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Laccase-Mediated Oxidation of Totarol

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Abstract: In this study, novel dimers of the biologically active phenolic compound totarol were synthesized using the phenol oxidase enzyme laccase, obtained from *Trametes pusbescens*, in organic solvent medium. Two dimeric products, linked either by carbon-carbon or by carbon-oxygen bonds, were isolated and characterized. The effect of changes in various parameters such as solvent, temperature, pH and buffer concentration on the conversion of totarol by laccase was investigated. The nature of the organic solvent, in particular, was found to affect the nature and the ratio of the products obtained.

Keywords: biocatalysis; biotransformations; laccase; oxidoreductases; phenols; totarol

Introduction

Enzymes are increasingly becoming an important tool in the oxidation of various substrates to produce novel derivatives for application as pharmaceuticals and, specifically, to modify natural compounds with phenolic substructures.[1,2] Chemical methods may be used for the oxidation of these molecules, but these protocols are often complex, costly, and difficult to predict or control. Thus, alternative methods which are more selective and require more gentle conditions are desirable.[1] Laccases catalyze the one-electron oxidation of a wide variety of organic substrates, including mono-, di- and polyphenols, aminophenols, methoxyphenols, aromatic amines and ascorbate, with the concomitant four-electron reduction of oxygen to water.[3] They have also been used in the biocatalytic oxidations of dyes, polymerization of lignin and lignosulfonates, bioremediation and bleaching.[4,5] In recent years, there has been an increasing interest in the application of laccases in organic solvents, the advantage being the increased solubility of their substrates in the reaction medium. More recently, organic solvents have also been shown to have an influence on the laccase enzyme selectivity, by changing the relative ratio of the dimers obtained in laccase-catalyzed oxidations.[6] Among the different laccases found in nature, the enzymes isolated from *Trametes* strains are generally stable biocatalysts. Some of them are readily available commercially, but they can also be produced simply, in the laboratory, by fermentation.[3]

(+)-Totarol (**1**) is a natural, phenolic, and highly hydrophobic diterpenoid which has been found to be a potent antibacterial. It is extracted from the New Zealand endemic hardwood tree, *Podocarpus totara*. This species is common and well known in New Zealand and most of the wood used for totarol production comes from discarded fencing, which, after as long as 150 years in the ground, is often found to be still in perfect condition at the core. The fact that the wood does not degrade is due to the valuable antimicrobial properties of **1**. The extract is presently used in a variety of cosmetic and personal care formulations (i.e., toothpaste and mouthwash) in New Zealand and Australia. More recently, totarol and its derivatives have been found to be highly effective as antibacterial and antiplasmodial agents with potential for application in treating malaria.[7] Site-specific derivatization of totarol has proved to be a challenging, but promising, route to new biologically active products.[8]

These observations prompted us to investigate the potential of biocatalysis for the conversion of totarol to other novel derivatives, as part of our current study aimed to the production of new biologically active compounds. As an outcome of this study, we report for the first time the oxidation of totarol (**1**) catalyzed by a laccase in the presence of significant amounts

(50% v/v) of organic cosolvents, to produce novel derivatives identified as carbon-carbon or carbon-oxygen linked dimers (**1a** and **1b**).

Results and Discussion

Extracellular laccase was produced by fermentation of the white rot fungal strain *Trametes pubescens* using an airlift reactor at a scale of 5 L. The laccase was produced as an extracellular enzyme and was readily isolated from the fermentation medium by precipitation with ammonium sulfate or acetone.[3b] The freeze-dried laccase produced in this way was used for the oxidation of totarol (**1**) dissolved in a homogeneous solution comprising sodium acetate buffer and a water miscible organic solvent (MeOH was arbitrarily chosen for the initial experiments) in a 1:1 v/v ratio.

