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# Reactions of nitric oxide and nitrogen dioxide with coenzyme Q: involvement of the isoprenic chain†

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The formation of a di-*tert*-alkyl nitroxide has been observed by Electron Spin Resonance during the exposure of coenzyme  $CoQ_{10}$ , in both the oxidized and reduced forms, to nitrogen dioxide (` $NO_2$ ) or to nitric oxide (`NO) in the presence of oxygen. The same kind of nitroxide has been observed also with  $CoQ_1$ ,  $CoQ_3$  or with 1-phenyl-3-methyl-2-butene, chosen as model compounds. In all cases, the formation of the nitroxide may be justified only by admitting the involvement of the isoprenic chain of the coenzymes and in particular the addition of ' $NO_2$  to the double bond. A mechanism which accounts for the formation of the nitroxide as well as the other compounds observed in the reactions is proposed and confirmed by a spectroscopic investigation (FT-IR, <sup>1</sup>H NMR, X-ray analysis) and by ESI-MS.

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### Introduction

Ubiquinones (or coenzymes CoQ) are hydrophobic molecules acting as essential electron carriers in the mitochondrial respiratory chain.<sup>1,2</sup> In particular, coenzyme CoQ<sub>10</sub> is the predominant endogenous form of ubiquinones in humans, which is synthesized in the mitochondrial inner membrane and comprises a quinoid head group attached to a long, hydrophobic tail of 10 isoprene units.<sup>2</sup> The long side chain ensures that native CoQ<sub>10</sub> is almost exclusively associated with phospholipid bilayers within the cell where it can fulfil its electron transport and antioxidant functions. The predominant redox forms of CoQ<sub>10</sub> are the oxidized ubiquinone (CoQ<sub>10</sub>) and the reduced ubiquinol (CoQ<sub>10</sub>H<sub>2</sub>) and they are easily interconverted by transfer of two electrons and two protons (Scheme 1). The redox activity of the ubiquinone moiety enables it to act as a



mobile electron carrier in the mitochondrial inner membrane where it is reduced to ubiquinol by several dehydrogenases and oxidized back by complex III.

On the other hand, the reduced  $\text{CoQ}_{10}\text{H}_2$  form has important protective functions directly as a chain breaking antioxidant, terminating lipid peroxidation in phospholipid bilayers by reacting, *via* hydrogen transfer, with carbon- and oxygencentered radicals<sup>3,4</sup> and also through vitamin E ( $\alpha$ -tocopherol) recycling from the tocopheroxyl radical.<sup>5,6</sup> Besides this interaction of CoQ<sub>10</sub>H<sub>2</sub> with lipid peroxidation derived radicals, a direct interaction of CoQ<sub>10</sub> and CoQ<sub>10</sub>H<sub>2</sub> with superoxide (O<sub>2</sub><sup>--</sup>) radical may also be important<sup>4,7</sup> within mitochondria, where O<sub>2</sub><sup>--</sup> is formed during the respiratory chain in the autoxidation of ubisemiquinone (CoQ<sub>10</sub><sup>--</sup>).<sup>8,9</sup>

The potential antioxidant role of ubiquinol is not only limited to its reactivity toward Reactive Oxygen Species (ROS) but also toward nitrogen monoxide (nitric oxide, `NO) and its metabolites (`NO<sub>2</sub>, <sup>-</sup>OONO, *etc.*). The reaction of nitric oxide with ubiquinol has been kinetically studied<sup>10</sup> as well as the oxidation of ubiquinol by peroxynitrite.<sup>11</sup> In both cases, it seems that nitrosative damage is prevented by a one electron oxidation of the  $CoQ_{10}H^-$  anion to give a protonated ubisemiquinone radical  $CoQ_{10}H^+$  by 'NO or <sup>-</sup>OONO even though these

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 $<sup>\</sup>dagger$  Electronic supplementary information (ESI) available: ESI-MS spectrum of (CoQ<sub>1</sub> + 'NO<sub>2</sub>), ESI-MS/MS of *m*/*z* 343 and 359, synthesis and characterization of 2-methyl-3-nitro-4-phenylbutan-2-ol **10**, thermochemical and activation parameters for H-abstraction and 'NO<sub>2</sub> addition. CCDC 909977 for compound **7**. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/ c2ob27198b

reactions are not so simple and are part of a complex regulatory mechanism with many implications for mitochondrial function and integrity.

The antioxidant activity of  $\text{CoQ}_{10}\text{H}_2$  is thus attributed almost exclusively to its redox properties and a possible involvement of the isoprenic chain in its reactivity has never been considered. The isoprenic unit contains a C=C double bond which may easily react with oxidant species such as 'NO and its metabolites<sup>12-14</sup> and could in this way contribute to the overall antioxidant power.

