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Laccase-mediated oxidation of the steroid hormone 17β-estradiol in organic solvents

Silvia Nicotra,[a] Annalisa Intra,[a,b] Gianluca Ottolina,[a] Sergio Riva[a,*] and Bruno Danieli[b,†]

[a]*Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Via Mario Bianco 9, 20131 Milano, Italy*
[b]*Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano, Via Venezian 21, 20133 Milano, Italy*

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Abstract—Laccase-mediated oxidation of the steroid hormone 17β-estradiol **1** in organic solvents or in a biphasic system allowed the isolation of the C–C and C–O dimers **1a**–**d**. Concerning the C–C dimers, the relative ratio of the symmetric 4-4′ **1c** and asymmetric 4-2′ **1d** products was influenced by the catalyst used. Both **1c** and **1d** were formed as an equimolar mixture of diastereomeric atropisomers.

1. Introduction

Laccases are copper-containing enzymes, which catalyze the oxidation of a variety of substrates with the concomitant reduction of molecular oxygen to water.[1] They have been used in bioremediation processes or as useful adjuvants in the textile, pulp, and paper industries[2] and, more recently, as 'green' catalysts for organic synthesis.[3] In this respect, the selective modifications of the target molecules have been achieved either by direct oxidation of aromatic OH or NH_2 groups,[3] or via the preliminary oxidation of the so-called chemical mediators (i.e., Tempo).[4]

Expanding our range of biocatalyzed selective modifications of natural compounds, we have recently started an investigation on the performances of these enzymes. As a first example, we have reported the laccase-mediated oxidation of the stilbenic phytoalexin resveratrol to give a *trans*-dehydrodimer as the only isolated product.[5] As a further significant target for these enzymes, we have chosen the steroid hormone β-estradiol **1**, as the reactions catalyzed by laccases might be representative of those occurring in vivo to metabolize this compound and thus lead to the identification of reaction products of biological relevance. Additionally, the very low water solubility of **1** made this compound an ideal target to assess the performances of these enzymes in non-natural organic media.[6]

2. Results and discussion

Following the protocol used for the oxidation of resveratrol,[5] the enzyme obtained from a *Mycelyopthora* strain (*ML*) was adsorbed on glass beads and added to a solution of **1** in a suitable organic solvent. After experimentation, a 1:3 mixture of dioxane and water-saturated toluene was chosen, and the suspension shaken for few days at 45 °C. As shown in Figure 1, laccase oxidation of **1** generates an oxygen radical that can delocalize to carbon-located radicals. Subsequent coupling of these reactive intermediates produces C–C or C–O dimers, which could be further oxidized to generate oligomers and polymers. Accordingly, in addition to a brown precipitate (due likely to the formation of insoluble oligomeric and polymeric derivatives of **1**), TLC analysis of the reaction showed the formation of two products more polar than β-estradiol. These compounds were isolated by flash chromatography and analyzed by mass spectrometry: the observed mass values of the molecular peaks (542 *m*/*z* in both cases) were consistent with a dimeric structure for all the derivatives obtained.

Preliminary ^{1}H NMR analysis indicated that both the isolated products were a mixture of compounds, while a more detailed investigation (using COSY correlation) allowed us to prove that the less polar product (R_f 0.25) was a mixture of the C–O dimers **1a** and **b** in a

* Corresponding author. Tel.: +39-02-2850-0032; fax: +39-02-2850-0036; e-mail: sergio.riva@icrm.cnr.it
† Co-corresponding author. E-mail: bruno.danieli@unimi.it

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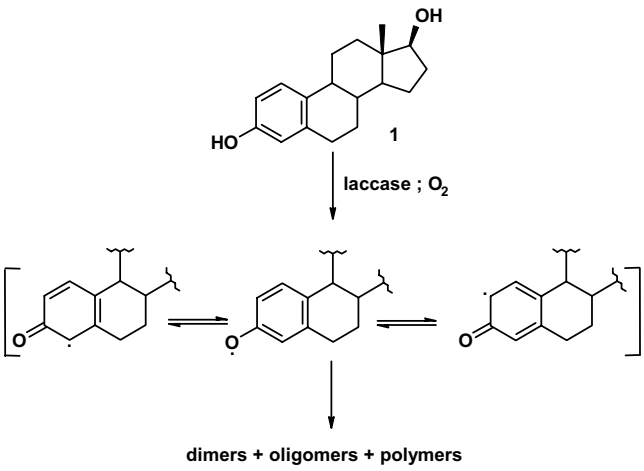

Figure 1. Laccase-mediated oxidation of β-estradiol **1**.