Using this reaction medium, totarol was converted by the laccase into two products (**1a** and **1b**), as resolved by TLC and HPLC. The more polar compound (**1a**, TLC R_f of 0.15) was the main product and was obtained at a 8 : 1 ratio with the minor one (**1b**, TLC R_f of 0.19). These compounds were purified by flash chromatography and analyzed using mass spectrometry and NMR. The ESI-MS values of their molecular peaks ($m/z = 593.43389$ and 593.43290, respectively) suggested that both the products were dimers of **1**, whereas their complete structure elucidation was achieved by ^{1}H NMR and ^{13}C NMR. Figure 1 compares the signals due to the aromatic protons (Figure 1 A) and to some of the aliphatic protons (Figure 1 B) of totarol and of the two dimers. In compound **1a** the aromatic AB system present in **1** (two doublets at $\delta =$ 7.03 and 6.54) was missing and only a singlet at $\delta =$ 7.03 was present. Instead the signals between $\delta = 1.90$ and 3.60, due to some of the aliphatic protons of **1** and **1a**, were almost superimposible, suggesting the presence of the symmetric C–C dimeric structure shown in Scheme 1. In contrast, the aromatic portion of the ^{1}H NMR spectrum of **1b** showed both the presence of two doublets and of one singlet (overall three protons), whereas all the signals between $\delta = 1.90$ and 3.60 were duplicated, thus suggesting the formation of a C–O linkage between two totarol units. The ^{13}C NMR spectra of **1a** and **1b**, as well as their bidimensional COSY and HSQC spectra further supported the proposed structures (spectra not shown; see Supporting Information). As shown in Scheme 1, the formation of dimeric products is attributed to the coupling of the radical intermediates produced by the laccase-mediated oxidation of the phenolic substrate.[3a,6b]

In order to optimize the reaction conditions, the effects of buffer composition, pH and concentration were investigated, based on previous reports that these parameters would affect enzyme stability and activity.[9] No significant improvements in the bioconversion were observed as a result of using either lower or higher salt concentrations. However, the pH of the reaction mixture did affect the bioconversion of totarol, the best performances being achieved at pH 4.5–5 (a result consistent with other reports on this laccase).[10] The oxidation of totarol at various temperatures was also investigated, and it was shown to increase with temperature up to an optimum temperature 30 °C, beyond which the conversion decreased. Under these optimized reaction conditions a 62.6% conversion of totarol was observed after 24 h, as demonstrated by HPLC. The incomplete transformation was mainly due to substrate precipitation even at the low concentration used (2 mg mL^{-1}). In order to improve products yields, several other water-miscible cosolvents were considered and, among them, acetonitrile and acetone proved to be the most suitable ones. Reactions were performed on a semi-preparative scale (100 mg of **1**) in the presence of 50% v/v of these solvents as well as of MeOH (for the sake of comparison). As reported in Table 1, the best results were obtained in the presence of acetone, which allowed an almost quantitative conversion of totarol (96.3%), as shown by the HPLC chromatogram (Figure 2). Even more remarkable, and unexpected, was the effect of the organic solvents on the products composition: the **1a**:**1b** ratio moved from 8.2 with MeOH to 16.8 with CH_3CN and to 25.8 with acetone (Table 1). In each case, we observed that the ratio **1a**:**1b** was not altered if the reaction was stopped at 3, 6 or 24 h.

Laccase-mediated oxidation of totarol was also attempted in biphasic systems, using reaction media comprising 1:1 v/v sodium acetate buffer with the non-miscible solvents AcOEt, $CHCl_3$, methyl *tert*-butyl ether, *t*-amyl alcohol or toluene. The reactions were observed to proceed very slowly, with far lower levels of conversion being achieved even after 4 days. Specifically, no products were observed using methyl *tert*-butyl ether, conversions were lower than 10% using AcOEt, $CHCl_3$ or toluene, and acceptable results (56% conversion, **1a**:**1b** ratio of 3.9) were achieved only with the reaction medium that contained *t*-AmOH. The slow rate of oxidation in a biphasic system can be attributed to insufficient mass transfer of the reactants to and products from the enzyme and between two phases, as suggested by Carrea[11] and Faber.[12]

The influence of the solvent on enzyme selectivity has been widely exploited to optimize the stereoselective performances of hydrolytic enzymes, mainly lipases and proteases, with the so-called "medium engineering".[13] More recently we observed a similar effect on the reaction outcomes of the oxidation of tetrahydro-2-naphthol or 17β-estradiol catalyzed by

