Significant and Unexpected Solvent Influence on the Selectivity of Laccase-Catalyzed Coupling of Tetrahydro-2-naphthol Derivatives

Annalisa Intra,[a,b] Silvia Nicotra,[a] Sergio Riva,[a,*] Bruno Danieli[b]

[a] Istituto di Chimica del Riconoscimento Molecolare, C. N. R., Via Mario Bianco 9, 20131 Milano, Italy
Fax: (+39)-02-2850-0036, e-mail: Sergio.riva@icrm.cnr.it
[b] Dipartimento di Chimica Organica e Industriale, Università di Milano, Via Venezian 21, 20133 Milano, Italy

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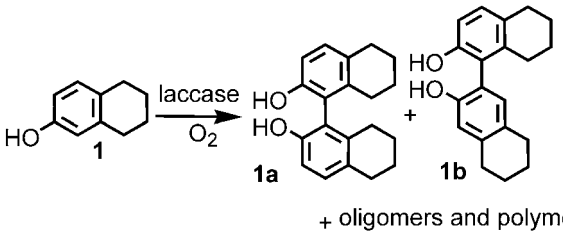
Abstract: The relative ratio of the dimers obtained by laccase-catalyzed oxidation of tetrahydronaphthyl derivatives was profoundly influenced by the nature of the organic solvent used. For instance, in the oxidation of 5,6,7,8-tetrahydronaphtalen-2-ol (**1**), the formation of the symmetrical 1,1′ product **1a** with respect to the 1,3′ dimer **1b** was highly favored in the aromatic solvents benzene and toluene.

Keywords: biotransformations; laccase; organic solvents; oxidation; phenols; radicals

Laccases are a group of ubiquitous enzymes belonging to the multinuclear copper-containing oxidases.[1] The search for new, environmentally benign processes for the textile and for the pulp and paper industries[2] has increased the interest for these "ideally green" enzymes that work with air and produce water as the only by-product in a catalytic cycle during which four substrate molecules suffer monoelectronic oxidation to the corresponding radicals.[3] Typical laccase substrates are amines (aliphatic and aromatic) and phenols. As the dimers of the latter compounds (e.g., BINOL[4] and H_8-BINOL[5]) are of paramount importance as ligands in modern asymmetric catalysis, laccases-catalyzed dimerization of naphthyl or of 5,6,7,8-tetrahydronaphthyl derivatives might open an access to new ligands.

In the late 1970s a laccase from *Polyporus versicolor* was shown to oxidize 17β-estradiol to a mixture of dimeric, oligomeric and polymeric products.[6] Being interested in reinvestigating this kind of biotransformation, initially a model substrate, 5,6,7,8-tetrahydronaphthalen-2-ol (**1**), was chosen to be submitted to the action of the available laccase from *Myceliophtora thermophyla* (MtL).[7] The reactions were performed in organic solvents,[8] while trying to overcome the extensive polymerization of the reactive radical intermediates (generated either by metal ions or by enzymes), and specifically exploiting the protocol developed years ago for the oxidative reactions catalyzed by tyrosinases.[9]

Accordingly, MtL was adsorbed on glass beads or on celite following standard procedures,[9,10] and suspended in different saturated organic solvents. Conversions were generally high (see Table 1), but a mixture of the dimeric derivatives **1a** and **1b** could be isolated only in up to 14% yields due to persisting significant polymerization. To our surprise, however, the relative ratio of the two dimers was profoundly influenced by the solvent used: the symmetrical 1,1′ dimer **1a** was the highly favored product in benzene and toluene, while a small selectivity was observed in *tert*-amyl alcohol (Table 1). In all cases the dimers **1a** and **1b** were formed as racemic mixture of enantiomeric atropisomers.



Scheme 1. Laccase-catalyzed dimerization of 5,6,7,8-tetrahydronaphthalen-2-ol (**1**).

The orienting effect of the aromatic solvents on the **1a/1b** ratio was confirmed by carrying out the oxidation in a series of substituted benzene derivatives: in all cases but one this ratio was higher than that obtained in the "best" non-aromatic solvent. Interesting data were also obtained by using mixture of solvents: the **1a/1b** ratio decreased smoothly on moving from 100% toluene (8.4) to 100% AcOEt (4.8) or methyl *tert*-butyl ether (2.9), while the addition of just 25% v/v of *tert*-amyl alcohol was enough to make the 75% v/v toluene ineffective (2.3 *vs.* 1.9 in pure *tert*-amyl alcohol).