On the other hand, nitric oxide/oxygen is a complex system which gives rise to metabolites such as  $^{1}NO_{2}$ ,  $N_{2}O_{3}$ ,  $N_{2}O_{4}$ ,  $NO^{+}/NO_{3}^{-}$ , very reactive species able to nitrate, nitrosate and oxidize many organic compounds<sup>15,16</sup> and to cause nitrosative and oxidative stress.<sup>17,18</sup>

For this reason, the interaction between  $CoQ_{10}$  and  $CoQ_{10}H_2$  and nitric oxide (in the presence and in the absence of oxygen) and with nitrogen dioxide was studied, with a particular attention focused on the role of the isoprenic chain in these reactions. A spectroscopic investigation carried out by ESR (Electron Spin Resonance), <sup>1</sup>H NMR, FT-IR and a spectrometric analysis by ESI-MS were undertaken not only on  $CoQ_{10}$ but also on its homologous  $CoQ_1$  and  $CoQ_3$  and on 1-phenyl-3-methyl-2-butene, chosen as model compounds. Most of the experiments reported here were performed on  $CoQ_1$  and 1-phenyl-3-methyl-2-butene for the sake of simplicity.

### **Results and discussion**

#### 1. Oxidation of CoQ<sub>10</sub>H<sub>2</sub> by 'NO and 'NO<sub>2</sub>

Pure 'NO was not able to oxidize  $CoQ_{10}H_2$ ; the reaction was repeated several times, either by bubbling 'NO gas in the CoQ<sub>10</sub>H<sub>2</sub> solution or by allowing the CoQ<sub>10</sub>H<sub>2</sub> solution to be in contact with an 'NO atmosphere, but in no cases the formation of CoQ<sub>10</sub> was observed. Both an electron transfer and a hydrogen abstraction process should be ruled out and changes in the isoprenic chain were also not observed. In the first case, the difference between the oxidation potential of CoQ10H2, ca. 1.0 V vs. NHE (Normal Hydrogen Electrode) by comparison with other activated phenols,<sup>19</sup> and the reduction potential of the couple 'NO/NO<sup>-</sup> [0.8 V vs. SCE<sup>20</sup> (Saturated Calomel Electrode)] is such that, according to the Marcus theory,<sup>21</sup> a simple electron transfer is unlikely to occur. On the other hand, 'NO is also a rather weak hydrogen abstractor: H-NO Bond Dissociation Energy (BDE) can be estimated as 49 kcal mol<sup>-1 22</sup> whereas the predicted BDE values for the two O-H groups in  $CoQ_{10}H_2$  are 82.0 and 81.6 kcal mol<sup>-1</sup>.<sup>23</sup>

Nitrogen dioxide, an 'NO metabolite formed after a rapid reaction with oxygen ( $k = 1.1 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ ),<sup>24</sup> can easily oxidize CoQ<sub>10</sub>H<sub>2</sub>. In fact, 'NO<sub>2</sub> is both a quite good oxidant ( $E^{\circ} \cdot_{\text{NO}_2/\text{-}NO_2} = 0.99 \text{ V}$ )<sup>25</sup> and a good hydrogen abstractor (the BDE of H–ONO is 80 kcal mol<sup>-1</sup>).<sup>22</sup> Hence, CoQ<sub>10</sub>H<sub>2</sub> could be oxidized both by an electron transfer process and by a hydrogen abstraction. In fact, when 'NO<sub>2</sub> and CoQ<sub>10</sub>H<sub>2</sub> were dissolved in an aprotic solvent and their solutions were mixed,



Fig. 1 The ESR spectrum of the reaction solution (CoQ $_{10}$  +  $\cdot$ NO $_2$ ) in benzene (A experimental, B simulated).

 $CoQ_{10}H_2$  was immediately oxidized to  $CoQ_{10}$  and a pale yellow water soluble precipitate was formed. The same precipitate was also obtained from the reaction between 'NO<sub>2</sub> and CoQ<sub>10</sub>.

# 2. Reaction between $CoQ_{10}H_2$ or oxidized CoQ and 'NO<sub>2</sub>: ESR measurements

In order to ascertain the presence of radical species eventually formed during the reaction, the mixture  $(CoQ_{10}H_2 + 'NO_2)$  was analyzed by ESR spectroscopy: an intense three lines spectrum typical of a di-*tert*-alkyl nitroxide was detected. The same signal was also observed when oxidized  $CoQ_{10}$  was treated with 'NO in the presence of oxygen or with 'NO<sub>2</sub> (Fig. 1) thus suggesting that 'NO<sub>2</sub> could interact with the isoprenic chain identical in the two redox forms of the coenzyme.

Also when the oxidized forms of  $\text{CoQ}_1$  and  $\text{CoQ}_3$  were reacted with 'NO<sub>2</sub> similar nitroxide radicals were observed, whereas no signal was recorded when 'NO<sub>2</sub> was added to duroquinone (2,3,5,6-tetramethyl-1,4-quinone). These results confirm that the isoprenic chain is required for the formation of the observed nitroxides. The nitrogen hyperfine coupling constants ( $a_N$ ) were determined and found in all cases to range between 14.5 and 15.0 Gauss while a value of 2.0063(7) was measured for the *g*-factor.

Recorded signals were characterized by rather large linewidths suggesting an overlapping of radicals of the same type. In particular, in the case of  $CoQ_1$  the presence of two different signals with *ca.* 1 G difference in the  $a_N$  value was clearly evident. At present, we have not identified yet the second radical observed but it may likely arise from the trapping of some other radicals by  $\beta$ -nitroso compound **3** (Scheme 2).

