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Evidence of an Electron-Transfer Mechanism in the Peroxynitrite-Mediated Oxidation of 4-Alkylphenols and Tyrosine

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The mechanism of interaction of the peroxynitrite with some 4-alkylphenols and tyrosine was mainly studied by means of ESR spectroscopy and product analysis. The radical intermediates, detected as spin adducts to the 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO), were identified as carbon-centered radicals to the benzene ring. The reaction seems to proceed via an electron transfer process (ET), most likely mediated by a NO*x* derivative, leading to the intermediacy of a phenoxyl-type radical as proved by the detection of the corresponding Pummerer-type ketone. No evidence of the formation of hydroxyl radicals, due to the homolytic cleavage of the peroxynitrite at physiological pH was obtained, even though DEPMPO hydroxyl spin adducts were detected: the latter most likely arises from the direct attack of the spin trap by the oxidant species. The possible involvement of HCO_3^-/CO_2, i.e., the formation of the nitrosoperoxycarbonate, $ONOOCO_2^{\bullet-}$, was also investigated.

Introduction

Peroxynitrite, $ONOO^-$, is formed in vivo by the reaction of nitric oxide, NO, with the superoxide, $O_2^{\bullet-}$, eq 1:[1−3]

$$NO + O_2^{\bullet-} \xrightarrow{k_1} {}^-OONO \qquad (1)$$

This reaction is diffusion controlled ($k_1 = (4.3-6.7) \times 10^9$ M^{-1} s^{-1}),[2] and the half-life of $ONOO^-$ under physiological conditions is reported to range between 20 ms[4] and 1 s,[3] as the anion is, at pH = 7.4, in equilibrium ($pK_a = 6.8$) with its conjugated acid, the peroxynitrous acid (HOONO), which spontaneously decays to give mostly the nitrate,[3] eq 2:

$${}^-OONO \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} HOONO \rightarrow H^+ + NO_3^- \qquad (2)$$

The biological relevance of this species was first pointed out by Beckman et al.,[5] who recognized that the peroxynitrite may be formed in significant quantities under pathological conditions, where both NO and $O_2^{\bullet-}$ are produced at high rates by phagocytic cells such as macrophages, since relatively small increases in rates of NO and $O_2^{\bullet-}$ production may greatly increase the rate of peroxynitrite formation, $v = k[NO][O_2^{\bullet-}]$, reaching potentially cytotoxic levels.

The role of the peroxynitrite as a cytotoxic species in vivo is still debated,[3] but, being in its associated form, i.e., the peroxynitrous acid, an oxidant, it may be able to destroy critical cellular targets. On the other hand, the oxidizing capability of peroxynitrite toward a variety of biomolecules has been widely investigated and demonstrated in vitro.[6] Moreover, peroxynitrite can perform nitration of tyrosine and this modification may have dramatic effects on proteins and enzyme functions.[2]

In 1952, Halpfenny and Robinson[7] proposed a radical mechanism for the reaction of peroxynitrous acid with aromatic compounds at pH 1.4, which led to nitrated and hydroxylated derivatives. In 1994, Van der Vliet et al.[8] reported that the phenylalanine and the tyrosine are nitrated and hydroxylated by peroxynitrite and proposed that the hydroxylation could be caused by hydroxyl radicals and the nitration by nitrogen dioxide. However, an ET mechanism has been also suggested[9,10] and the intermediacy of a phenoxyl radical has been claimed several times,[9−11] even if never proved directly.

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TABLE 1. ESR Parameters of DEPMPO Adducts, in Buffer Phosphate Solution (0.2 M, pH 7.4) at Room Temperature[a]

substrate	spin adduct	hfc (mT) a_P	hfc (mT) a_N	hfc (mT) $a_{H\beta}$
1	**1a**	4.683	1.462	2.125
2	**2a**	4.685	1.450	2.020
3	**3a**	4.660	1.430	2.105
4	**4a**	4.730	1.430	2.000
1−4	**5**	4.680	1.400	1.350

[a] *g*-Factors are lying in the range 2.0061 ± 0.0002.