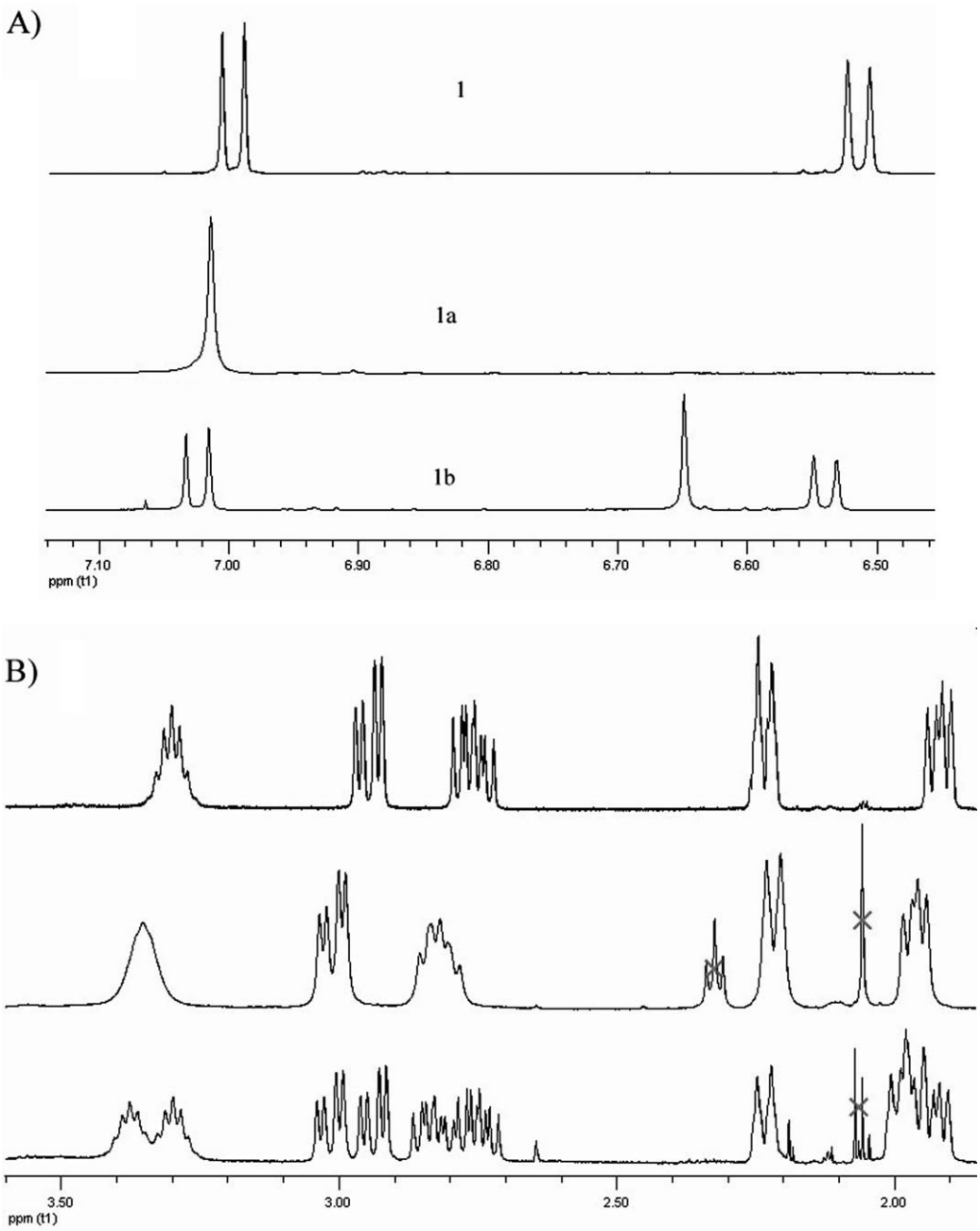
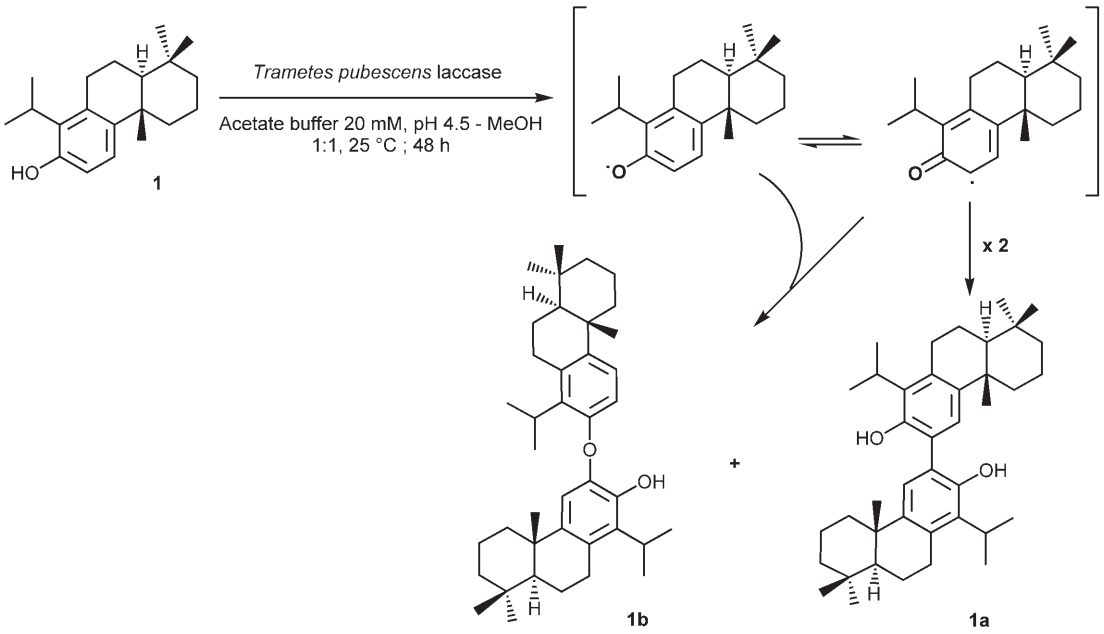