No other ESR signals different from di-*tert*-alkyl nitroxides were detected. In particular, formation of oxynitroxyl radicals arising from addition of nitrogen dioxide to the double bonds of the quinone or of the iminoxyl and acylnitroxide radicals deriving from their decomposition<sup>26</sup> was not observed.

A mechanism to account for the formation of a di-*tert*-alkyl nitroxide is shown in Scheme 2 and is in agreement with lite-rature reports:<sup>12</sup> addition of 'NO<sub>2</sub> to the carbon–carbon

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double bond in **1** gives a  $\beta$ -nitroalkyl radical **2** which couples with 'NO (formed during the decomposition of a nitro-nitrite intermediate, see Scheme 3) to afford the nitroso compound **3**. Because of its spin trapping properties, **3** can trap another radical **2** giving the di-*tert*-alkyl nitroxide **4**.

These results prompted us to investigate the reaction of  $CoQ_{10}$  and its analogues by means of FT-IR, <sup>1</sup>H NMR and, in particular, by ESI-MS.

#### 3. Reaction between CoQ and 'NO2: FT-IR measurements

Changes occurring upon addition of  ${}^{N}O_2$  to CCl<sub>4</sub> solutions of CoQ<sub>1</sub>, CoQ<sub>3</sub> and CoQ<sub>10</sub> were monitored by FT-IR spectroscopy. After the addition, all CoQ studied showed the same absorptions at 1650 and 1611 cm<sup>-1</sup> for the quinoid moiety and an intense band at 1554 cm<sup>-1</sup> typical for the nitro group but of different intensities in the three cases. The 1650 cm<sup>-1</sup> band was normalised to the same intensity in the three spectra so that the intensity of the 1554 cm<sup>-1</sup> band was proportional to NO<sub>2</sub> concentration. In fact, going from CoQ<sub>1</sub> to CoQ<sub>10</sub> (Fig. 2), the intensity of the band considerably increased thus indicating that a larger number of NO<sub>2</sub> groups added to CoQ<sub>10</sub> than to CoQ<sub>1</sub> and CoQ<sub>3</sub> further confirming that the isoprenic chain was involved in the reaction of the various CoQ with  ${}^{N}O_2$ .

#### 4. Reaction between CoQ and 'NO<sub>2</sub>: <sup>1</sup>H NMR study

<sup>1</sup>H NMR spectra recorded on CDCl<sub>3</sub> solutions of CoQ<sub>1</sub>, CoQ<sub>3</sub> and CoQ<sub>10</sub> after addition of submolar amounts of  $\cdot$ NO<sub>2</sub> indicated its addition to the double bond of the isoprenic unit. In



Fig. 2 The FT-IR spectrum of the reaction solutions (CoQ with  $NO_2$ ): red CoQ<sub>1</sub>; black CoQ<sub>3</sub>; blue CoQ<sub>10</sub>.



Fig. 3 (a) The  $^1H$  NMR spectrum of pure CoQ1; (b) the  $^1H$  NMR spectrum of CoQ1 +  $^1NO_2$  solution.

fact, in all cases the typical resonances of the two methoxyl (doublet at  $\delta$  = 3.98 ppm) and of the nuclear methyl group (singlet at  $\delta$  = 2.01 ppm) remained almost unchanged thus indicating that the quinoid moiety did not take part in the reaction, whereas the signals due to the isoprenic chain completely changed. This was particularly evident with CoQ1 (Fig. 3): the two isoprenic methyl groups at  $\delta = 1.67$  and 1.74 ppm shifted to higher fields,  $\delta = 1.35$  and 1.42 ppm respectively, the multiplet at  $\delta$  = 4.91–4.95 ppm due to the vinyl hydrogen was partially destroyed while the signals of the quinone moiety remained unchanged. With CoQ<sub>3</sub> and CoQ<sub>10</sub> partially unresolved spectra were recorded upon addition of 'NO<sub>2</sub>, because of the presence of multiple double bonds all able to add NO<sub>2</sub> groups. In these cases, the resonances due to the nuclear methyl and methoxy groups did not change and were still well resolved at the same chemical shifts.

#### 5. Reaction between CoQ and 'NO<sub>2</sub>: ESI-MS study

After dilution in a methanolic solution of ammonium acetate (3 mM), the reaction medium ( $CoQ_1 + 'NO_2$ ), either in benzene or  $CDCl_3$ , was electrosprayed and gave rise to a complex mass spectrum displaying multiple signals (see Fig. S1, ESI<sup>+</sup>).