To verify the possible involvement of radical species in the peroxynitrite-mediated oxidation of phenols, the interaction with tyrosine **1** and 4-alkylphenols such as 4-methylphenol **2**, 4-ethylphenol **3**, and 4-*tert*-butylphenol **4**, which mimic the tyrosine framework, has been studied. Experiments, carried out in a buffer phosphate solution at pH = 7.4, were performed using the ESR spin trapping technique, which is one of the most appropriate methods available for assessing free radical formation in biological systems. For these experiments the 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide, DEPMPO, was used; a spin trap suitable for biological milieu because of its good solubility in water, and due to its ability to trap carbon, sulfur and oxygen-centered radicals, which are quite persistent spin adducts.[12]

Results and Discussion

When substrates **1**−**4** were investigated by ESR spectroscopy, two paramagnetic species were detectable: **1a**−**4a** characterized by hyperfine coupling constants typical[12] of a DEPMPO spin adduct deriving from the trapping of a carbon-centered radical, the other identified as the DEPMPO− hydroxyl radical spin adduct **5**, Table 1.

The relative amount of carbon-centered/hydroxyl adducts was depending on the nature of the substrate investigated. However, to account for these results it was necessary to be more acquainted with the chemical behavior of DEPMPO. In particular, it was necessary to verify if it could act as a trap for nitrogen-centered radicals, such as NO or NO_2, possible radical intermediates, and/or react directly with the peroxynitrite. Toward this goal, test experiments were conducted. When DEPMPO was reacted with pure NO or NO_2, in the absence of CO_2 and at pH 7.4, no evidence of the formation of spin adducts due to the trapping of nitrogen-centered radicals was obtained. The DEPMPO too, as the majority of the most common spin traps, was unable to trap nitrogen-centered radicals. In contrast, when the peroxynitrite was reacted with DEPMPO, a radical species characterized by ESR spectroscopic parameters coincident with those of the DEPMPO-hydroxyl radical spin adduct was detected. Actually, this result was analogous to that evidenced when either the DMPO[13,14] (5,5-dimethyl-1-pyrroline *N*-oxide) or the TMIO[15] (2,2,4-trimethyl-2*H*-imidazole 1-oxide) or the CDMIO[15] (4-carboxy-2,2-dimethyl-2*H*-imidazole 1-oxide) were used as spin traps in the study of the peroxynitrite decomposition. But, truly surprising was to note that conducting experiments with the same amount of peroxynitrite, but different quantity of DEPMPO,[12b] the corresponding hydroxyl radical spin adduct was detectable at different elapse of time: the delay was inversely proportional to the amount of spin-trap used.[16] The same test experiments were then curried out adding bicarbonate, i.e., in the presence of the nitrosoperoxycarbonate, $ONOOCO_2^-$, which is believed the real reacting species with biological targets,[17] but no radical species were detected by ESR.

Besides the carbon-centered radical adducts, the detection and the identification in all experiments of the DEPMPO-hydroxyl spin adduct, independently of the substrate investigated, could in principle support the involvement of freely hydroxyl radicals as one of the reacting species, Figure 1. Its formation could be accounted for, as usually invoked, by the homolytic cleavage of the −O−O− bond of the peroxynitrite, and the detection of **5** could thus be validated invoking a direct trap of the hydroxyl radical, Scheme 1. Carbon-centered radicals too could be accounted for by the presence of hydroxyl radicals. For example, a benzylic-type radical arising from the *p*-alkyl group of the substrates, via the direct abstraction of hydrogen, could be involved. However, this hypothesis seems not tenable. In fact, the radical **4a**, which arises from **4**, a substrate that does not have hydrogens on the benzylic carbon of the *p*-alkyl substituent, shows the same ESR spectroscopic parameters (hfc) as **1a**−**3a**, Scheme 1.

Thus, for the formation of carbon-centered radicals a different pathway has to be invoked. For instance, a phenoxyl radical intermediate could be hypothesized, but, of course, a completely different reaction mechanism has to be taken into consideration (see later for discussion).