Figure 1. ^{1}H NMR spectra of compounds **1**, **1a** and **1b**, showing (**A**) the aromatic protons and (**B**) a portion of the aliphatic protons.

the laccases from *Trametes pubescens* or *Myceliophthora thermophyla*, where the relative ratios of the dimeric products were strongly affected by the nature of the organic solvent present in the reaction solution.[6a] However, the values of the **1a**:**1b** ratio obtained with totarol, by changing the solvent (3.9, 8.2, 16.8, and 25.8 with *t*-AmOH, MeOH, CH_3CN and acetone, respectively) could have simply originated by different interactions of the different solvent molecules with the radical intermediates during their coupling reactions, without any real interference with the laccase oxidative action. If this had been the case, a similar effect would have been observed using chemical oxidants. To verify this hypothesis, control experiments were run (in triplicate) using $FeCl_3$ (homogeneous solutions) or MnO_2 (heterogeneous system) as catalysts. The results, summarized in Table 2, (visualized by the TLC) clearly showed that the laccase-catalyzed reaction was much more efficient and "cleaner", and that the "solvent effect" on the products ratio was either absent (MnO_2) or very minor ($FeCl_3$) in comparison with the data obtained by enzymatic cat-



Scheme 1. Synthesis and structures of the dimeric products **1a** and **1b** obtained by the laccase-catalyzed oxidation of totarol (**1**).

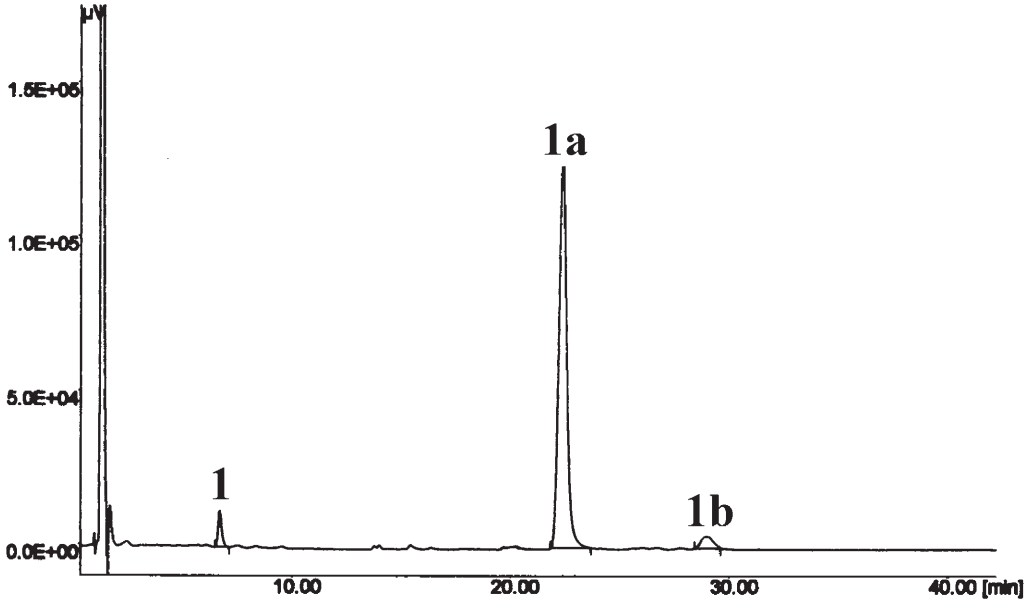


Figure 2. HPLC profile of the T. *pubescens* laccase-catalyzed oxidation of totarol in acetate buffer-acetone 50% v/v after 24 h reaction time.

alysis (Table 2). Additionally, it should be noted that the formation of considerable amounts of unidentified by-products using chemical oxidants makes less significant the comparison of the relative ratio of just two of them (**1a** and **1b**), as one of these two dimers could have undergone a preferential subsequent oxidation by the chemical catalyst (something that, apparently, did not occur in the presence of the laccase). Therefore it could reasonably be concluded that the solvent used for the laccase-catalyzed oxidation of totarol had indeed a significant effect on the course of the enzymatic oxidation, both in terms of degree of conversion and of products composition.