1.6:1 ratio. In addition to the signals due to two intact A-rings (two doublets, with $J=8.4$ and 2.8 Hz, respectively, and one doublet of doublet), the ^{1}H NMR spectrum of the mixture showed an additional AB system (structure **1a**) and two singlets (structure **1b**) for the corresponding A′-rings (Fig. 2). The more polar product (R_f 0.15) gave a very complex ^{1}H NMR spectrum (see the following discussion) and was instead a mixture of the C–C dimers **1c**–**d** in a 2.1:1 ratio.

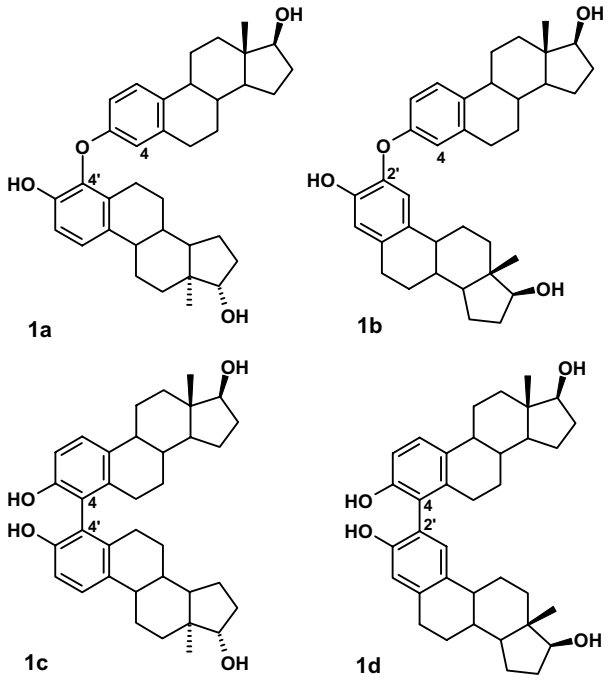

Figure 2. Structures of dimeric products **1a**–**d**.

Qualitatively similar results were obtained using a different laccase, isolated from a *Trametes* strain (*TL*). In this case the reaction was performed in a biphasic system made of buffer and AcOEt, as this enzyme did not retain its activity once adsorbed on glass beads.[5]

All the subsequent attempts to separate the two C–O dimers **1a** and **b** were unsuccessful, while the C–C dimers were easily split by RP-HPLC. Figure 3 shows the RP-HPLC chromatogram of the mixture of **1c** and **1d** dimers obtained from the reaction catalyzed by *TL* (part A). For comparison, the results obtained with MnO_2, a typical chemical oxidant, are also reported (Fig. 3, part B). A similar equimolar formation of four products was also observed with other chemical oxidants like $Mn(OAc)_3$ or $K_3Fe(CN)_6$.[7]

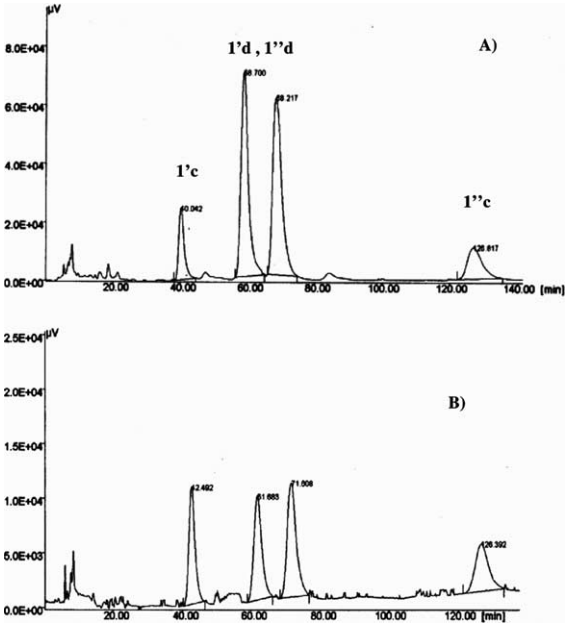

Figure 3. RP-HPLC of **1c**–**d** mixtures obtained from reactions catalyzed by *T. pubescens* laccase (part A) or MnO_2 (part B).