Control experiments were run using chemical oxidants.[11] As an example, Table 1 (last column) shows the results obtained with MnO_2 suspended in six differ-

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Table 1. Oxidation of **1** in different organic solvents.[a]

Solvent	% conversion[b]	**1a/1b** ratio[c]	
		Mt laccase[d]	MnO_2[e]
Benzene	93.1	11.5	2.3
Toluene	95.3	9.3	1.8
$CHCl_3$	51.8	6.0	2.1
AcOEt	18.6	4.7	1.6
Methyl *tert*-Butyl Ether	13.2	3.9	2.6
tert-Amyl Alcohol	66.5	2.4	1.6
1,2-Dichlorobenzene	64.5	9.7	
Nitrobenzene	46.8	9.1	
Chlorobenzene	63.1	7.7	
Propylbenzene	62.0	6.7	
tert-Butylbenzene	65.5	4.1	

[a] A mixture of **1** (15 mg), organic solvent (1 mL), tetradecane (as internal standard for GC analysis, 5 μL) and an appropriate amount of enzyme or chemical catalyst was shaken for a definite time. Each reaction was repeated at least three times.
[b] Determined after 24 h by GC analysis: initial *T*: 120 °C, initial *t*: 1 min, rate: 2 °C/min, final *T*: 180 °C (t_r, tetradecane: 14.0 min, **1**: 16.1 min).
[c] Determined by GC: initial *T*: 200 °C, initial *t*: 1 min, rate: 5 °C/min, final *T*: 300 °C (t_r: **1a**: 14.1 min, **1b**: 16.1 min).
[d] The organic solvents were presaturated with a 50 mM TRIS buffer at pH 6.5 and 10 μL of the same buffer (1% v/v) were also added. Enzyme: 50 mg of MtL adsorbed on glass powder (very similar results were obtained with 20 mg of MtL adsorbed on celite); *T*: 45 °C.
[e] MnO_2, 5 mg (heterogeneous system), room temperature.

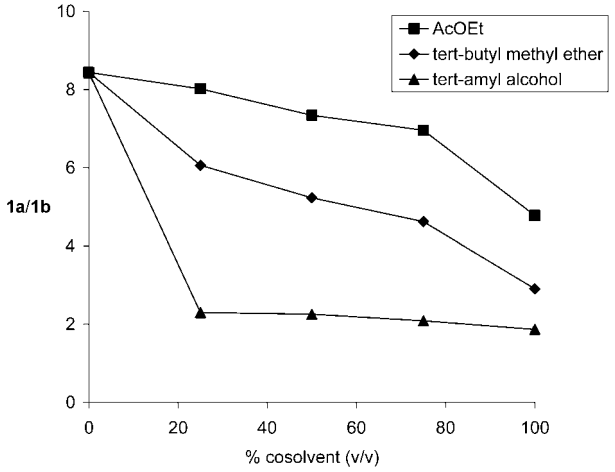


Figure 1. Oxidation of **1** catalyzed by MtL in mixtures of toluene and organic cosolvents (each reaction was repeated twice).

ent solvents: as expected the **1a/1b** ratio (slightly in favor of the symmetrical dimmer) was not appreciably effected by the solvent used. Other oxidants like $K_3Fe(CN)_6$ or $Mn(OAc)_3$ behaved similarly, and the same results were obtained using the latter oxidant dissolved in the reaction solvents by adding a minimum volume of AcOH. Furthermore, to exclude a possible influence of the used materials, the MnO_2-catalyzed oxidation of **1** was performed in the presence of "nude" glass beads or of albumin adsorbed on glass beads: in all cases the **1a**/**1b** values were similar to the data previously reported in the last column of Table 1. Finally, we also considered the possibility that the two dimers were formed at similar rates and then further oxidized and degraded by MtL with different rates in the different solvents. This hypothesis was also ruled out by monitoring the MtL-catalyzed oxidation of **1** in toluene: the **1a**/**1b** ratio did not change as a function of the reaction's progress.

This set of experimental data clearly indicates that organic solvents influence the outcome of laccases-mediated oxidation of phenol derivatives. This finding was quite unexpected. In fact, while it is commonly accepted that substrate oxidation takes place in the active site at the so-called T-1 copper atom of the metal cluster and that the subsequent radical coupling takes place "out of sphere" (therefore without any orienting effect due to the protein structure), our data indicate that the enzyme molecule is also involved in the coupling of the phenolic radicals.