Table 1. Conversion of totarol and products composition of the laccase-catalyzed oxidation in the presence of different cosolvents.[a]

Cosolvent (50% v/v)	Conversion [%]	Products [%] **1a**	**1b**	Ratio of **1a**:**1b**
MeOH	62.6	49.1	6.0	8.2
CH_3CN	71.3	60.3	3.6	16.8
Acetone	96.3	85.0	3.3	25.8

[a] Detected by HPLC after 24 h.

Table 2. Relative ratio of the dimers **1a** and **1b** obtained by totarol oxidation with different catalysts.[a]

Cosolvent (50% v/v)	Ratio of **1a**:**1b**		
	T. pubescens laccase	MnO_2	$FeCl_3$
MeOH	8.2	5.2	13.8
CH_3CN	16.8	5.2	18.6
Acetone	25.8	6.6	18.9
t-AmOH	3.9	n.d.	n.d.

[a] Detected by HPLC after 24 h.

Conclusions

This study has described the synthesis of novel C–C and C–O linked dimers of the biologically active compound totarol *via* oxidative reactions catalyzed by a laccase from *Trametes pubescens* in biphasic or homogenous aqueous-organic media. The dominant product was a symmetrical C–C linked dimer, which was obtained in unusually high yields under optimized reaction conditions and exploiting the "medium engineering" approach; to the best of our knowledge these are by far the best results obtained in the biocatalyzed coupling of phenols to give a product with a defined chemical structure. The laccase-catalyzed oxidation of **1** was achieved under very mild reaction conditions and gave significantly better results in comparison with similar reactions obtained with chemical oxidants. It can be considered a significant example of "sustainable chemistry" performed with environmentally benign protocols.

Experimental Section

Growth of *Trametes Pubescens* for Laccase Production

T. pubescens (CBS 696.94) was grown on agar plates containing 50 g/L malt extract agar, supplemented with laccase inducer [1% phenol mixture (phenol: 82.8 mM, *p*-cresol: 24.99 mM, *m*-cresol: 25.8 mM, *o*-cresol: 77.03 mM)].[14] *T. pubescens* mycelial blocks were aseptically inoculated on the plates and incubated at 28 °C for 6 days. *T. pubescens* was also cultured in *Trametes* Defined Medium (liquid medium) containing 10 g glucose, 10 g peptone bacteriological, and 2 g KH_2PO_4 per liter.[15] The autoclaved medium was distributed into 1000-mL Erlenmeyer flasks and inoculated with a homogenized mycelial plug taken from a solid dish preculture (see above). Cultures were incubated at 28 °C with agitation, for 9 days. These cultures were used to inoculate two 4-L airlift bioreactors each containing 10 g/L glucose, 10 g/L peptone, 0.03% antifoam and 2 g/L KH_2PO_4.[14] After 5 days the medium in the bioreactors was supplemented with 30 mL of phenol mixture and 1 g of glucose. Subsequently 30 mL of phenol mixture and 1 g of glucose were added to the medium daily. The laccase activity was assayed after 6 days (see below). After 13 days, the medium, containing laccase with the activity of 2–3 U mL^{-1}, was harvested from the bioreactors and kept at −20 °C or 4 °C till further use. Some samples were freeze- dried and stored at −20 °C until needed.

Laccase Assay

Laccase activity was determined with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) as the substrate.[16] The assay mixture contained 0.33 mL of 5 mM ABTS, 2.5 mL of 0.1 M sodium acetate buffer (pH 5), and 0.17 mL aliquots of culture fluid. Oxidation of ABTS was monitored by following the increase in absorbance at 420 nm ($\varepsilon = 36\,000\ M^{-1}\,cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize 1 μmol of ABTS per min at 25 °C.

