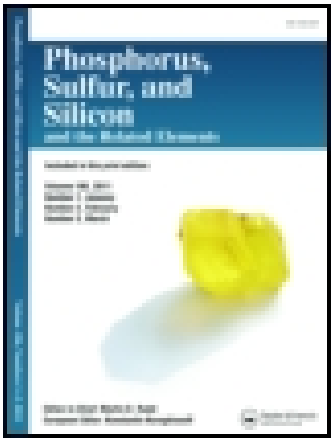

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SYNTHESIS AND CHARACTERIZATION OF NEW AROMATIC THIONITRITES

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SYNTHESIS AND CHARACTERIZATION OF NEW AROMATIC THIONITRITES

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Thionitrites (*S*-nitrosothiols) play an essential biological role as nitric oxide (NO˙) carriers. Here, we present the synthesis, the characterization and the stability studies in solution of new aromatic thionitrites **1b–4b** as potent nitric oxide donors. The four thionitrites were characterized by ^{1}H NMR, UV-visible and IR spectroscopies. Their decomposition occurs within a few minutes in dichloromethane, and yields quantitatively the corresponding disulfide. NO˙ and the thiyl radicals coming from their decomposition were trapped by distinct spin traps to give characteristic EPR signals. **4b** possesses the di-*tert*-butylphenol moiety responsible for the antioxidant properties of BHT and of the structurally-related drug probucol.

Keywords: Nitric oxide; *S*-Nitrosothiols; NO donors; antioxidant; probucol; BHT

INTRODUCTION

Nitric oxide (NO˙), considered until recently as a toxic radical, appears now to play a key role as messenger implicated in a wide range of biological processes including immune, cardiovascular or nervous systems.[1] Nitric oxide is biosynthesized by a two-step oxidation from L-arginine by NO-synthase (NOS), a NADPH-dependent enzyme.[2] Chemical reagents that can release NO˙ in physiological conditions are good candidates to mimic the activity of NOS. A potential therapeutic application lies in their possible use as vasodilators or as drugs for treatment of angina, and a chemical application in their use as storage medium for NO˙ gas which is often difficult to handle due to its high reactivity

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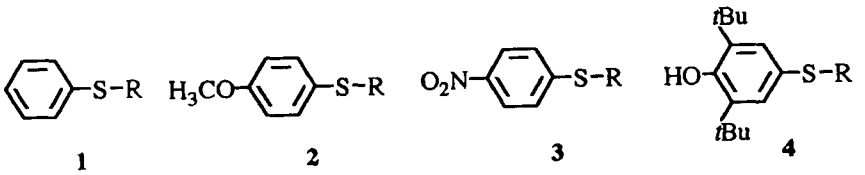

1 2 3 4

a: R = -H
b: R = -NO
c: R = -S-Ar

FIGURE 1 Structures of thiols (**a**), thionitrites (**b**) and disulfides (**c**).

and toxicity. The NO• donors can be classified into the main following categories: organic nitrate[3] (glyceryl nitrate, isosorbide dinitrate), organic nitrite[4] (amyl nitrite) or furoxane derivatives[5] associated with a thiol as cofactor, iron-nitrosyl complexes[6] (sodium nitroprusside), the NOR[7] and NOC[8] series (hydroxyimino and hydroxy-oxo-alkyltriazine compounds respectively), and *S*-nitrosothiols[9] (*S*-nitroso-*N*-acetyl-D,L-penicillamine, SNAP; *S*-nitrosoglutathione, GSNO; *S*-nitrosocysteine derivatives, *etc*). *In vivo*, high and low molecular weight *S*-nitrosothiols, including some protein thiols, are currently postulated to be carriers of NO•. Most of the synthetic *S*-nitrosothiols are relatively unstable and spontaneously break down to produce quantitatively NO• and the corresponding disulfide (equation (1)).

$$2R\text{-}S\text{-}NO \rightarrow R\text{-}S\text{-}S\text{-}R + 2NO^{\bullet} \quad (1)$$

However, some of them exhibit significant stability in solution, particularly those with bulky substituents[10] (*tert*-butyl thionitrite or triphenylmethyl thionitrite for example). Among the most stable thionitrites are the well known NO• donors SNAP[11] and GSNO[12] that are able to generate slowly NO• in physiological conditions, and thus might display pharmacological effects as potential vasodilator or neuroprotector. Some parameters (light, temperature) have been shown to affect the stability of thionitrites. In particular, the trace amounts of Cu^{2+} and Fe^{2+} present in the solutions would catalyze the decomposition of thionitrites to give the corresponding disulfide and NO•, and thus, the addition of ethylenediaminetetraacetic acid (EDTA) dramatically reduces the decomposition.[13]