When the nitrosoperoxycarbonate was reacted,[17] eq 3,

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow NO_2 + CO_3^{\bullet -} \quad (3)$$

to account for spectroscopic results, i.e., the detection of DEPMPO-carbon-centered adducts and the DEPMPO-hydroxyl adduct, the homolysis of the −O−O− bond could still be invoked. The formation of **5** could be justify via the direct trap of the $CO_3^{\bullet -}$ radical, while for

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(16) The experiments were conducted in a buffer phosphate solution 0.2 M (pH 7.4), at room temperature, in the absence of CO_2, and the peroxynitrite concentration set at 0.84×10^{-3} mol/L. When the DEPMPO concentration used was 5.0×10^{-2} mol/L, the spin adduct **5** was detectable almost immediately; dropping the concentration to 1.0×10^{-2} and 5.0×10^{-3} mol/L, the delay was ca. 2 and 10 min, respectively; when the concentration was 2.0×10^{-3} mol/L no radical species were detected within 40 min of investigation.

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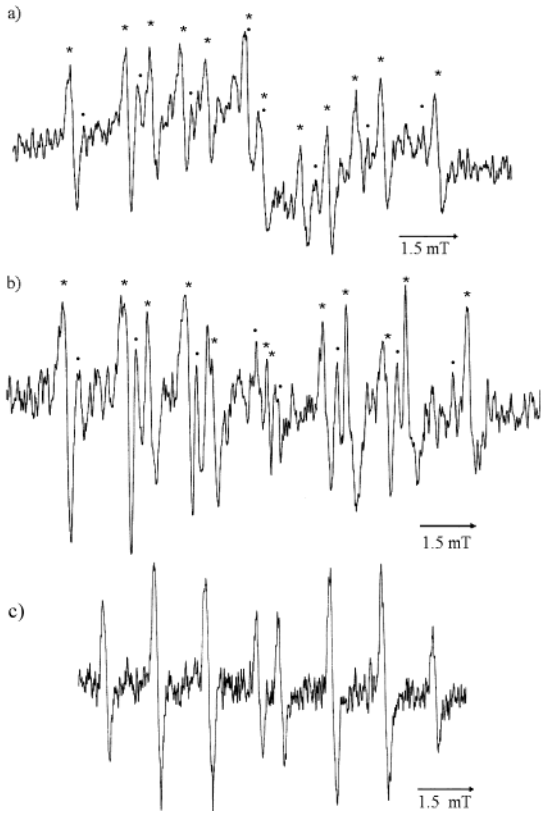
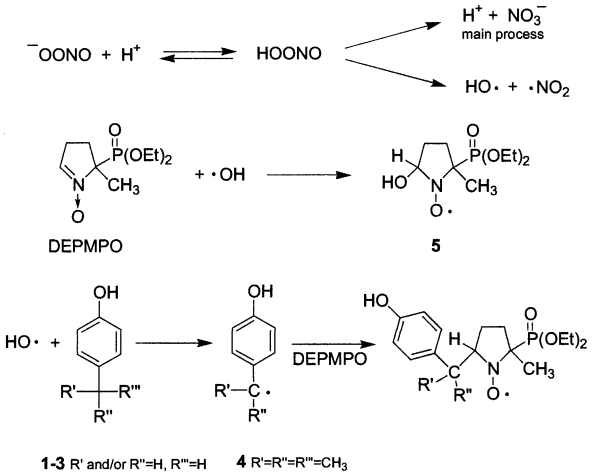


FIGURE 1. ESR spectra obtained at room temperature in a buffer phosphate solution (pH 7.4): (a) reacting **1**; radicals **1a** (*****) and **5** (•) are detected; (b) reacting **2**; radicals **2a** (*****) and **5** (•) are detected; (c) radical **5** obtained from the Fenton's reaction in the presence of DEPMPO.

SCHEME 1



carbon centered radicals an electron-transfer process between $CO_3^{\bullet-}$ and the substrate should be hypothesized, Scheme 2.

As a matter of fact, the detection of $^{13}CO_3^{\bullet-}$, obtained reacting ^{13}C-bicarbonate and peroxynitrite, has been reported,[18] but, as stated before, blank experiments conducted between DEPMPO and the peroxynitrite in the presence of CO_2 did not allow any radical species to be detected.