HPLC Analysis

Laccase-catalyzed oxidations of totarol were analyzed by HPLC using a JASCO 880-PU instrument equipped with a Jasco 870 UV detector. Samples were analyzed on a Lichrosper® 100 RP-18 column (4×125 mm Particle SizePore 5.0 μm; Merck). Eluent: from acetonitrile-H_2O 80:20 to acetonitrile-H_2O 100:0 in 10 min; same eluent (100% CH_3CN) for 40 min; flow rate 1 mL min^{-1}; UV detection at 280 nm. Retention times: **1**, 6.72 min; **1a**, 22.34 min; **1b**, 28.87 min.

Laccase-Catalyzed Oxidation of Totarol

A) In methanol/buffer medium under non-optimized conditions: Totarol (**1**, 200 mg) dissolved in 32 mL methanol was added to 32 mL acetate buffer 20 mM pH 4.5 in which the laccase from *Trametes pubescens* (100 mg, 60 U) had been previously dissolved. The milky solution was incubated at 25 °C under mild shaking, following the conversion by TLC (eluent: petroleum ether-AcOEt, 19:1) and HPLC. After 48 h a 39% conversion to two products in an 8:1 ratio was achieved, with the formation of a precipitate that was recovered by filtration. The solution was extracted with AcOEt and the solvent was evaporated to give a residue that showed a composition similar to the precipitate. The overall crude material (200 mg) was purified by flash chromatography (eluent: petroleum ether) to give the products **1a** (yield: 37 mg; 18.6%) and **1b** (yield: 2.5 mg; 1.3%), together with unreacted **1** (109 mg). R_f in TLC (eluent: petroleum ether-AcOEt, 100:0.5): **1**, 0,09; **1a**, 0,15; **1b**, 0,19.

B) In other monophasic reaction media and under ioptimized conditions (see Table 1): Totarol (**1**, 100 mg) dissolved in 16 mL water-miscible organic solvent (methanol, acetonitrile or acetone) was added to 16 mL acetate buffer 20 mM pH 4.5 in which the laccase from *Trametes pubescens* (100 mg, 60 U) had been previously dissolved. The solution was incubated at 30 °C under mild shaking, following the conversion by TLC and HPLC. After 24 h the solution was extracted with AcOEt (which dissolved also most of the solid precipitate) and the solvent was evaporated. The crude material (~100 mg) obtained using acetone as cosolvent was purified by flash chromatography (eluent: petroleum ether-AcOEt, 100:0.5) to give the products **1a** (62 mg) and **1b** (2 mg) with a yield of 64 % and 2 %, respectively.

C) In biphasic system: The reaction mixture contained 10 mg totarol, 40 U *Trametes pubescens* laccase, 0.5 mL AcOEt or $CHCl_3$ or methyl *tert*-butyl ether or *t*-AmOH or toluene and 0.5 mL acetate buffer 20 mM pH 4.5. The reactions were incubated at 30 °C with shaking at 200 rpm, the progress of the reactions being monitored by TLC and HPLC. After 5 days the degrees of conversion (evaluated by HPLC) were 7, 7, 0, 56 or 8 % in AcOEt, $CHCl_3$, methyl *tert*-butyl ether, *t*-AmOH or toluene, respectively. The reaction with *t*-AmOH was scaled-up. Totarol (100 mg) was dissolved in 8 mL *t*-AmOH and added to 16 mL acetate buffer 20 mM pH 4.5 with the laccase from *Trametes pubescens* (400 U). The reaction was incubated at 30 °C by shaking at 220 rpm for 4 days. Following phase separation and extraction of the water phase with $CHCl_3$, the organic phases were mixed, and the solvent was evaporated. The crude material was purified by flash chromatography (eluent: petroleum ether-AcOEt, 25:1) to give a mixture of **1a** and **1b** (34 mg; 34 % overall yield) and two additional new minor by-products (a yellow one, ~1 mg, R_f 0.34 in TLC with the eluent: petroleum ether-AcOEt, 19:1; an orange one, ~1.5 mg, R_f 0,45 in TLC), whose attempted structural characterization resulted unsuccessful.

Effect of Buffer Salts Concentration on the Bioconversion of Totarol

The buffer concentrations were varied from 20 to 100 mM. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *Trametes pubescens* laccase, 1 mL methanol and 1 mL sodium acetate buffer with concentration of 20, 50 or 100 mM at pH 5. The reactions were incubated at 30 °C with shaking at 200 rpm. The progress of the reaction was monitored by HPLC.

Effect pH on the Bioconversion of Totarol

The pH of the reaction medium was varied from 4 to 7 using 20 mM sodium acetate buffer. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *Trametes pubescens* laccase, 1 mL methanol and 1 mL 20 mM sodium acetate buffer.