A low abundance m/z 251 ion suggested the presence of residual CoQ<sub>1</sub>, produced in the gas phase as a protonated molecule. This assumption was supported by MS/MS experiments, showing the same dissociation pattern as compared to CID (Collition Induced Dissociation) of  $[CoQ_1 + H]^+$  obtained from a pure CoQ<sub>1</sub> solution (data not shown). In addition, three main sets of MS signals were clearly evidenced and, according to their accurate m/z values, assigned to compounds resulting from the addition of two NO<sub>2</sub> moieties ( $[M + H]^+$  at m/z 343 and  $[M + NH_4]^+$  at m/z 360), of one NO<sub>2</sub> and one NO<sub>3</sub> group  $([M + H]^+$  at m/z 359 and  $[M + NH_4]^+$  at m/z 376), and of one NO<sub>2</sub> and one OH ([M + H]<sup>+</sup> at m/z 314 and [M + NH<sub>4</sub>]<sup>+</sup> at m/z331). The three protonated molecules at m/z 343, m/z 359 and m/z 314 were further submitted to CID experiments, allowing validation of the proposed structures. Briefly, CID of  $[CoQ_1 +$  $2NO_2 + H^{\dagger}$  at m/z 343 (ESI Fig. S2<sup>+</sup>) would generate the [CoQ<sub>1</sub> + H]<sup>+</sup> at m/z 251 upon a concerted elimination of two nitrogen dioxide species (loss of 92 Da) in a radical process. Another informative dissociation pathway would consist of a sequential release of two 47 Da neutral fragments assigned to HNO<sub>2</sub> and HO-N=O, respectively to produce m/z 296 and m/z 249 from m/z 343. The MS/MS spectrum of m/z 359, assigned to  $[CoQ_1 +$  $NO_2 + NO_3 + H^{\dagger}$  (ESI Fig. S3<sup>+</sup>), indicated that the precursor ion mostly dissociates via the loss of a 46 Da radical (i.e., 'ONO released from the nitrate group) to yield m/z 313, followed by the elimination of a 47 Da neutral (*i.e.*, HNO<sub>2</sub>) to generate m/z266. All proposed dissociation reactions are detailed in ESI.<sup>†</sup>

The identified products all derived, as discussed below, from a homolytic addition of  ${}^{\circ}NO_2$  to the double bond of the isoprenic chain. As for the nitronitrate derivative (*m*/*z* 359 and 376) it may arise from nitrosation by nitrosyl nitrate to give a nitrosonitrate and hence a nitronitrate (2  ${}^{\circ}NO_2 \leftrightarrow N_2O_4 \leftrightarrow$ ONOONO).<sup>27</sup>

Electrospray ionization of the reacting medium containing CoQ<sub>3</sub> and 'NO<sub>2</sub> also gave rise to a complex mass spectrum in which the following compounds derived from CoQ<sub>3</sub> were observed in their protonated form:  $[CoQ_3 + NO_2 + OH + H]^+$  at m/z 450,  $[CoQ_3 + 2NO_2 + H]^+$  at m/z 479,  $[CoQ_3 + NO_2 + NO_3 + NO_3$  $H^{+}_{1}$  at m/z 495,  $[CoQ_{3} + 2NO_{2} + 2OH + H^{+}_{1}]^{+}$  at m/z 513, and  $[CoQ_3 + 4NO_2 + H]^+$  at m/z 571. These assignments were further supported by CID data. First, all MS/MS spectra exhibited an intense peak at m/z 197, a typical product ion of [CoQ<sub>3</sub> + H]<sup>+</sup>, formed after the release of the isoprenic chain. Then, diagnostic neutral losses previously described for CoQ<sub>1</sub> derivatives were systematically observed. For example,  $[CoQ_3 + NO_2 +$  $OH + H^{\dagger}$  at m/z 450 was shown to expel a hydroxyl radical prior to releasing HNO<sub>2</sub>, while compounds arising from the addition of two 'NO<sub>2</sub> (m/z 479) or four 'NO<sub>2</sub> (m/z 571) were observed to release two or four 47 Da neutrals, respectively.



Fig. 4 The ESI-MS spectrum of the reaction medium of Q10 with 'NO<sub>2</sub>.

The ESI mass spectrum obtained after the CoQ10 reaction with  $NO_2$  exhibited numerous signals in the 800-1500 m/zrange (Fig. 4). Molecules were all produced as ammonium adducts in the gas phase, showing that proton affinity of these species decreases as their size increases. Unreacted CoQ<sub>10</sub> was observed as an ammonium adduct at m/z 880.7 and the most intense peak detected at m/z 988.7 could be assigned to [CoQ<sub>10</sub> + NO<sub>2</sub> + NO<sub>3</sub> + NH<sub>4</sub>]<sup>+</sup>. Multiple combinations of CoQ<sub>10</sub> with OH, NO2 and NO3 added moieties were also observed and a product resulting from the addition of up to eight NO2 groups on CoQ10 was detected, although no MS/MS experiments could be performed on this ion due to its too low abundance and multiple isotopic interferences. The most favoured dissociation reaction of all ionic species scrutinized in MS/MS was shown to be the elimination of the isoprenic chain, yielding an intense m/z 197 product ion. The whole dissociation pathway previously described for CoQ1 and CoQ3 derivatives to enable all added groups to be identified did not efficiently compete, in the case of  $CoQ_{10}$ , with the reaction yielding m/z197. Nonetheless, MS peak assignments could still be validated in MS/MS experiments, based on rules previously established for CoQ1 and CoQ3 derivatives. Indeed, compounds containing only NO2 groups always first experienced a 47 Da neutral loss, those containing both NO2 and NO3 moieties always first eliminated a 46 Da neutral loss, and release of a 17 Da radical was systematically observed as the first reaction from CoQ derivatives holding an OH group.