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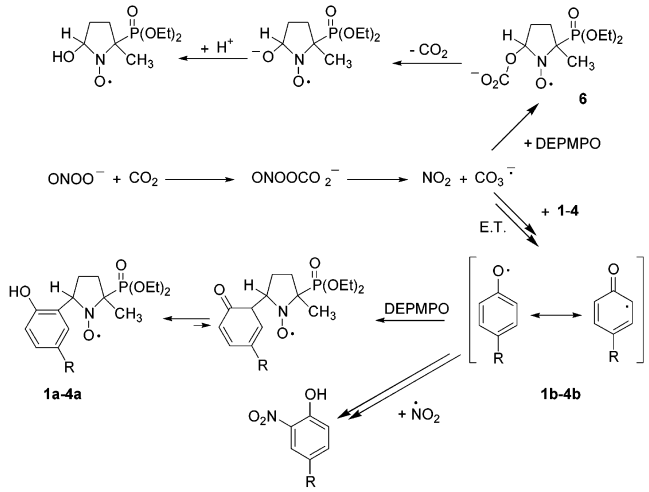
SCHEME 2



However, the homolytic cleavage of the peroxynitrite or the nitrosoperoxycarbonate, which can be considered the competing process to the isomerization to HNO_3 and to NO_3^-/CO_2, respectively, takes place in a percentage ranging between 10 and 25% maximum,[19,20] and that led us to consider the amount of free-radical species, such as •OH or $CO_3^{\bullet-}$, available for reacting, really poor. Furthermore, the detection in blank experiments with DEPMPO and the peroxynitrite, but not with the peroxycarbonate, only of the DEPMPO-hydroxyl spin adduct, and mainly the delay of its detectability depending on the quantity of spin trap used, leads us to hypothesize a completely different reaction mechanism. To account for this behavior, in fact, one needs to hypothesize that the DEPMPO itself is oxidized by the peroxynitrite to a diamagnetic species, which spontaneously, but slowly, decays to a quite persistent radical species characterized by hfc coincident with those of **5**.[21] This behavior is definitely in agreement with that showed in studies on the decomposition of peroxynitrite in the presence of other spin traps.[13−15] In particular, when the DMPO was used, the authors[13,14] concluded that although a weak signal corresponding to the DMPO-hydroxyl radical spin adduct is observed this does not constitute proof of the presence of free hydroxyl radicals. As well, when TMIO or CDMIO reacts with peroxynitrite, the authors[15] state that even though the same spin adduct as after the reaction with OH-radicals is detected, it seems to be formed under direct oxidation of the spin trap by peroxynitrite and not under the reaction with OH-radicals, leading to exclude the involvement of free hydroxyl radicals deriving from the homolytic cleavage of peroxynitrite.

Therefore, these results support also the hypothesis that radical species deriving from the homolytic decay

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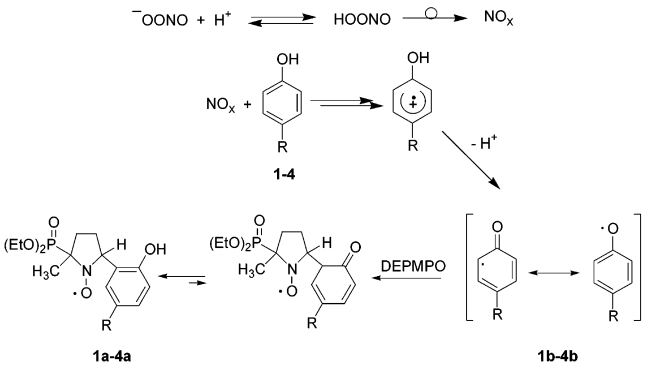
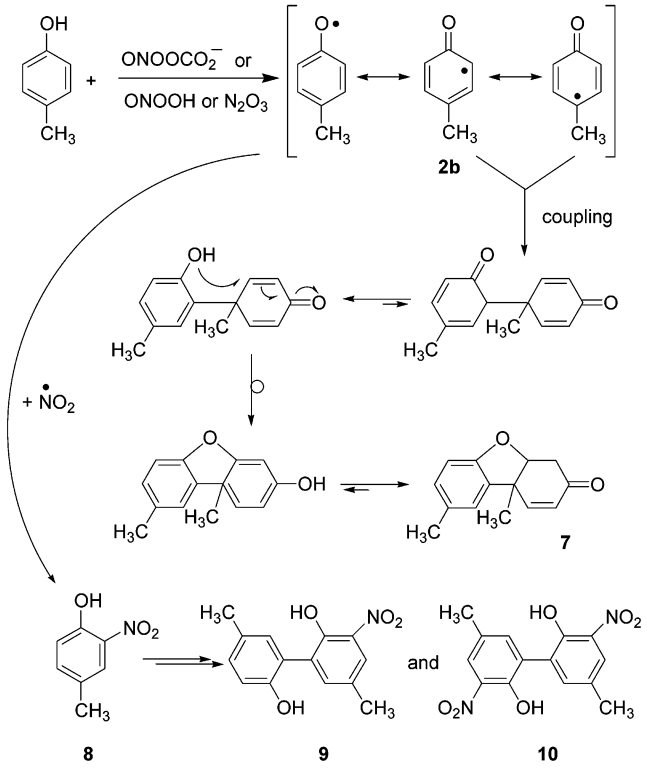
(21) The delay in the ESR detection of **5**, as a function of the concentration of spin trap, could be explained assuming that reducing the DEPMPO concentration the amount of the radical-precursor decreases as well, and then a longer period of time is necessary to get to an ESR-detectable concentration of the radical species, via accumulation.