In this paper, we report the synthesis, the characterization by ^1H NMR, UV-visible and IR spectroscopies, the EPR studies and the stability studies of a series of aromatic thionitrites **1b–4b** (Figure 1). While a large body of research

work has been reported in aliphatic series, little attention has been paid to aromatic thionitrites: *S*-nitrosothiophenol and *p*-methyl-*S*-nitrosothiophenol have been synthesized by reaction with dinitrogen tetroxide, but were not fully characterized.[14] The aromatic ring of coupounds **1**, **2** and **3** corresponds to a phenyl, a *p*-methoxyphenyl and a *p*-nitrophenyl group respectively, whereas **4** possesses the di-*tert*-butylphenol moiety responsible for the biological activity of the lipophilic antioxidant 3,5-di-*tert*-butyl-4-hydroxytoluene (BHT) and of the structurally-related antiatherogenic drug probucol (4,4′-[(1-methylethylidene)*bis*-(thio)]*bis*[2,6-*bis*(1,1-dimethylethyl)]phenol), which is known to decrease high blood cholesterol levels,[15] and possesses antioxidant properties.[16]

RESULTS AND DISCUSSION

Synthesis and spectral properties of thionitrites 1b–4b

Thiols precursors **1a–3a** of thionitrites **1b–3b** were commercially available, whereas thiol **4a** was obtained in two steps from 2,6-di-*tert*-butyl-phenol, which was first converted into the corresponding thiocyanate derivative by treatment with thiocyanogen, followed by a reduction with $LiAlH_4$. Thionitrites **1b–4b** were synthesized in mild conditions by electrophilic nitrosation of the corresponding parent thiols **1a–4a** with 1 equivalent of *tert*-butyl nitrite in dichloromethane at room temperature. They all gave a deep red (**1b**, **3b**) or green (**2b**, **4b**) colour in solution which characterizes the *S*-nitroso group. Nitrosation reactions were completed after 10 to 15 minutes and were quantitative as shown by ^1H NMR spectroscopy. The thionitrites were kept in solution at low temperature for short periods because none of them could be stored as a solid.

The thionitrites were characterized by ^1H NMR, UV-visible and IR spectroscopies. For the NMR experiments, thiols **1a–4a** were allowed to react *in situ* with 1 equivalent of *tert*-butyl nitrite in $CDCl_3$ at room temperature and spectra were immediatly recorded. ^1H NMR aromatic *ortho* and *meta* (relative to the sulfur atom H_o and H_m, respectively) protons shift values of thionitrites **1b–4b** are given in the Table. Compared to the parent thiols, the introduction of a nitroso group caused slight changes in the aromatic proton resonance. The protons H_o of the aromatic ring were slightly shifted downfield for the compound **1b** (0.30 ppm) and the nitro derivative **3b** (0.10 ppm), and upfield for electron-donating substituent compounds **2b** (0.20 ppm) and **4b** (0.19 ppm), whereas a deshielding effect was observed for all corresponding disulfides **1c–4c** (between 0.12 and 0.27 ppm, data not shown). Surprisingly, the four aromatic protons of *p*-methoxy-*S*-nitrosothiophenol **2b** were magnetically equivalent (singlet at 7.07

TABLE ^{1}H NMR, UV, IR and EPR data of thionitrites

		1b	*2b*	*3b*	*4b*
^{1}H NMR[a] (ppm)	H*o*	7.53–7.57 (m)	7.07 (s)	7.42–7.46 (d)	6.99 (s)
	H*m*	7.20–7.27 (dd)	7.07 (s)	8.37–8.41 (d)	–
UV[b] (λ_{max}, nm)		374 (1776)	376 (583)	380 (757)	378 (640)
		570 (53)	577 (68)	567 (15)	567 (50)
$t_{1/2}$ (min)[c]		7	9	12	14
EPR[d] (G)	a_N	13.6	13.6	13.8	14.0
	a_H	1.8	–	2.3	–

[a] ^{1}H NMR chemical shifts δ values recorded at 250 MHz for *ortho* (H*o*) and *meta* (H*m*) protons (relative to the sulfur atom). The values are reported in ppm relative to the residual peak of the solvent ($CDCl_3$). The multiplicity of the signals is indicated in parentheses. [b]Solvent: dichloromethane (ε in $M^{-1}cm^{-1}$ is indicated in parentheses). [c]Half-life time in minute of thionitrites in dichloromethane (85 mM). [d]EPR constant values of thiyl radicals trapped by BPN.

ppm), suggesting that the -SNO and -OCH_3 groups have in this case a comparable apparent shielding effect. In the spectrum of compound **1b**, a *para* proton shift (0.3 ppm, downfield) was detectable. After one hour, the ^{1}H NMR spectra of **1b**, **2b** and **3b** displayed the signals of the corresponding stable disulfides **1c**, **2c** and **3c**, respectively, while the decomposition of **4b** gave the corresponding disulfide **4c**, which quickly decomposed to a mixture of at least four products as shown by thin layer chromatography.