# 6. Reaction of the isoprenic chain with $^{\circ}NO_2$ and $^{\circ}NO$ in the presence of oxygen

Coenzymes  $CoQ_1$ ,  $CoQ_3$  and  $CoQ_{10}$  in their oxidised forms were reacted with pure 'NO in the presence of oxygen or with 'NO<sub>2</sub>. From these reactions several products were formed, all derived from the interaction between nitrogen dioxide and the isoprenic chain, and it was very difficult to isolate and identify any of them. As reported in the literature, the reaction between alkenes and nitrogen dioxide is rather complex and the products may arise from competing mechanisms such as addition, substitution, oxidation or C–C bond cleavage. This



Fig. 5 The molecular structure of compound 7, with displacement ellipsoids drawn at the 30% probability level.

reaction usually affords a complex mixture of dinitro, nitronitrito and nitro-nitrato compounds, often accompanied by further degradation products.<sup>13,14,28-30</sup> In the present case, it is not difficult to imagine how complex our reaction mixtures were, especially when CoQ<sub>3</sub> or CoQ<sub>10</sub>, with their multiple double bonds, were used. On the other hand  $CoQ_1$ , with only one isoprenic unit and one double bond, could have been a good candidate to be used as a model compound. However, it is a rather expensive chemical and since the quinone moiety is not involved in the reaction with 'NO2, we preferred to use 1-phenyl-3-methyl-2-butene 5 and to study its reactivity toward nitrogen dioxide. Again, the typical three line signal similar to those recorded with the other coenzymes was observed when the crude mixture was analyzed by ESR spectroscopy and it was also possible to isolate two compounds, the dinitro derivative 7 and the nitroalcohol 10 (Scheme 3).

The structure of compound 7 (Fig. 5) was elucidated by X-ray analysis, whereas the structure of the nitroalcohol **10** was confirmed by comparison with an authentic sample prepared starting from  $\beta$ -nitrostyrene following the reaction sequence described in Scheme S3 (ESI<sup>†</sup>).

In compound 7 (Fig. 5), bond lengths and angles are not unusual. The plane through the isopropyl chain forms a dihedral angle of  $67.79(7)^{\circ}$  with the phenyl ring, and is tilted by  $53.79(15)^{\circ}$  and  $78.27(12)^{\circ}$  with respect to the planes of the nitro groups including the N1 and N2 nitrogen atoms, respectively. The crystal packing is stabilized only by van der Waals interactions, no hydrogen bonds, C–H… $\pi$  or  $\pi$ … $\pi$  stacking interactions are observed.

The two isolated products both derived from the initial rapid reversible addition of nitrogen dioxide (as a monomer  $^{1}NO_{2}$  or as a diamagnetic dimer  $N_{2}O_{4}$ )<sup>13,14,31</sup> to the double bond<sup>32</sup> to give a tertiary radical which may further react with another  $^{1}NO_{2}$  giving the dinitro derivative 7 or, by reacting with  $^{1}ONO$ , the nitro-nitrite 8 (Scheme 3). This latter possibility arises from the fact that the unpaired electron of nitrogen dioxide can be delocalized throughout the molecule, as the mesomeric structures in Fig. 6 demonstrate, so that the tertiary radical may couple with both the nitrogen and the oxygen atom of  $^{1}NO_{2}$ .



Fig. 6 Resonance structures of nitrogen dioxide ('NO<sub>2</sub>)

Compound 8 could not be isolated and likely decomposed, in agreement with the literature reports,<sup>14</sup> giving the nitroalcohol **10**, the other product obtained from the reaction. During the decomposition of **8**, nitric oxide (nitrogen monoxide) could likely be formed and this is consistent with the detection of a nitroxide radical when the reaction mixture was analysed by ESR spectroscopy (see Scheme 2). In order to support the formation of `NO, as shown in Scheme 2,  $CoQ_{10}$  was reacted with *tert*-butyl-nitrite in the presence of oxygen: the formation of the nitroxide was observed also in this case.<sup>33</sup>

#### 7. DFT calculations

Appropriate DFT calculations were performed with the aim to verify the feasibility of the mechanistic proposal drawn in Scheme 3. Products obtained from the reaction with the model compound 1-phenyl-3-methyl-2-butene and the results of the spectroscopic measurements performed on CoQ clearly indicate that 'NO<sub>2</sub> addition to the isoprenic double bond occurs and is a very likely reaction pathway, even though hydrogen abstraction cannot be ruled out. The methylene group directly bonded to the quinoid moiety is in a "bis-allylic" position and the C-H BDH (Bond Dissociation Enthalpy) is expected to be very low. Indeed, a value of 71.34 kcal mol<sup>-1</sup> was obtained from our calculations (see Scheme S4, ESI<sup>+</sup>) prompting us to look for products derived from a hydrogen abstraction from this allylic position, 'NO2 being a very good hydrogen abstractor.14,32 However, even if we cannot exclude this pathway, products of this kind were not unambiguously observed.

