medium-controlled. In addition, the product is generally a laccase substrate that can react further, leading to complex mixtures through polymerisation. The well-known laccases catalysed oxidation of sinapinic acid (SA) and ferulic acid (FA) offer good example of such a complexity. With SA as substrate, a single dimeric compound (a bis-lactone lignan, Scheme 1) is obtained before it reacts further to give a complex mixture as judged by HPLC [11]. With FA as substrate, two main dimeric products are initially formed due to the possibility of C–O coupling (Scheme 1) but the bis-lactone lignan is not detected [12].

In this paper, we report on the use of biphasic media to run laccase catalysed transformation of SA and FA. Our objective was to improve both the yield and the selectivity through a simple adaptation of the reaction medium.

2. Material and methods

2.1. General

Melting points were determined on an Electrothermal 9300 capillary melting point apparatus and are uncorrected. IR spectra were measured on KBr plates on a Bruker IFS25 spectrophotometer. NMR spectra were recorded on a Bruker Avance DPX-300 spectrophotometer (^{1}H, 300 MHz; ^{13}C, 75.5 MHz), chemical shifts (δ) are reported in ppm relative to the NMR solvent. Microanalyses were performed on a Thermo Finnigan EA 1112 apparatus. Flash column chromatography was carried out with silica gel 60 (MERCK, particle size 230–400 mesh). HPLC analysis were conducted using a LiChrospher 100 RP-18 (5 μm) column from Agilent Technologies, and an HP 1100 series system (DAD). Solvent flow: 0.5 mL/min. Solvent gradient: 100% A to 40% A–60% B in 10 min and then to 100% B in 20 min (A: 90/10/0.1, water/acetonitrile/acetic acid; B: 90:10:0.1, acetonitrile/water/acetic acid). The detection was per-

* Corresponding author. Tel.: +33 491 282 856; fax: +33 491 983 208.
E-mail address: gilles.iacazio@univ-cezanne.fr (G. Iacazio).

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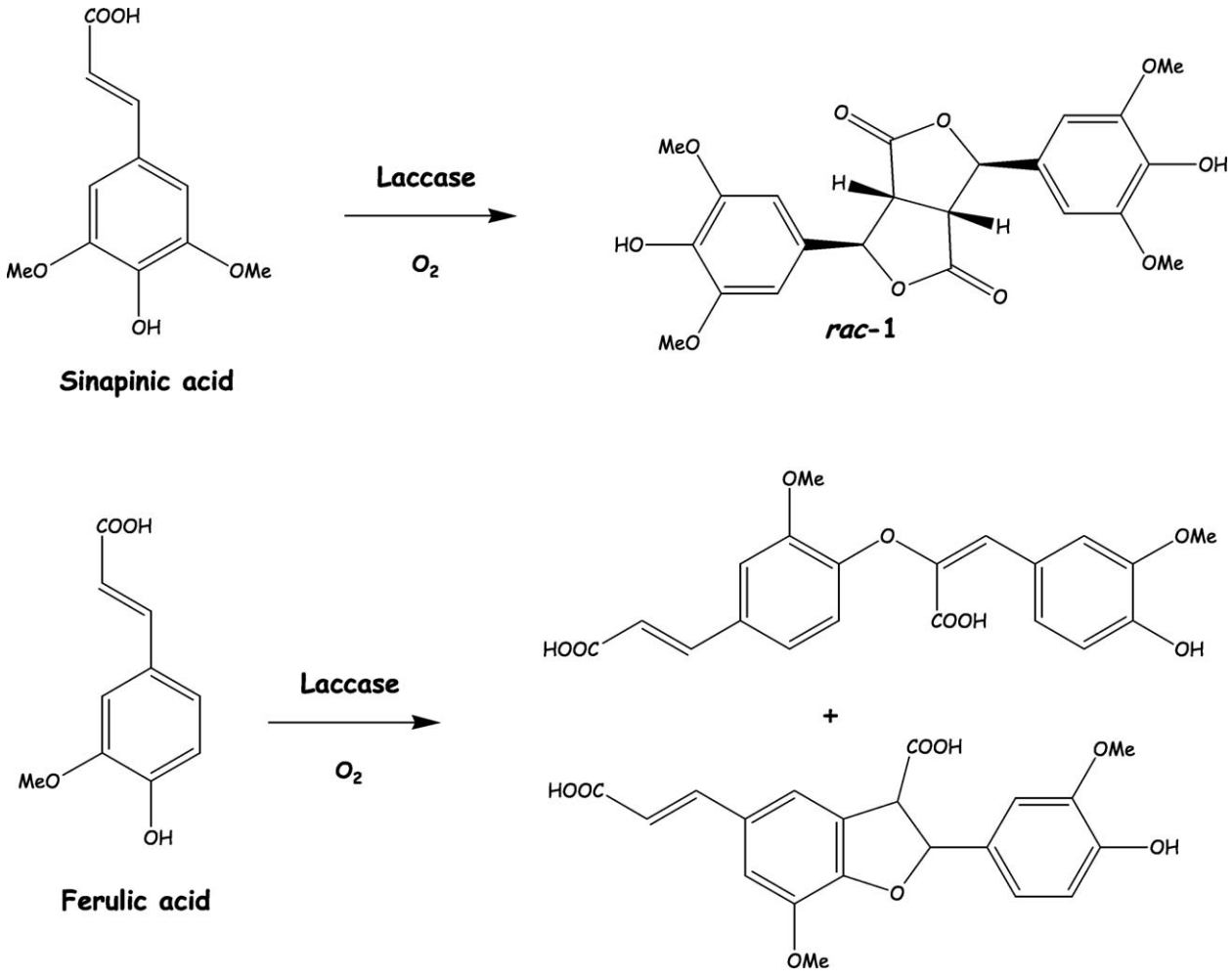