SCHEME 3



SCHEME 4



of the peroxynitrite cannot account for the formation of carbon-centered radical intermediates. In contrast, a reaction mechanism[22] via an ET process seems to be more tenable. To validate the results, the involvement of a NO_x species has been invoked too, Scheme 3.

In particular, the intermediacy of N_2O_3[23] has been hypothesized to be responsible for the oxidation of phenolic substrates. Actually, we have recently demonstrated[24] that, under the same experimental conditions, the 4-methylphenol reacts with N_2O_3, via an ET process, leading to the same products obtained when peroxynitrite was used; in particular, the recover of the Pummerer's ketone **7**[25] is considered a definitive prove of the involvement of an ET process (Scheme 4).

It is worth noting that the conversion of **2** was found to be dependent on the presence of CO_2;[17a,b] in particular, at pH 7.4, the conversion was more than 80% when HCO_3^-/CO_2 was added. The product analysis showed the formation of **7** and **8**, yield 12.4% and 21.6%, respectively, as the main reaction products, but no hydroxylated derivatives. Furthermore, compounds due to the oxidation of **8**, **9**, and **10** (<1%) and phenolic polymeric products were also identified, Scheme 4. When the reaction was conducted in the absence of CO_2, the conversion was much lower, ca.10%, and the detected products were **7**, yield 5%, and a trace amount of **8.** The formation of these products definitely supports the involvement of **2b**, which can be considered the precursor of all the identified compounds and, of course, of the radical **2a**, Schemes, 2 and 3. Product analysis for the reaction of peroxynitrite with **3** was also conducted, and analogous results were obtained.

Conclusions

Although in the reaction between the tyrosine, tyrosine-type phenols, and the peroxynitrite or the peroxycarbonate the involvement of a homolytic −O−O− cleavage, and then freely diffusing hydroxyl, $CO_3^{\bullet-}$ and NO_2 radicals, cannot be completely excluded, it seems definitively most plausible that an interaction via an ET process, leading to the formation of a phenoxyl intermediate, takes place, as confirmed by ESR spectroscopy and product analysis. In particular, the detection of the dimeric Pummerer's ketone can be considered a definitive prove of the ET mechanism. In this light, also the hypothesis of the intermediacy of N_2O_3, arising from the peroxynitrite, as the reacting species, seems tenable. Finally, the DEPMPO-hydroxyl spin adduct most probably results to be formed under direct oxidation of the spin trap, but not under the reaction with OH-radical. The detection of **5** cannot be considered a prove of the homolytic decay of the peroxynitrite.