We hypothesized that the presence of four peaks (two by two equimolar in the enzymatic reactions) might be due to the atropoisomerism resulting from the restricted rotation around the bond connecting the A- and A′-rings of the two dimers and the presence of several other stereogenic centers generating couples of diastereomeric atropoisomers differently eluted by the nonchiral RP-column. To confirm this hypothesis and to characterize the compounds, the **1c** and **1d** mixtures were separated by semi-preparative RP-HPLC. The less retained peak and the more retained one were isolated in pure form and proved to be two atropoisomers of the symmetric C–C dimer **1c**, that is **1′c** and **1″c**. Their NMR spectra were, as expected, equivalent and showed the presence of just one set of signals due to the symmetry of the structures. Two doublets, integrating two protons each, due to the AB systems on the A- and A′-ring, and one triplet integrating two protons, due to H-17 and H-17′, were the more significant signals. Figure 4 shows the CD spectra of the two compounds that, as expected, looked like the ones of the two pseudo-enantiomeric compounds. From the respective CD spectra, using the methodology proposed by Mason et al.,[8] it was possible to tentatively assign the structures **1′c** and **1″c** (Fig. 5A) to the less retained and more retained peak, respectively.

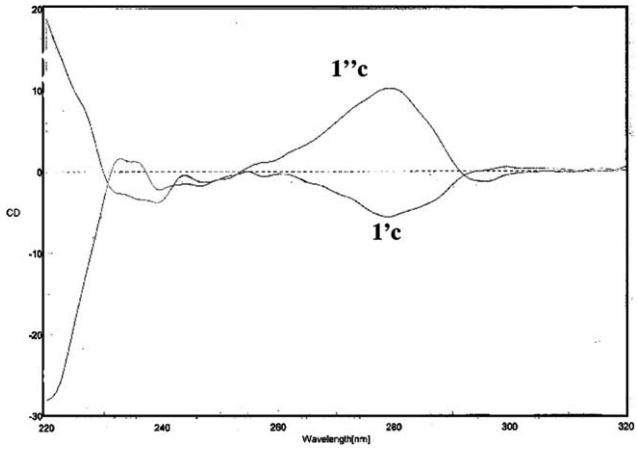

Figure 4. CD spectra of the diastereomeric atropoisomers **1′c** and **1″c**.

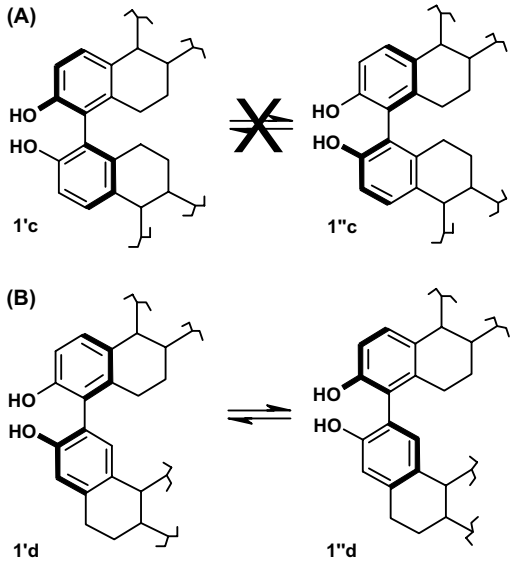

Figure 5. Interconversion of the dimeric atropoisomers of **1c** and **1d**.

On the contrary, the two atropoisomers of the asymmetric C–C dimer **1d** could not be obtained in pure form. Despite the fact that the corresponding peaks were differently retained in the RP-column, compounds **1′d** and **1″d** (Fig. 5B) were isolated as a mixture at the end of the chromatographic process. In fact, the NMR spectrum showed the presence of the two atropoisomers in an equimolar ratio: most of the signals due to the aromatic protons were split and therefore H-2 was present as two doublets at 6.89 and 6.88 ppm ($J = 8.5$ Hz), H-1′ as two singlets at 7.03 and 7.02, and H-4′ as two singlets at 6.80 and 6.78.