Effect of Reaction Temperature on the Bioconversion of Totarol

In order to determine the impact of temperature on totarol conversion by a particular dose of enzyme, reactions were conducted at various temperatures. The reaction mixture contained 1 mg totarol, 10 mg (6 U) *Trametes pubescens* laccase, 1 mL methanol and 1 mL 20 mM sodium acetate pH 4.5. The reactions were incubated at 20, 30, 40, and 50 °C , with all other conditions remaining the same.

Product Characterization

NMR spectra were recorded with a Bruker AC400 (400 MHz). High resolution electrospray mass spectra (HR-ESI-MS) were acquired with an FT-ICR (Fourier Transfer Ion Cyclotron Resonance) APEX™ II model (Bruker Daltonics) equipped with a 4.7 Tesla cryo-magnet (Magnex). Samples were dissolved in CH_3CN and injected into the instrument equipped with its own ESI source. Spectra were recorded in the HR mode with resolutions ranging from 20000 to 30000.

Totarol (**1**)*:* TLC, R_f (eluent: petroleum ether-AcOEt 100:0.5): 0.09; HPLC, R_t: 6.72 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.03 (1 H, d, J = 8.5 Hz, H-11), 6.54 (1 H, d, J = 8.5 Hz, H-12), 3.31 (1 H, sept, J = 7.0 Hz, H-15), 2.96 (1 H, dd, J_1 = 17.1 Hz, J_2 = 6.3 Hz, H-7$_{eq}$), 2.78 (1 H, ddd, J_1 = 17.1 Hz, J_2 = 11.5 Hz, J_3 = 7.8 Hz, H-7$_{ax}$), 2.25 (1 H, dt, J_1 = 12.7 Hz, J_2 = 3.7 Hz, H-1$_{eq}$) and 1.36 (1 H, td, J_1 = 12.8 Hz, J_2 = 3.8 Hz, H-1$_{ax}$), 1.94 (1 H, ddt, J_1 = 13.3 Hz, J_2 = 7.8 Hz, J_3 = 1.8 Hz, H-6$_{eq}$) and 1.67 (1 H, m, H-6$_{ax}$), 1.75 (1 H, qt, J_1 = 13.7 Hz, J_2 = 3.4 Hz, H-2$_{ax}$) and 1.61 (1 H,d quint, J_1 = 13.8 Hz, J_2 = 3.7 Hz, H-2$_{eq}$), 1.49 (1 H, ddt, J_1 = 13.2 Hz, J_2 = 3.7 Hz, J_3 = 1.4 Hz, H-3$_{eq}$) and 1.22 (1 H, td, J_1 = 13.4 Hz, J_2 = 4.1 Hz, H-3$_{ax}$), 1.29 (1 H, dd, J_1 = 12.7 Hz, J_2 = 2.2 Hz, H-5), 1.38 and 1.36 (3 H each, d each, J = 7.1 Hz, CH_3-16 and CH_3-17), 1.20 (3 H, s, CH_3-20), 0.97 (3 H, s, CH_3-18) 0.94 (3 H, s, CH_3-19); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 152.6 (C-13), 144.0 (C-9), 134.7 (C-8), 131.7 (C-14), 123.7 (C-11), 115.0 (C-12), 50.3 (C-5), 42.3 (C-3), 40.4 (C-1), 38.4 (C-10), 33.9 (C-18), 29.4 (C-7), 27.8 (C-15), 25.8 (C-20), 22.3 (C-19), 21.1 (C-16 and C-17), 20.2 (C-2), 20.1 (C-6) [for COSY and HSQC spectra see Supporting Information].