Moreover, although calculations indicate that H-abstraction by 'NO<sub>2</sub> is a more exergonic process than its addition to the isoprenoid C=C double bond (see Scheme S5, ESI<sup>†</sup>), the comparison of the Activation Energies ( $E_{att}$ ) for these reactions leaves no doubt about the fact that 'NO<sub>2</sub> addition is much faster than H-abstraction, probably by several orders of magnitude, in agreement with the reaction product distribution. In conclusion, it can be assumed that the whole process is under kinetic rather than thermodynamic control.

#### Conclusions

The data reported in this paper show that  $^{\circ}NO$  is not able to oxidise the reduced  $CoQ_{10}H_2$ , whereas  $^{\circ}NO_2$ , formed from  $^{\circ}NO$  in the presence of oxygen, is an efficient oxidant agent for  $CoQ_{10}H_2$ . It has also been demonstrated that  $^{\circ}NO_2$  adds to the isoprenic double bond of  $CoQ_{10}$  by direct observation of the corresponding derivatives detected by ESI-MS,  $^{1}H$  NMR, FT-IR and ESR; these results have been confirmed by studying the same reaction with model compounds such as  $CoQ_1$ ,  $CoQ_3$  and 1-phenyl-3-methyl-2-butene. DFT calculations demonstrate

that addition of 'NO2 to the double bond represents a kinetically favoured process with respect to hydrogen abstraction from the allylic position, even if the BDH for the allylic hydrogen is relatively low. At present, we do not have any indication about the rate constant for this addition reaction which depends on the type of the double bond. From the rate constant values found in the literature of 0.17  $M^{-1} s^{-1}$  and  $8 \times 10^4$ M<sup>-1</sup> s<sup>-1</sup> for the addition of 'NO<sub>2</sub> to 1-hexene and 2,5dimethyl-2,5-hexadiene, respectively<sup>34</sup> or even higher with polyunsaturated compounds like arachidonate  $(10^6 \text{ M}^{-1} \text{ s}^{-1})^{35}$ it can be deduced that the rate constant should significantly increase if the addition to the double bond gives a tertiary radical, as in our case. An indirect evidence for the formation of a tertiary radical is given by the observation of the persistent ESR signal, which is strictly similar to that of di-tert-butyl nitroxide.

### Experimental

Melting points are uncorrected and were measured with an Electrothermal apparatus. <sup>1</sup>H NMR spectra were recorded in CDCl<sub>3</sub> on Varian Gemini 200 or Varian 400 spectrometers. J values are given in Hz and residual protic solvent CHCl<sub>3</sub> ( $\delta_{\rm H}$  7.26 ppm) was used as the internal reference. <sup>13</sup>C NMR spectra were recorded in CDCl3 at 100 MHz on a Varian 400 spectrometer, using the central resonance of CDCl<sub>3</sub> ( $\delta_{\rm C}$  77.0 ppm) as the internal reference. ESR spectra were run on a Bruker EMX ESR spectrometer equipped with an XL microwave frequency counter, Model 3120 for the determination of g-factor and on a Varian E4 spectrometer. The spectra were recorded with the following instrumental settings: modulation frequency 100 kHz, modulation amplitude 0.4 G, sweep width 40 G, microwave power 5 mW, time constant 1.28 s, receiver gain  $5 \times 10^3$ . Computer simulation of ESR spectra was calculated using the WinSim program in the NIEHS public ESR software tools package (www.epr.niehs.nih.gov/).<sup>36</sup> IR spectra were recorded in CCl<sub>4</sub> solution on a Perkin-Elmer Fourier Transform Infrared 20-SX spectrometer equipped with a Spectra Tech. Mass spectra were recorded with a QStar Elite (Applied Biosystems SCIEX) mass spectrometer with a QTOF detector.

Gaseous nitrogen dioxide (98.5% purity) was purchased from Fluka. It was used without purification in the volumetric preparation of the solutions: a 20 mL volumetric flask was filled with the chosen solvent and weighed. Gaseous nitrogen dioxide was bubbled for a few seconds and the flask was weighed again: the difference between the two weights gave the amount, and hence the number of moles, of  $^{\rm NO}_2$  dissolved in 20 mL; nitrogen dioxide was also prepared by decomposition of lead(n) nitrate. Gaseous nitrogen monoxide (nitric oxide,  $^{\rm NO}$ ) (98.5% purity) was purchased from Aldrich or was generated from the autodecomposition of nitrous acid obtained from sodium nitrite and monochloroacetic acid.  $^{\rm NO}$  was purified from its higher oxides by passing it through a deaerated 20% aqueous KOH solution under argon. Coenzymes  $CoQ_1$ ,  $CoQ_3$  and  $CoQ_{10}$  were purchased from Sigma-Aldrich. 1-Phenyl-3-methyl-2-butene was obtained from Frinton Laboratories, Inc. (USA). Solvents were Carlo Erba reagents, ACS grade and were used without further purification.