Scheme 1. Literature reported laccase catalysed biotransformations of sinapinic and ferulic acids.

formed at 280 nm. Synthetic methyl-3,4,5-trimethoxybenzoate was used as internal standard in all cases. Laccase preparations from *Trametes versicolor* and *Agaricus bisporus* were purchased from Fluka. Lac1 and Lac3 from *Trametes* C30 were obtained as previously described [13,14]. Crude laccase preparation from *Melanocarpus albomyces* was a kind gift from Dr. Kristiina Kruus, VTT, Finland. Sinapinic and ferulic acids were purchased from Aldrich and were used without purification.

2.2. Analytical biotransformations

2.2.1. Buffered medium alone

9.5 mL of 50 mM buffer (phosphate or acetate) at the required pH, and 0.5 mL of a substrate solution (sinapinic or ferulic acids) dissolved in CH_3CN (final substrate concentration 0.1 mg/mL) were stirred with a magnetic stirrer in a 20 mL open round bottomed flask at room temperature. The reaction was initiated by addition of a concentrated laccase solution to reach a final activity of 150 U/L for SA and 375 U/L for FA (ABTS as substrate). To follow the reaction, periodically withdrawn aliquots were analysed by HPLC (sinapinic acid $t_r = 12.6$ min; bis-lactone **1** $t_r = 16.0$ min; ferulic acid $t_r = 15.4$ min; bis-lactone **2** $t_r = 23.9$ min).

2.2.2. Biphasic system

Two millilitres of 50 mM buffer (phosphate or acetate) at the required pH, and 8 mL of a substrate solution (sinapinic or ferulic acid) dissolved in the requested non-miscible solvent (final substrate concentration 0.1 mg/mL) were vigorously stirred with a magnetic stirrer in a 20 mL open round bottomed flask at room temperature. The reaction was initiated by addition of a concentrated laccase solution to reach a final activity of 150 U/L for SA and 375 U/L for FA (ABTS as substrate). To follow the reaction, periodically withdrawn aliquots were analysed by HPLC.

2.3. Preparative biotransformations

Twenty millilitres of 50 mM buffer (phosphate or acetate) at the required pH and 80 mL of a substrate solution (sinapinic or ferulic acids) dissolved in ethyl acetate (final substrate concentration 1.25 mg/mL for SA and 1 mg/mL for FA) were vigorously stirred with a magnetic stirrer in a 250 mL open round bottomed flask at room temperature. The reaction was initiated by addition of a concentrated laccase solution to reach a final activity of 1500 U/L for SA and 3750 U/L for FA (ABTS as substrate). At the end of the reaction, the organic phase was withdrawn and the aqueous phase extracted once with ethyl acetate. The combined