Apparently the energy needed to interconvert the two **1d** isomers was much lower than the one needed to interconvert **1′c** and **1″c**. To confirm this experimental result, the energies of interconversion of the atropoisomers of **1d** and of **1c** were evaluated in the vacuum by a computational approach. Due to the high number of atoms, the dimeric structures were studied using a semiempirical method at the AM1 theory level.[9] The atropoisomers of **1d** can be interconverted via two distinct transition states, the first one having in the same plane the C-3 OH of the A-ring and the C-3′ OH of the A′-ring (*sin*-transition state, TS-1), and the second one overlapping the C-3′ OH of the A′-ring and the C-6 methylene of the B-ring (*trans*-transition state, TS-2). The AM1 energy profiles were obtained by varying the dihedral angle through rotation around the biaryl axis. The thus obtained transition states and minima states were then fully optimized and characterized. The ΔE values, related to the calculated minimum energy value, were found to be 27.67 and 32.79 kcal/mol, respectively. A similar calculation on the interconversion of the atropoisomers of **1c** gave values of 41.93 and 39.54 kcal/mol for the ΔE needed to reach the respective transition states TS-1 and TS-2. Avoiding to overestimate the significance of the absolute values of these data, in agreement with the experimental results the energetic gap to be matched in order to reach the TS-1 for the atropoisomers of **1d** resulted clearly lower than the one needed to reach both the TS-1 and the TS-2 for the atropoisomers of the symmetric dimer **1c**.

Finally, by exploiting the slightly different chemical shifts of the H-2 signals of **1′d** and **1″d** (2.66 Hz in d_6-DMSO at 50 °C), dynamic NMR experiments allowed us to evaluate at 100 °C the coalescence temperature (T_C) for the two atropoisomers. From that value (and from the corresponding rate constant k_C)[10] it was possible to estimate within 21 kcal/mol the value of the free energy of activation ($\Delta G^{\ddagger}$) for the interconversion process. These values, and in particular the relatively low T_C, suggest that the interconversion of **1′d** and **1″d** might happen during solvent evaporation after the chromatographic step.

3. Conclusions

Herein it has been shown that laccases from different sources can be used in biphasic systems or in water-saturated organic solvents. 17β-estradiol has been oxidized by two different enzymes while the chemical identities of the products have been elucidated. The oxidation of **1** by a laccase from *Polyporus versicolor* was described more than 30 years ago in one of the first papers on the use of enzymes in biphasic systems,[11] but at that time the structures of the reaction products were poorly characterized and not unequivocally determined.

As far as the products' composition concerns, Figure 3 shows that while the chemical reactions gave the two C–C dimers in equimolecular amounts, the **1c**/**1d** ratio was 1:3 when the oxidation was catalyzed by *TL* (and 1:2 in the reaction catalyzed by *ML*, chromatogram not shown). Different reaction outcomes were also obtained when the chemical and the laccase-mediated oxidations of simpler phenolic derivatives (i.e., 5,6,7,8-tetrahydro-β-naphthol)[6] were compared. These findings, apart from suggesting a participation of the enzyme both in the formation and in the coupling of the radical intermediates, are of synthetic relevance for the selective preparation of C–C phenolic dimers that are well-known

components of metal organic catalysts.[12] Quite recently,[13] it has been shown that a Ti(IV)-H_8-BINOL complex is a very effective catalyst to obtain highly enantioselective hetero-Diels–Alder reaction of aldehydes with Danishefsky's diene. The better results obtained in comparison with other BINOL catalysts were ascribed to the increased rigidity and wider dihedral angle of the axial biaryl groups in the titanium complex, due to the steric effect of the hydrogen of the far-side cyclohexane rings. In this respect the two symmetric dimers **1′c** and **1″c**, once their stereochemical structures have been unambiguously determined, might be even more efficient ligands. Work is currently in progress to verify this hypothesis.

4. Experimental

4.1. Materials and methods

The laccase from a Myceliophtora strain (*ML*) was from Novozymes, while the laccase from *Trametes pubescens* (*TL*) was provided by Prof. Haltrich (Universitat fur Bodenkultur, Wien, 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 Licrospher 100 RP-18 (5 μm, Merck) reverse phase analytical column. HPLC purification: Partisil 10 ODS-3 column (Whatman). ^{1}H- and ^{13}C NMR spectra at 300 and 75.2 MHz were recorded on a Bruker AC-300. CD spectra were recorded on a Jasco J500A spectropolarimeter. Mass spectra were recorded on a VG 70-70-EQ instrument.