The finding that the nature of the organic solvents profoundly influences enzyme's selectivity was a significant breakthrough for the synthetic application of biocatalysis.[12] This so-called "medium engineering" approach has been widely exploited as a very simple tool to optimize the performances of lipases and proteases in the kinetic resolutions of racemates and in the desymmetrization of *meso* compounds,[12] and scant data have also been reported on the effect on the regioselectivity of the same enzymes.[13] However, despite the reasonable claim that the influence of organic solvents on enzyme selectivity should be a phenomenon of general validity, up to now all the reports have been related to hydrolases.

The search for an exhaustive rationale for this effect is still the object of scientific debate. Nevertheless, signifi-

cant evidence at a molecular level came from a series of crystallographic data reported by Klibanov.[14] In these works it was shown that molecules of the organic solvents were able to penetrate into the active site of the protease subtilisin, displacing some of the water molecules present. Quite significantly, molecules of different solvents were found to be located in different regions of the active site and, additionally, this happened not only in pure organic solvents but also in mixtures of organic solvents and water (i.e., dioxane-water 40:60 v/v).[14b] This latter important observation prompted us to study the oxidation of **1** in biphasic systems, where the laccase would have been solubilized (and therefore confined) in the water phase. Any solvent effect on the **1a**/**1b** ratio might only be due to molecules of solvents associated to the enzyme's active site, as the results previously obtained with MnO_2 (Table 1) already ruled out a solvent influence on the radical coupling in the organic phase. The hypothesis that even in biphasic systems some solvent molecules might be adsorbed into the enzyme's active site was not in contrast to the crystallographic data of a different laccase (from *Coprinus cinereus*), which showed that the organic substrate binding site is very close to the surface of the protein and is easily accessible to the solvent.[15]

As shown in Table 2, we were pleased to find that the preferential formation of **1a** in aromatic solvents was still clearly measurable and, additionally, similar data were obtained with another laccase from the fungus *Trametes pubescens* (TpL).[16]

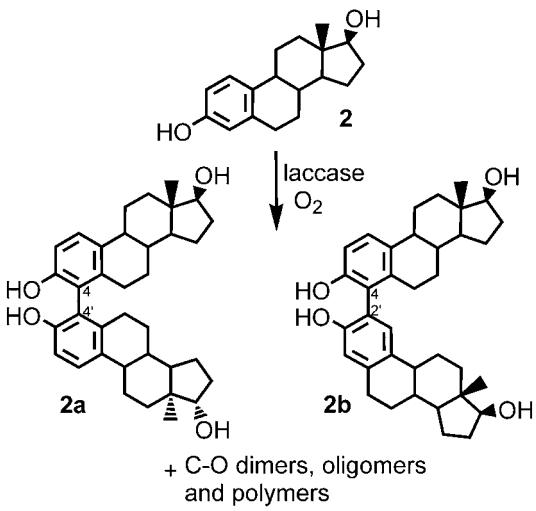
With these data in hand, we moved our attention to the laccase-catalyzed oxidation of 17β-estradiol (**2**), which was performed in a biphasic system using AcOEt as organic phase because of the low solubility of this steroid in other solvents. A mixture of C–O and C–C dimers was obtained, from which the latter could be isolated in 13% yield.[17]

As far as the C–C dimers **2a** and **2b** are concerned, their ratio was in favor of the 2,4′ dimer **2b** in the MtL-catalyzed reaction and even more so in the TpL-mediated oxidation (**2b**/**2a** ratios were 2.1 and 3.6, respectively). Therefore, at variance with **1**, the unsymmetrical 2,4′ dimer prevailed, thus outlining the influence of the substrate structure on the reaction outcome. On the other hand, the MnO_2-catalyzed chemical oxidation gave, again, a mixture of the two dimers almost without selectivity (**2b**/**2a** ratio was 1.3).

Table 2. Oxidation of **1** in biphasic systems.[a]

Organic phase	**1a/1b** ratio[b]	
	Mt laccase[c]	Tp laccase[d]
Benzene	7.2	6.6
Toluene	6.2	5.6
$CHCl_3$	5.6	5.0
AcOEt	2.0	1.0
Methyl *tert*-Butyl Ether	1.3	1.2
tert-Amyl Alcohol	1.3	1.2

[a] A biphasic system made of the respective organic solvent (1 mL) containing **2** (15 mg), and of a buffer (1 mL), containing the enzyme (25 units), was gently shaken for 48 h. Each reaction was repeated three times.
[b] Determined by GC, see footnotes to Table 1.
[c] Water phase: 50 mM TRIS buffer, pH 6.5.
[d] Water phase: 20 mM acetate buffer, pH 4.5.