organic phases were dried over anhydrous sodium sulfate and concentrated in vacuo. For SA, the formed product did not need further purification: 120 mg (97%) of **1** were obtained as a purple solid. Mp: 198 °C (decomposition), IR (KBr, cm^{-1}): 3441, 2963, 2851, 1776, 1523, 1325, 1156, 1116, 700, 610. ^{1}H NMR (DMSO-D6) δ: 8.61 (s, 2H), 6.71 (s, 4H), 5.77 (s, 2H), 4.29 (s, 2H), 3.81 (s, 12H). ^{13}C NMR (DMSO-D6) δ: 175.6, 148.1, 136.2, 128.1, 104.0, 82.2, 56.1, 48.0. Anal. calc. for $C_{22}H_{22}O_{10}$: C, 59.2; H, 4.9. Found: C, 59.0; H, 5.5.

In the case of FA, the product was purified by flash chromatography (ethyl acetate/pentane: 90/10): 36 mg (36%) of **2** were obtained as a yellow solid. Mp: 191 °C (decomposition), IR (KBr, cm^{-1}): 3444, 2924, 2850, 1788, 1520, 1267, 1231, 1185, 824, 627. ^{1}H NMR (DMSO-D6) δ: 9.25 (s, 2H), 6.98–6.73 (m, 6H), 5.73 (s, 2H), 4.2 (s, 2H), 3.8 (s, 6H). ^{13}C NMR (DMSO-D6) δ: 175.3, 147.8, 147.3, 128.9, 119.1, 115.4, 110.6, 82.0, 55.7, 48.0. Anal. calc. for $C_{20}H_{18}O_{8}$: C, 62.2; H, 4.7. Found: C, 62.6; H, 4.9.

3. Results

We first studied the laccase catalysed oxidation of SA as the steric hindrance of the two methoxy groups of this compound limit the complexity of the reaction mixture to a single product (Scheme 1) resulting from a C–C coupling. A commercial laccase preparation from *T. versicolor* was used for preliminary studies. HPLC analysis of the reaction conducted in 50 mM phosphate buffer, pH 5, revealed a rapid formation of a major compound (maximum yield 85% after 20 min, Table 1). As expected from a previously published study, this compound (later identified as the bis-lactone lignan **1**, see below), disappeared gradually from the reaction medium (24% yield after 2 h, Fig. 1) [11].

Keeping the same enzyme and substrate concentrations, we tested the influence of different biphasic systems on the reaction. Four organic water non-miscible solvents (diisopropyl ether, toluene, ethyl acetate, dichloromethane) were independently used in a 1/4: buffer/organic solvent ratio. Except for dichloromethane (no conversion observed), whatever the co-solvent used, only compound **1** was detected by HPLC.

From the different reaction conditions tested, highest yield for the conversion of SA to bis-lactone **1** by laccase were obtained in the presence of either ethyl acetate or toluene as co-solvent. Bis-lactone **1** appeared to be more stable when ethyl acetate rather than toluene was used. Diisopropyl ether did not allow to stabilise bis-lactone **1** enough and dichloromethane did not allow any conversion of the substrate at all. For further studies we thus selected ethyl acetate as co-solvent.

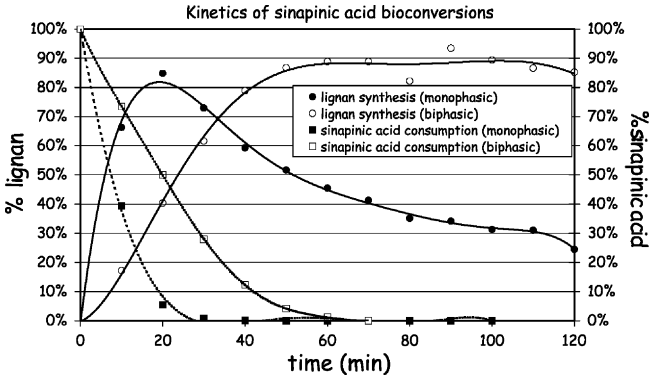


Fig. 1. *Trametes versicolor* catalysed bioconversion of sinapinic acid in mono- and biphasic (buffer/ethyl acetate) system.

In order to test the general ability of these newly established conditions, we tested four other laccase preparations (*Trametes* C30 Lac 1, *Trametes* C30 Lac 3, *A. bisporus* laccase and *M. albomyces* laccase) under the previously established conditions. Results of biotransformation are reported in Table 2.