Experimental Section

Materials. Tyrosine, 4-methylphenol, 4-ethylphenol, and 4-*tert*-butylphenol were purchased from Aldrich and used as received. The DEPMPO[26] and the peroxynitrite[27] were synthesized according to the literature methods. For the synthesis of the latter, a solution of H_2O_2 30% (10.0 mL) was diluted to 50 mL with water, chilled at 4 °C, and then added with 18 mL of NaOH (5.0 M) and 5.0 mL of 0.04 M diethylenetriaminepentaacetic acid (DTPA) in NaOH (0.05 M); the resulting mixture was diluted to 100 mL, total volume, with water (the final pH was around 13.5). Subsequently, the solution was allowed to react with an equimolar amount of isoamyl nitrite (12.3 mL) under vigorous stirring for 3−4 h at ca. 15 °C. The aqueous phase was then washed with chloroform (5 × 75 mL) to remove the contaminating isoamyl alcohol and the unreacted nitrite. The resulting yellow solution was treated with an excess of MnO_2 in order to destroy the unreacted H_2O_2 and then filtered. The yield of $ONOO^-$ was determined from its absorption, $\epsilon_{302} = 1670\ M^{-1}\ cm^{-1}$.[28] Solutions kept at −18 °C showed little decomposition over several weeks.

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Analysis Procedures. ESR analyses were carried out on a spectrometer equipped with an X-band microwave bridge, 100 kHz modulation, and with a variable-temperature apparatus. An aliquot of the reaction mixture obtained by addition to a buffer phosphate solution (pH 7.4), the peroxynitrite (final concentration 3.0 mM), the substrate (15−35 mM), and the spin trap, DEPMPO (7.5 mM), was introduced in a quartz flat-cell placed inside the cavity of the ESR spectrometer and immediately analyzed. GC−MS analyses were performed using a gas chromatograph equipped with a methyl silicone plus 5% phenyl silicone capillary column and fid integration. All compounds were identified by comparison of their retention times with those of authentic samples and/or by their mass spectra. TLC chromatography was conducted as usual; ^{1}H NMR and mass spectra identified separated products.

Product Analysis. Experiments were conducted on substrates **2** and **3**.

(a) In the Presence of CO_2. At room temperature, to 20 mL of a 0.2 M buffer phosphate solution were added 0.87 mmol of $NaHCO_3$ (∼25mM, i.e., close to the concentration in plasma), 1.2 mmol of substrate, and under vigorous stirring, 1.2 mmol of peroxynitrite; the pH (7.4) was adjusted by adding HCl (37%) dropwise. The resulting mixture was stirred for further 15 min, and the organic phase was extracted with ether (3 × 25 mL), washed twice with water (20 mL), and then dried with Na_2SO_4. In particular, when the 4-methylphenol was reacted a high conversion yield was obtained. The GC−MS analysis led to the identification of, besides a low quantity of unreacted starting material, the 4-methyl-2-nitrophenol (21.6% yield) and the 8,9b-dimethyl-4a,9b-dihydroxy-4*H*-dibenzofurane-3-one (Pummerer's ketone) (12.4% yield). However, through an accurate quantitative TLC chromatography, minor quantities (<1%) of the 2,2′-dihydroxy-5,5′-dimethyl-3-nitrobiphenyl (**9**) and the 2,2′-dihydroxy-5,5′-dimethyl-3,3′-dinitrobiphenyl (**10**) were recovered; the remaining converted product was identified as a polymeric (phenolic) derivative. When the 4-ethylphenol (**3**) was reacted, besides some unreacted substrate, the Pummerer-like ketone and the 4-ethyl-2-nitrophenol were identified. The yield of the latter was higher compared with that obtained reacting (**2**), and that at the expenses of dimeric products.

(b) In the Absence of CO_2. To keep the presence of bicarbonate/carbon dioxide as low as possible, the buffer phosphate solution, containing the substrate and the spin trap, was sonicated for at least 50/60 min and purged by bubbling of N_2 for an additional 1 h just before use. The peroxynitrite, which also was purged in advance by bubbling of N_2, was then added to the mixture and the reaction carried out under nitrogen atmosphere. When **2** was reacted, the conversion yield was very poor. The GC−MS analysis showed the presence of the 4-methyl-2-nitrophenol (∼1% yield) and the 8,9b-dimethyl-4a,9b-dihydroxy-4*H*-dibenzofuran-3-one (5% yield), besides, of course, unreacted starting material. Similarly, when **3** was investigated the 4-ethyl-2-nitrophenol and the corresponding Pummerer's-like ketone were detected.

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