#### Oxidation of $CoQ_{10}H_2$ by `NO and $`NO_2$

 $CoQ_{10}H_2$  (100 mg in 5 mL of *n*-hexane) was dissolved in a test tube and degassed with argon. While degassing, purified 'NO was introduced into the  $CoQ_{10}H_2$  hexane solution. The bubbling continued for 30 min and the reaction was followed by tlc: no changes were observed. The same reaction was also repeated by dissolving  $CoQ_{10}H_2$  (100 mg in 5 mL of *n*-hexane) in one arm of a small Y-reactor together with sodium nitrite (3 mmol) and monochloroacetic acid (3 mmol) in hexane (5 mL) in the other arm. The solutions were accurately degassed by using the freeze-pump-thaw degassing method (the cycle was repeated for at least three times), mixed and stirred for 2 h. After this time,  $CoQ_{10}H_2$  remained unchanged.

The reaction was repeated by bubbling gaseous  $^{\circ}NO_2$  into the CoQ<sub>10</sub>H<sub>2</sub> hexane solutions but in this case a very rapid change in the colour of the solution indicated that the reaction was instantaneous and CoQ<sub>10</sub> was formed. The reaction solution was analyzed by ESR spectroscopy and an intense three line signal was recorded characterized by the following spectral parameters:  $a_N = 15.06$  G and g-factor = 2.0063(9).

## Reactions of CoQ<sub>1</sub>, CoQ<sub>3</sub> and CoQ<sub>10</sub> with 'NO<sub>2</sub>: ESR, FT-IR and <sup>1</sup>H NMR measurements

 $CoQ_1$ ,  $CoQ_3$  and  $CoQ_{10}$  15 mM solutions were prepared using benzene,  $CCl_4$  or  $CDCl_3$  as the solvents. A 'NO<sub>2</sub> solution was prepared, as described above, in the same solvents. The concentration of the 'NO<sub>2</sub> solutions varied each time they were prepared so the volumes to be used for the reactions were determined according to the molarity of the 'NO<sub>2</sub> solution.

To 500  $\mu$ L of CoQ solutions in benzene some drops of 'NO<sub>2</sub> solution were added (CoQ was in each case in excess compared to 'NO<sub>2</sub>). The reaction mixture was immediately transferred into a 50  $\mu$ L capillary tube and inserted in the ESR cavity for the measurements.

 $CCl_4$  solutions were used for FT-IR measurements. In this case, the proper volume of 'NO<sub>2</sub> solution was used in order to have a stoichiometric ratio or just a slight excess of CoQ (2 molecules of 'NO<sub>2</sub> were considered for each double bond of the isoprenic chain).

To 0.7 mL of  $CDCl_3$  solution of each CoQ the proper volume of  $^{\circ}NO_2$  solution in  $CDCl_3$  was added: equimolar amounts of the two species were used in the case of  $CoQ_1$ ; with  $CoQ_3$ , the ratio  $CoQ_3$ :  $^{\circ}NO_2$  was 2:1 whereas with  $CoQ_{10}$  a 3:1 ratio was used. The reaction mixture was then submitted to  $^{1}H$  NMR spectroscopy.

#### Reactions of CoQ<sub>1</sub>, CoQ<sub>3</sub> and CoQ<sub>10</sub> with 'NO<sub>2</sub>: ESI-MS/MS

High resolution MS and MS/MS experiments were performed with a QStar Elite mass spectrometer (Applied Biosystems SCIEX, Concord, ON, Canada) equipped with an electrospray

ionization source operated in the positive ion mode. The capillary voltage was set at +5500 V and the cone voltage at +75 V. In this hybrid instrument, ions were measured using an orthogonal acceleration time-of-flight (oa-TOF) mass analyzer. A quadrupole was used for selection of precursor ions to be further submitted to collision induced dissociation (CID) in MS/MS experiments. In MS, accurate mass measurements were performed using two reference ions from a poly(ethylene glycol) or a poly(propylene glycol) internal standard, according to a procedure described elsewhere.<sup>37</sup> Air was used as the nebulizing gas (10 psi) while nitrogen was used as the curtain gas (20 psi) as well as the collision gas. Collision energy was set according to the experiments. Instrument control, data acquisition and data processing of all experiments were achieved using Analyst software QS 2.0 provided by Applied Biosystems. Reaction media were diluted (dilution factor: 1/10 to 1/100) in a methanolic solution of ammonium acetate (3 mM) and sample solutions were introduced into the ionization source at a 5  $\mu$ L min<sup>-1</sup> flow rate using a syringe pump.

#### Reaction of 1-phenyl-3-methyl-2-butene 5 with 'NO2

1-Phenyl-3-methyl-2-butene was dissolved in benzene (268 mg, 2 mmol in 5 mL) and was reacted with two equivalents of 'NO<sub>2</sub> in benzene added dropwise, at room temperature, under stirring and in the absence of oxygen. In 5 minutes, the solution turned from colourless to pale-yellow and then to palegreen. At this point 500 µL of the reaction solution was tested by ESR: a spectrum of two superimposed three line signals was recorded. The two di-tert-alkyl nitroxides have the same g-factor 2.0062(8) and slightly different coupling constants,  $a_{\rm N}$  = 14.33 G and  $a_{\rm N}$  = 13.77 G. The reaction solution was left for 12 h under stirring at room temperature and then evaporated to dryness. The residue was repeatedly chromatographed by preparative SiO<sub>2</sub> tlc by eluting with cyclohexane-ethyl acetate 8:2, two compounds were isolated: a compound with  $R_{\rm f}$  = 0.36 identified as the dinitroadduct 7 on the basis of its X-ray analysis and of its spectroscopic data, and a compound with  $R_{\rm f} = 0.13$  identified as the nitroalcohol **10** for comparison with an authentic sample (see ESI<sup>+</sup>).