From Table 2 it could be concluded that, quasi-quantitative yields were obtained with all laccase preparation used, suggesting that the reaction is independent of the enzyme preparation. For a full characterisation of the formed product, we ran a preparative reaction with 125 mg of SA up-scaling all other parameters accordingly. Based on results presented in Table 2, the *T. versicolor* laccase preparation was chosen as it is easily available commercially. Spectroscopically pure bis-lactone **1** was obtained in quantitative yield (97%) after separation of the organic phase at the end of the reaction (30 min), evaporation of ethyl acetate and drying under vacuum. X-ray diffraction analysis of crystals of bis-lactone **1** obtained in acetonitrile confirmed the previously assigned relative stereochemistry (Fig. 2).

Based on our results of laccase-catalysed oxidation of SA in biphasic system we then tested these reaction conditions for the oxidation of FA. As this was described previously, the lack of a methoxy group adjacent to the reactive *p*-phenolic group in FA allow various carbon–carbon and carbon–oxygen couplings and therefore multiple reaction products were expected [12]. When *T. versicolor* laccase catalysed oxidation of FA was

Table 1
Influence of added co-solvent on the biotransformation of sinapinic acid catalysed by *T. versicolor* laccase

Added co-solvent	Maximum yield **1**/time	Yield after 2 h (%)
None	85%/20 min	24
Diisopropyl ether	77%/30 min	2
Ethyl acetate	90%/60 min	85
Toluene	97%/50 min	68
Dichloromethane	0%	0

Table 2
Influence of various laccase preparations on the biotransformation of sinapinic acid in buffer/ethyl acetate biphasic medium

Laccase preparation	ABTS laccase unit added	Maximum yield **1**/time
Trametes versicolor	0.320	90%/60 min
Agaricus bisporus	0.311	97%/90 min
Trametes C30 Lac 1	0.365	100%/50 min
Trametes C30 Lac 3	0.374	98%/70 min
Melanocarpus albomyces	0.365	98%/30 min

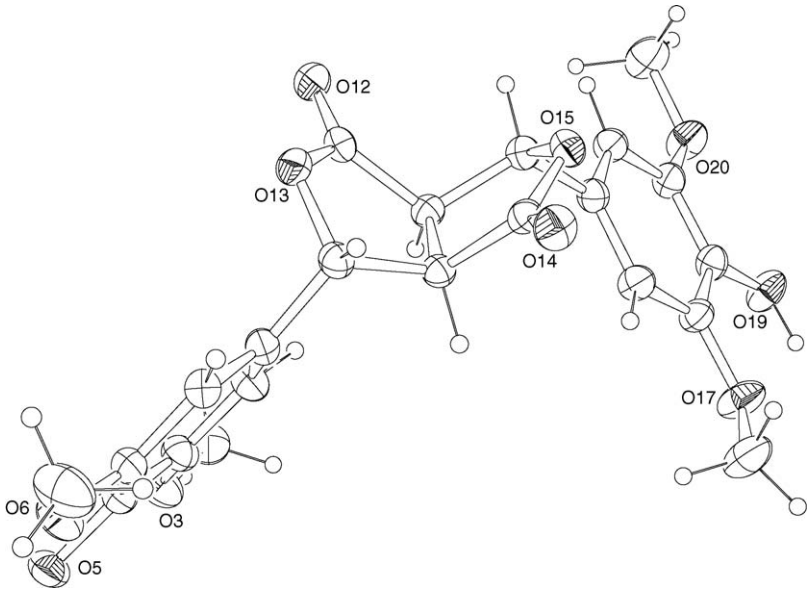


Fig. 2. Perspective view of bis-lactone **1**.