Scheme 2. Laccase-catalyzed dimerization of 17β-estradiol (**2**).

As a final remark, it has to be noted that the selective preparation of compounds like **2a** and **2b** is of biomedical interest to study the action of estrogen metabolites and of synthetic relevance for the production of new potential ligands for metal organic catalysts and scaffolds for supramolecular chemistry.[18]

In conclusion, in this communication we have described, for the first time, a significant and unexpected solvent influence on the coupling of phenols catalyzed by laccases, thus confirming the general validity (not limited to hydrolases) of the "medium engineering" approach to influence enzymatic performance. Finally, it has to be pointed out that the isolated yields of the dimers **1a, b** and **2a, b**, albeit quite low, compare well and are even higher than the data reported for other chemical or enzymatic oxidations.[11,18]

Experimental Section

Materials and Methods

The laccase from *Myceliophthtora thermophyla* (MtL) was from Novozymes, while the laccase from *Trametes pubescens* (TpL) was provided by Prof. Haltrich (Vienna University, Austria). TLC: precoated silica gel 60 F_{254} plates (Merck). Flash chromatography: silica gel 60 (70–230 mesh, Merck). HPLC analysis: Jasco HPLC instrument (model 880-PU pump, model 870-UV/VS detector, λ: 200 nm) and a Lichrospher 100 RP-18

(5 μm, Merck) reverse phase analytical column or a Chiralcel OD column. HPLC purification: Partisil 10 ODS-3 column (Whatman). ^{1}H and ^{13}C NMR spectra at 300 MHz and 75.2 MHz were recorded on a Bruker AC-300. GC analyses were performed using a capillary methylsilicone column (HP-1 Crosslinked Methyl Silicone Gum, 25 m × 0.32 mm ID × 0.52 μm film thickness, Hewlett-Packard) and a Hewlett-Packard 5890 series II instrument.

Evaluation of Laccase Activity

MtL activity was evaluated spectrophotometrically by measuring the increase of adsorbance at 530 nm of a solution of syringaldazine. In a 3-mL cuvette were charged: 2.63 mL of a 25 mM phosphate buffer at pH 6.5; 0.22 mL of a 0.224 mM solution of syringaldazine [obtained by diluting with the same buffer a 0.56 M (202 mg/mL) mother solution in EtOH], 0.15 mL of the enzymatic solution to be tested (typically obtained by dissolving 5 mg of the MtL preparation in 0.5 mL of buffer and diluting 1 μL of this solution in 1 mL of a 5% solution of PEG-5000 in the same buffer).

TpL activity was evaluated spectrophotometrically by measuring the increase of adsorbance at 436 nm of a solution of ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt]. In a 1-mL cuvette were charged: 0.89 mL of a 20 mM acetate buffer at pH 3.5, 0.1 mL of a 10 mM solution of ABTS, 0.01 mL of the enzymatic solution to be tested.

Adsorption of the Laccase from *M. thermophyla* on Glass Beads

MtL was dialyzed against 25 mM phosphate buffer pH 6.5 and lyophilized. The enzyme was redissolved in the same buffer (60 mg/mL, ~ 600 U/mL) and the solution was dropped onto glass beads (1 mL/3 g). The slurry was mixed and left to dry at room temperature with occasional mixing for nearly two days, to get a solid with ~ 0.2 U/mg.

Oxidation of 5,6,7,8-Tetrahydronaphthalen-2-ol (1) Catalyzed by MtL

The substrate (500 mg) dissolved in 33 mL of toluene (presaturated with 50 mM TRIS buffer, pH 6.5), 330 μL of the same buffer (1% v/v) and tetradecane (33 μL, internal standard for GC analysis) were added to 1 g of MtL supported onto glass beads and the suspension was shaken at 45 °C. The reaction was monitored by GC analysis and after 24 hours the glass beads, covered by a brown precipitate, were separated by filtration, the solvent was evaporated and the crude residue purified by flash chromatography (eluent, petroleum ether-AcOEt, 9:1) to give 70 mg of a mixture of **1a** and **1b** (14% yield). The ratio between the dimers **1a** and **1b** was determined by GC and by RP-HPLC (analytical, eluent, acetonitrile-water, 1:1, flow rate 1 mL/min, λ = 281 nm, t_r, **1**: 2.4 min, **1a**: 9.7 min, **1b**: 11.0 min). They were isolated as pure compounds by preparative RP-HPLC (eluent, acetonitrile-water, 1:1; flow rate 4 mL/min, λ = 281 nm, t_r **1**: 26.2 min, t_r **1a**: 81.1 min, t_r **1b**: 91.2 min), obtaining 26 mg of **1a** and 3 mg of **1b**.