4.2. Evaluation of laccases activity

ML activity was evaluated spectrophotometrically by measuring the increase of adsorbance at 530 nm of a solution of syringaldazine. In a 3 mL cuvette: 2.63 mL of a 25 mM phosphate buffer at pH 6.5; 0.22 mL of a 0.224 M 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 *ML* 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).

TL activity was evaluated spectrophotometrically by measuring the increase in 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: 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.

4.3. Adsorption of the laccase from a Myceliophtora strain on glass beads

ML was dialyzed against a 25 mM phosphate buffer pH 6.5 and lyophilized. The enzyme was dissolved again in the same buffer (60 mg/mL, ~600 U/mL) and the solution 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 give a solid with ~0.2 U/mg.

4.4. Oxidation of β-estradiol 1

4.4.1. Catalyzed by Myceliophtora laccase. β-Estradiol **1** (250 mg) was dissolved in 12.5 mL of dioxane. Toluene (37.5 mL) saturated with 50 mM Tris buffer at pH 6.5, 0.5 mL of the same buffer (1% v/v) and 500 mg of *ML* supported onto glass beads were added and the suspension shaken at 45 °C. After two days, 200 mg of enzyme and 0.5 mL of buffer were added. After seven days the solid (glass beads, enzyme and a yellow precipitate) was filtered off, the 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 **1a** and **1b** mixture (8% yields) and 20 mg of a **1c** and **1d** mixture (8% yields).

4.4.2. Catalyzed by *T. pubescens* laccase. β-Estradiol **1** (150 mg) was dissolved in 15 mL 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 to give 21 mg of a **1a** and **1b** mixture (14.0% yields) and 19 mg of a **1c** and **1d** mixture (12.7% yield).

4.4.3. Catalyzed by MnO_2. β-Estradiol **1** (150 mg) was dissolved in 3 mL of dioxane and 27 mL of chloroform after which MnO_2 (27 mg) was added. The system was shaken at room temperature for 48 h. MnO_2 was filtered on a Celite layer, the organic solvent evaporated, and the crude residue was purified by flash to give 20 mg of a **1a** and **1b** mixture (13.3% yields) and 12 mg of a **1c** and **1d** mixture (8.0% yield).

TLC (eluent: petroleum ether–AcOEt–MeOH 7:3:0.5): R_f **1**, 0.38; **1a** and **1b**, 0.25; **1c** and **1d**, 0.15. RP-TLC (eluent: acetonitrile–H_2O 6:4): R_f **1**, 0.38; **1a** and **1b**, 0.05; **1′c**, 0.22; **1″c**, 0.11; **1′d** and **1″d**, 0.19 and 0.17. RP-HPLC, analytical (eluent: acetonitrile–H_2O 1:1, flow rate 0.5 mL/min, λ 290 nm): t_R **1**, 3.7 min; **1a** and **1b**, 38.0 min; **1′c**, 6.4 min; **1″c**, 14.6 min; **1′d** and **1″d**, 9.2 and 11.4 min. RP-HPLC, preparative (eluent: acetonitrile–H_2O 1:1, flow rate 4 mL/min, λ 290 nm): t_R **1′c**, 42 min; **1″c**, 126 min; **1′d** and **1″d**, 61 and 71 min; (eluent: acetonitrile–H_2O 7:3, flow rate 4 mL/min, λ 290 nm): t_R **1a** and **1b**, 60 min.