(3-Methyl-2,3-dinitrobutyl)benzene 7. M.p. 67–68 °C (crystals from evaporation of the CDCl<sub>3</sub> solution); IR (cm<sup>-1</sup>, neat) 3032, 1555, 1538, 1365, 700; <sup>1</sup>H NMR (400 MHz) δ 1.76 (3H, s), 1.82 (3H, s), 2.99 (1H, dd, J = 1.7 and 14.5 Hz), 3.35 (1H, dd, J = 11.5 and 14.5 Hz), 5.41 (1H, dd, J = 1.7 and 11.5 Hz), 7.14–7.17 (2H, m), 7.28–7.34 (3H, m); <sup>13</sup>C NMR (100 MHz) δ 21.8, 25.0, 35.3, 88.5, 94.4, 128.2, 128.9, 129.0, 129.3, 134.5; HRMS calcd for C<sub>11</sub>H<sub>18</sub>N<sub>3</sub>O<sub>4</sub> [M + NH<sub>4</sub>]<sup>+</sup> 256.1292, Found: 256.1589.

**2-Methyl-3-nitro-4-phenylbutan-2-ol 10.** Colorless oil; IR (cm<sup>-1</sup>, neat) 3445, 3032, 1550, 1456, 1372, 700; <sup>1</sup>H NMR (400 MHz)  $\delta$  1.37 (3H, s), 1.40 (3H, s), 3.18 (1H, dd, J = 3.0 and 14.5 Hz), 3.38 (1H, dd, J = 11.5 and 14.6 Hz), 4.66 (1H, dd, J = 3.0 and 11.5 Hz), 7.14–7.17 (2H, m), 7.25–7.32 (3H, m); <sup>13</sup>C NMR (100 MHz)  $\delta$  26.1, 27.6, 35.0, 71.3, 98.3, 127.7, 128.2, 128.9, 135.8; HRMS calcd for C<sub>11</sub>H<sub>19</sub>N<sub>2</sub>O<sub>3</sub> [M + NH<sub>4</sub>]<sup>+</sup> 227.1390, Found: 227.1408.

#### Single crystal X-ray analysis of compound 7

Crystallographic data were collected at room temperature on a Siemens AED diffractometer using Cu-Ka radiation  $(\lambda = 1.54178 \text{ Å})$ . Data were corrected for Lorentz and polarization effects but not for absorption. The structures were solved by direct methods using SIR9738 and anisotropically refined for all the non-H atoms. The refinements were performed on  $F^2$  using SHELXL-97.<sup>39</sup> All H atoms were placed at calculated positions and refined using a riding model approximation, with C-H = 0.93-0.98 Å, and with  $U_{iso}(H) = 1.2 U_{eq}(C)$  or 1.5  $U_{eq}(C)$  for methyl H atoms.  $C_{11}H_{14}N_2O_4$ ,  $M_r = 238.24$ , monoclinic, space group  $P2_1/n$ , a = 11.0598(8), b = 9.6504(6), c = 12.3144(10) Å,  $\beta = 112.315(4)^{\circ}$ , V = 1215.90(15) Å<sup>3</sup>, Z = 4,  $\rho_{\text{calc}} = 1.301 \text{ g cm}^{-3}, \mu = 0.842 \text{ mm}^{-1}$ ; pale yellow block, crystal dimensions  $0.07 \times 0.15 \times 0.18$  mm, T = 294(2) K, 154 parameters, R = 0.046,  $wR_2 = 0.122$  (for 2294 unique reflections, I > 0), S = 1.081,  $\Delta \rho(\min/\max) = -0.16/0.12 \text{ e} \text{ Å}^{-3}$ .

#### **DFT calculations**

Density Functional Theory<sup>40,41</sup> calculations were carried out using the GAUSSIAN 09 suite of programs<sup>42</sup> on an IBM SP-6 at Cineca Supercomputing Center.43 All calculations on paramagnetic species were carried out with the unrestricted formalism, giving  $\langle S^2 \rangle = 0.7501 \pm 0.0003$  for spin contamination (after annihilation). Thermodynamic quantities have been computed at 298 K by means of frequency calculations performed employing the M06-2X<sup>44</sup> functional in conjunction with the 6-31+G(d,p) basis set, starting from molecular geometries computed at the B3-LYP/6-31G(d) level of theory. In addition, in the corresponding frequency calculations, imaginary (negative) values were never found, confirming that the computed geometries were always referred to a minimum. On the other hand, Transition State optimizations have been performed by means of the Quadratic Synchronous Transit (STQN)<sup>45,46</sup> approach (Gaussian QST3 keyword) employing the MPW1K functional,<sup>47</sup> employing the 6-31+G(d,p) basis set for both optimizations and frequency calculations; in these last runs, the consistency of the negative (imaginary) value found with the desired TS structure has been confirmed by visualization of the associated molecular mode by means of appropriate visualization programs.

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