HPLC analysis using a Chiralcel OD column (eluent, hexane-*i*-PrOH, 9:1, flow rate 0.5 mL/min, λ = 254 nm) allowed the separation of the respective enantiomeric atropoisomers: **1a** at 11.67 and 13.17 min; **1b** at 11,67 and 12.98 min, present as equimolar (racemic) mixtures.

Characterization of 1a and 1b

1a: ^{1}H NMR ($CDCl_3 + D_2O$): δ = 7.12 (d, 2H, J = 8.36 Hz, ArH), 6.84 (d, 2H, J = 8.18 Hz, ArH), 4.49 (s, 2H, OH), 2.80–1.60 (m, 16H, aliphatic H); ^{13}C NMR ($CDCl_3$, APT): δ = 151.35 (C-2 and C-2′), 137.13, 130.12, 118.78 (C-1 and C-1′, C-9 and C-9′, C-10 and C-10′), 131.09, 112.95 (C-4 and C-4′, C-3 and C-3′), 29.22, 27.10, 22.98 (C-5 and C-5′, C-6 and C-6′, C-7 and C-7′, C-8 and C-8′).

1b: ^{1}H-NMR ($CDCl_3 + D_2O$): δ = 7.01 (d, 1H, J = 8.2 Hz, ArH), 6.83 (d, 1H, J = 8.8 Hz, ArH), 6.78 (s, 1H, ArH), 6.70 (s, 1H, ArH), 4.72 (s, 1H, OH), 4.61 (s, 1H, OH), 2.8–1.5 (m, 16H, aliphatic H); ^{13}C NMR ($CDCl_3$, APT): δ = 151.69, 151.13 (C-2 and C-2′), 139.62, 137.42, 130.20, 129.72, 120.38, 117.65 (C-3′, C-1, C-9, C-9′, C-10, C-10′), 131.02, 130.78, 115.82, 112.87 (C-4, C-4′, C-3, C-1′), 29.44, 29.27, 28.53, 27.60, 23.32, 23.03 22.94 (C-5, C-5′, C-6, C-6′, C-7, C-7′, C-8, C-8′).

Oxidation of 5,6,7,8-Tetrahydronaphthalen-2-ol (1) Catalyzed by TpL

The substrate (200 mg) was dissolved in 14 mL of toluene, while the laccase (266 U) was dissolved in 14 mL of 20 mM acetate buffer, pH 4.5; tetradecane (70 μL) was added as internal standard for GC analysis. The biphasic system was shaken at room temperature for 48 hours. The organic solvent was evaporated and the crude residue was purified by flash chromatography (eluent: petroleum ether-AcOEt, 9:1) to give 15 mg of a mixture of **1a** and **1b** (8% yield).

Chemical Oxidation of 5,6,7,8-Tetrahydronaphthalen-2-ol (1)

The substrate (1 g) was dissolved in THF (33 mL) and a solution of $K_3Fe(CN)_6$ (0.37 M) in NaOH 0.1 M (18.5 mL) was added. The biphasic system was shaken at room temperature. After 24 hours the reaction was stopped, the water phase was acidified with 2 N HCl and extracted with ethyl acetate, the organic phase was evaporated and the crude residue was purified by flash chromatography (eluent: petroleum ether-ethyl acetate, 9.1) to give 67 mg of a mixture of **1a** and **1b** (7% yield). The two dimers were separated by preparative reverse phase HPLC to give 11 mg of **1a** and 18 mg of **1b**.

Determination of the 1a/1b Ratio of the Products Obtained by Chemical Oxidation of 1

In the general protocol the reaction mixtures, containing 5,6,7,8-tetrahydronaphthalen-2-ol (15 mg), the organic solvent (1 mL) and an appropriate amount of the oxidizing agent, were shaken at room temperature. The chemical oxidant was MnO_2 (5 mg, heterogeneous system), or $Mn(OAc)_3$ (9 mg, heteroge-

neous system, or 9 mg dissolved in 100 μL AcOH, homogeneous system) or MnO_2 in the presence of glass powder and albumin (5 mg of MnO_2 and 50 mg of glass powder or 50 mg of albumin adsorbed on glass powder).