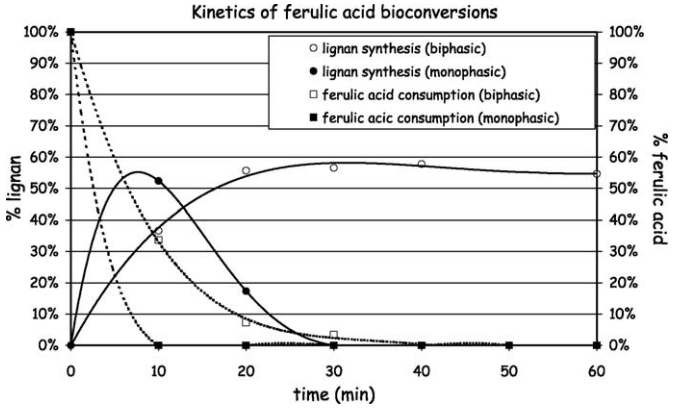
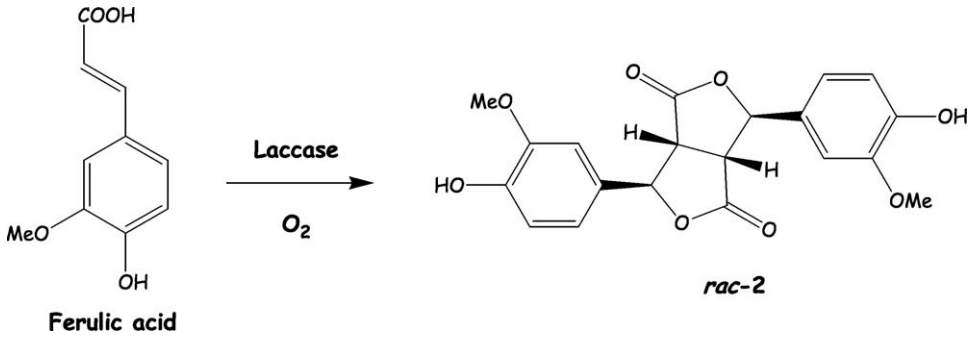


Fig. 3. *Trametes versicolor* laccase catalysed bioconversion of ferulic acid in mono- and biphasic (buffer/ethyl acetate) system.

first conducted in buffer alone, a main product among several minor ones was detected by reverse-phase HPLC. The major compound was found to co-eluate with synthetically obtained bis-lactone **2** [15]. When the same reaction was conducted in the previously described biphasic system, the reaction was found to be excessively slow as compared to results obtained with SA (the conversion was still not complete after 2 h of reaction). Changing the buffer from phosphate (50 mM, pH 5) to acetate (50 mM, pH 4) while keeping ethyl acetate as non-miscible solvent allowed a faster transformation of FA (Fig. 3) and apparently improved selectivity as less minor compounds were detected by HPLC (not shown).

In both cases (two different pH), **2** was found to be the main product formed (Scheme 2).

The systematic generation of **2** as a major product under our operating conditions contrast with the results of Carunchio et al. who reported the obtention of two main products from a *Pyricularia oryzae* laccase catalysed FA oxidation (0.07 M acetate buffer, pH 6, ethanol 9%), both products arising from a C–O coupling (Scheme 1). It is not known whether the difference in product selectivity of the two reactions is due to the use of a different enzyme preparation or to the different physicochemical properties of the two reaction media. In this context it should nevertheless be stressed that when other laccase preparations (*Trametes* C30 Lac 3 and *M. albomyces* laccase) are used to oxidise FA in biphasic system, compound **2** is always



Scheme 2. *Trametes versicolor* laccase catalysed biotransformation of ferulic acid.

formed predominantly. The scaled up reaction using 100 mg of FA allowed the purification of 36 mg of compound **2** (36% yield). Enzymatically generated compound **2** was found to be spectroscopically identical to its chemically generated counterpart [16], thus assessing the shown relative stereochemistry. Although the overall yield we obtained for the synthesis of compound **2** is low, to the best of our knowledge, 36% is the highest yield reported so far either from chemical or enzymatic synthesis.

4. Conclusion

From this work we conclude that a biphasic system can be used to improve both the selectivity and the yield of laccase catalysed reactions. Removal of the formed product(s) from the aqueous phase is probably important in impairing their further transformation by the enzyme. Successful use of a biphasic system for laccase-catalysed biotransformation have been reported previously [17]. Recently dimerisation of 17β-estradiol [7] and resveratrol [8] have been obtained with a laccase preparation from *Trametes pubescens* in a buffered/ethyl acetate biphasic system. Unfortunately, both the very long reaction time (48 h) used and the absence of data in an homogeneous aqueous solvent in the two reports make the comparison with our data difficult. However, from these examples and from our own data, we believe that, providing a necessary optimisation for each enzyme/substrate couple, the use of laccases in biphasic systems will widen the synthetic applicability of these very interesting and robust oxidases that use inexpensive molecular dioxygen as co-oxidant. On the other hand, it should be noted that the synthesis of bis-lactone lignans is of particular interest since they are convenient precursor for the obtention of furofuranones, dihydroxyfurofurans and furofurans [15].

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