Totarol main product **1a** *(symmetrical C–C dimer):* TLC, R_f (eluent: petroleum ether-AcOEt, 100:0.5): 0.15; HPLC, R_t: 22.34 min; ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.03 (2 H, s, H-11 and H-11′), 3.35 (2 H, m, H-15 and H-15′), 3.02 (2 H, dd, J_1 = 17.2 Hz, J_2 = 6.4 Hz, H-7$_{eq}$ and H-7′$_{eq}$), 2.82 (2 H, m, H-7$_{ax}$ and H-7′$_{ax}$), 2.25 (2 H, br d, J = 12.6 Hz, H-1$_{eq}$ and H-1′$_{eq}$), 1.97 (2 H, br dd, J_1 = 13.2 Hz, J_2 = 7.9 Hz, H-6$_{eq}$ and H-6′$_{eq}$), 1.72 (4 H, m, H-6$_{ax}$, H-6′$_{ax}$, H-2$_{ax}$, H-2′$_{ax}$), 1.60 (2 H, br d quint, J_1 = 13.9 Hz, J_2 = 3.4 Hz, H-2$_{eq}$ and H-2′$_{eq}$), 1.49 (2 H, br d, J = 13.1 Hz, H-3$_{eq}$ and H-3′$_{eq}$), 1.40–1.20 (6 H , m, H-1$_{ax}$, H-1′$_{ax}$, H-3$_{ax}$, H-3′$_{ax}$, H-5, H-5′), 1.40 and 1.38 (6 H each, d each, J = 7.1 Hz, CH_3-16 and CH_3-16′, CH_3-17 and CH_3-17′), 1.22 (6 H, s, CH_3-20 and CH_3-20′), 0.99 (6 H, s, CH_3-18 and CH_3-18′), 0.95 (6 H, s, CH_3-19 and CH_3-19′); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 150.4 (C-13 and C-13′), 144.0 (C-9 and C-9′), 135.3 (C-8 and C-8′), 132.7 (C-14 and C-14′), 125.1 (C-11 and C-11′), 121.5 (C-12 and C-12′), 50.3 (C-5 and C-5′), 42.3 (C-3 and C-3′), 40.3 (C-1 and C-1′), 38.5 (C-10 and C-10′), 34,0 (C-4 and C-4′), 33.9 (C-18 and C-18′), 29.5 (C-7 and C-7′), 28.4 (C-15 and C-15′), 26.0 (C-20 and C-20′), 22.3 (C-19 and C-19′), 20.9 (C-16 and C-16′, C-17 and C-17′), 20.2 (C-2 and C-2′), 20.1 (C-6 and C-6′) [for COSY and HSQC spectra see Supporting Information]; ESI-MS, positive mode: m/z = 593.43389 $[M+Na]^+$, calcd.: 593.43290.

Totarol by-product **1b** *(C–O dimer):* TLC, R_f (eluent: petroleum ether-AcOEt, 100:0.5): 0.19; HPLC, R_t: 28.87 min; ^{1}H NMR (400 MHz, $CDCl_3$, selected data): $\delta = 7.04$ (1H, d, $J = 8.8$ Hz, H-11), 6.67 (1H, s, H-11′), 6.56 (1H, d, $J = 8.8$ Hz, H-12), 3.38 and 3.30 (1 H each, m, H-15 and H-15′), 3.02 and 2.95 (1 H each, dd each, $J_1 = 16.9$ Hz, $J_2 = 6.1$ Hz, H-7$_{eq}$ and H-7′$_{eq}$), 2.84 and 2.75 (1 H each, ddd each, $J_1 = 16.9$ Hz, $J_2 = 9.1$ Hz, $J_3 = 5.3$ Hz,, H-7$_{ax}$ and H-7′$_{ax}$), 1.42 and 1.41, 1.40 and 1.38 (3 H each, d each, $J = 7.1$ Hz, CH_3-16 and CH_3-16′, CH_3-17 and CH_3-17′), 1.22 and 1.14 (3 H each, s, CH_3-20 and CH_3-20′), 0.99 and 0.96 (3 H each, s, CH_3-18 and CH_3-18′), 0.95 and 0.91 (3H each, s, CH_3-19 and CH_3-19′); ^{13}C NMR (100 MHz, $CDCl_3$): $\delta = 148.2$ and 146.1 (C-13 and C-13′), 145.0 and 143.0 (C-9 and C-9′), 135.7 and 134.8 (C-8 and C-8′), 132.4 (C-14). 128,9 (C-14′), 123.9 (C-11), 115.7 (C-12), 113.3 (C-11′), 50.5 and 50.3 (C-5 and C-5′), 42.3 (C-3 and C-3′), 40.3 (C-1 and C-1′), 38.6 (C-10 and C-10′), 34.0 (C-4 and C-4′), 33.9 (C-18 and C-18′), 29.5 and 29.1 (C-7 and C-7′), 28.5 and 28.2 (C-15 and C-15′), 25.8 (C-20 and C-20′), 22.3 (C-19 and C-19′), 21.8 and 20.9 (C-16 and C-16′, C-17 and C-17′), 20.1 (C-2 and C-2′, C-6 and C-6′) [for COSY and HSQC spectra see Supporting Information]; ESI-MS, positive mode: $m/z = 593.43130$ $[M+Na]^+$, calcd.: 593.43290.

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