Oxidation of 17β-Estradiol (2) Catalyzed by TpL

17β-Estradiol (**2**, 150 mg) was dissolved in 15 mL of AcOEt, while the laccase (100 U) was dissolved in 15 mL of 20 mM acetate buffer, pH 4.5. The biphasic system was shaken at room temperature for 48 h. The two phases were separated, the organic solvent evaporated and the crude residue purified by flash chromatography (eluent: petroleum ether-AcOEt-MeOH, 7:3:0.5) to give 20 mg of a mixture of **2a** and **2b** (13.3% yield) and 20 mg of other by-products. TLC: R_f **2**: 0.38; **2a** and **2b**: 0.15.

Compounds **2a** and **2b** could be separated by RP-HPLC; each of them consisted of a couple of diastereomeric atropoisomers that could also be separated. RP-HPLC, analytical (eluent, acetonitrile-H_2O, 1:1; flow rate 0.5 mL/min, λ = 280 nm): t_r **2**: 3.7 min; **2a′**: 6.4 min; **2a″**: 14.6 min; **2b′**: 9.2 min; **2b″**: 11.4 min. RP-HPLC, preparative (eluent, acetonitrile-H_2O, 1:1; flow rate 4 mL/min, λ = 290 nm): t_r **2a′**: 42 min; **2a″**: 126 min; **2b′**: 61 min; t_r **2b″**: 71 min.

The symmetrical dimeric atropoisomers **2a′** and **2a″** could be isolated as pure compounds, while the asymmetrical dimeric atropoisomers **2b′** and **2b″** interconverted during the isolation process.[17]

Characterization of Products 2

2a′: ^{1}H NMR ($CDCl_3 + D_2O$): δ = 7.33 (d, 2H, J = 8.7 Hz, H-1 and H-1′), 6.87 (d, 2H, J = 8.7 Hz, H-2 and H-2′), 3.73 (t, 2H, J = 8.4 Hz, H-3 and H-3′), 0.78 (s, 6H, CH_3-18 and CH_3-18′); EI-MS: m/z = 542 (M^+), 524.

2a″: ^{1}H NMR ($CDCl_3 + D_2O$): δ = 7.31 (d, 2H, J = 8.6 Hz, H-1 and H-1′), 6.86 (d, 2H, J = 8.6 Hz, H-2 and H-2′), 3.72 (t, 2H, J = 8.4 Hz, H-3 and H-3′), 0.78 (s, 6H, CH_3-18 and CH_3-18′). MS (EI, 70 eV): m/z = 542 (M^+).

2b: ^{1}H NMR ($CDCl_3 + D_2O$): δ = 7.33 (d, 2H, J = 8.61 Hz, H-1), 6.89 (d, 1H, J = 8.54 Hz, H-2 in **2b′**), 6.88 (d, 1H, J = 8.60 Hz, H-2 in **2b″**), 7.03 (s, 1H, H-1′ in **2b′**), 7.02 (s, 1H, H-1′ in **2b″**), 6.80 (s, 1H, H-4′ in **2b′**), 6.78 (s, 1H, H-4′ in **2b″**), 4.70 (s, 4H, phenolic OH's both in **2b′** and **2b″**), 3.75 (t, 2H, J = 3.39 Hz, H-17 and H-17′), 0.75 (12H, s, CH_3-18); ^{13}C-NMR ($CDCl_3$, APT): δ = 127.848, 127.504 (C-1′, **2b′** and **2b″**), 127.126 (C-1), 115.894, 115.802 (C-4′, **2b′** and **2″**), 112.75 (C-2, **2b′** and **2b″**), 115.748 (C-3 and C-3′), 139.376, 137.342, 133.807, 133.192, 117.592 (C-4, C-5, C-10, C-2′, C-5′, C-10′), 81.755 (C-17 and C-17′), 50.027 (C-14 and C-14′), 44.240, 44.051 (C-9, C-9′), 38.738, 38.308 (C-8, C-8′), 11.072 (C-18 and C-18′), 43.208, 36.717, 29.528, 28.450, 26.973, 26.489 26.355, 23.131, 23.073; MS (EI, 70 eV): m/z = 542 (M^+).

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