Compounds **1a** and **1b**. ^{1}H NMR ($CDCl_3 + D_2O$): δ **1a**, 7.22 (d, 1H, J = 8.4 Hz, H-1); 7.12 (d, 1H, J = 8.5 Hz, H-1″); 6.84 (d, 1H, J = 8.5 Hz, H-2′); 6.65 (dd, 1H, J_1 = 8.4 Hz, J_2 = 2.8 Hz, H-2); 6.60 (d, 1H, J = 2.7 Hz, H-4); 3.74–3.72 (2t, 2H, J = 3.2 Hz, H-17 and H-17′); 0.75–0.74 (2s, 3H each, CH_3-18 and CH_3-18′). Compound **1b**: 7.24 (d, 1H, J = 8.4 Hz, H-1); 6.83 and 6.77 (2s, 1H each, H-1′ and H-4′); 6.80 (dd, 1H, J_1 = 7.7 Hz, J_2 = 2.8, H-2); 6.73 (d, 1H, J = 2.7 Hz, H-4); 3.74–3.72 (2t, 2H, J = 3.2 Hz, H-17 and H-17′); 0.75–0.74 (2s, 3H

each, CH_3-18 and CH_3-18′). ^{13}C NMR ($CDCl_3$): APT, δ 154.89, 146.66, 138.85, 138.63, 135.10, 134.44, 133.65, 133.49, 130.91, 126.69 (C-1a and C-1b), 122.78 (C-1′a), 116.97 and 115.92 (C-1′b and C-4′b), 116.84 (C-4b), 114.76 (C-4a), 114.28 (C-2′b), 113.05 (C-2′a), 112.03 (C-2a), 81.89 (C-17a, C-17′a, C-17b, C-17′b); 50.06, 44.04, 43.24, 38.69, 38.26, 36.69, 30.60, 29.73, 29.18, 27.14, 26.51, 26.25, 23.84, 23.11, 11.07 (C-18a, C-18′a, C-18b, C-18′b). EI-MS: 542 (M^+), 524, 286.

Compound **1′c**: ^1H 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: 542 (M^+), 524. Compound **1″c**: ^1H 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′). EI-MS: 542 (M^+), 524.

Compounds **1′d** and **1″d**. ^1H NMR ($CDCl_3 + D_2O$): δ 7.33 (d, 2H, $J = 8.5$ Hz, H-1 of **1′d** and **1″d**), 7.03 and 7.02 (s each, 1H each, H-1′ of **1′d** and **1″d**), 6.89 and 6.88 (d each, 1H each, $J = 8.5$ Hz, H-2 of **1′d** and **1″d**); 6.80 and 6.78 (s each, 1H each, H-4′ of **1′d** and **1″d**), 3.75 (m, 4H, H-17 and H-17′ of **1′d** and **1″d**), 0.75 (s, 12H, CH_3-18 and CH_3-18′ of **1′d** and **1″d**). ^1H NMR (d_6-DMSO): δ 7.04 (d, 2H, $J = 9.2$ Hz, H-1 of **1′d** and **1″d**), 6.75 and 6.73 (s each, 1H each, H-1′ of **1′d** and **1″d**), 6.65 and 6.64 (d each, 1H each, $J = 9.2$ Hz, H-2 of **1′d** and **1″d**); 6.544 and 6.541 (s each, 1H each, H-4′ of **1′d** and **1″d**). ^{13}C NMR ($CDCl_3$): APT, δ 151.75, 139.38, 137.34, 133.81, 133.25, 127.85 and 127.50 (C-1′ of **1′d** and **1″d**), 127.13 (C-1 of **1′d** and **1″d**), 117.60, 115.89 and 115.80 (C-4′ of **1′d** and **1″d**), 115.75, 112.75 (C-2 of **1′d** and **1″d**), 81.75 (C-17 and C-17′ of **1′d** and **1″d**), 50.02, 44.24, 44.05, 43.94, 43.21, 38.74, 38.31, 38.22, 36.72, 36.64, 30.51, 29.53, 28.45, 27.51, 27.18, 26.97, 26.45, 26.36, 23.13, 23.07, 11.07 (C-18 and C-18′ of **1′d** and **1″d**). EI-MS: 542 (M^+), 524.

4.5. Computational methods

Calculations were carried out using the Gaussian 98 program,[14] while all the geometries were fully optimized with a semiempirical method using the AM1 Hamiltonian. Initial transition state geometries were obtained from several relaxed PES scans and refined with the eigenvalue-following methodology. The vibrational frequency calculations were performed to characterize the stationary points as either minima or first-order saddle points.

5. Note added in proof

The proposed structures for the isolated atropoisomers **1′c** and **1″c** were confirmed by comparison of their CD spectra with the CD spectrum of an authentic sample of (*R*)-H_8-BINOL.

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