UV-visible spectroscopy of thionitrites **1–4b** in dichloromethane gave two bands at about 370 (with absorption coefficients of 600–1800 $M^{-1}cm^{-1}$) and 570 nm (ε = 15–70 $M^{-1}cm^{-1}$) (see Table), which are characteristic of the S − N = O group, responsible for the red or green colour of thionitrites in solution.

IR spectroscopy is a convenient method for the characterization of the NO moiety in NO-containing compounds. Thionitrites possess generally NO strechings frequencies in the range of 1490–1700 cm^{-1}. Other higher values (1900–1910 cm^{-1}) that seem apparently erratic have been reported. The four thionitrites **1b–4b** displayed a strong band at 1620 cm^{-1} that corresponds to N = O stretch. Weak absorption bands at 1000–1100 cm^{-1} corresponding to the S-N bond were barely detectable. Other unidentified bands at 1560 cm^{-1} (for **1b**), 1710 cm^{-1} (for **3b**) and 1540 cm^{-1} (for **4b**) were observed.

Stability of the thionitrites 1b–4b

The decomposition studies of the thionitrites **1b** to **4b** were carried out by UV-visible spectroscopy at room temperature noting the disappearance of the absorbance at 370 nm or at 570 nm that characterize the presence of a *S*-nitrosothiol group. The thionitrites were generated *in situ* by addition of 1 equivalent of *tert*-butyl nitrite to a solution in dichloromethane of the corre-

sponding thiol (85 mM). The half-life times of thionitrites **1b–4b** in these conditions were 7, 9, 12 and 14 minutes, respectively. These data show that there is no significant effect of the electron donating substituents of the aromatic group on the kinetic of the homolytic cleavage of the S-N bond. At 1 mM in the same solvent, the half-lives were comprised between 2 and 7 minutes suggesting that the four compounds present a relative stability, even at very low concentration.

EPR spectroscopy studies

Both thiyl radical intermediates RS$^{\bullet}$ and NO$^{\bullet}$ originating from the decomposition of thionitrites **1b–4b** were indirectly detected by EPR spectroscopy. The unstable thiyl radical intermediates RS$^{\bullet}$ coming from the homolytic cleavage of the corresponding thionitrites were detected at room temperature in toluene after reaction with *N-tert*-butyl-α-phenylnitrone (BPN) used as spin trap. EPR spectrum for compound **1b** is depicted in Figure 2A. Hyperfine splitting constants of thiyl radical-BPN spin adducts a_N and a_H are given in Table. a_N values vary from 13.6 to 14 G and a_H from 1.8 (**1b**) to 2.3 G (**3b**). Similar constant values for aromatic thiyl-BPN adducts in benzene were reported.[17] For **2b** and **4b**, a_H constants were not observable due to the poor resolution of the EPR signals. Otherwise, NO$^{\bullet}$ was allowed to react with a dithiocarbamate-Fe^{2+} complex [Fe(II)$(MGD)_2$]. Although paramagnetic, NO$^{\bullet}$ is not directly detected by EPR spectroscopy in solution or even in the solid state. However, it can be trapped in aqueous or organic solution at room temperature by the ferrous complex to give a characteristic three-line EPR signal (with an hyperfine splitting constant a_N = 12.85 G). We observed this triplet when thionitrites **1b–4b** were generated *in situ* in the presence of [Fe(II)$(MGD)_2$] at room temperature in dimethylformamide as solvent (Figure 2B). The same signal was observed with NO$^{\bullet}$ gas in identical experimental conditions. Besides this characteristic triplet, the NO-trapped spectra for the four thionitrites displayed a weak signal (Figure 2B) which could be due to the corresponding thiyl radical trapped by [Fe(II)$(MGD)_2$]. Further experiments will be carried out to determine the nature of these latter species.

4b: a NO$^{\bullet}$ releasing agent with antioxidant activity

Although NO$^{\bullet}$ is a relatively stable radical, it can react rapidly with a variety of biological species.[18] Among these reactions, it reacts with the superoxide radical anion $O_2^{\bullet -}$, to form peroxynitrite anion $ONOO^-$ (equation (2)) which may oxidize a great diversity of biomolecules including DNA and lipids within the

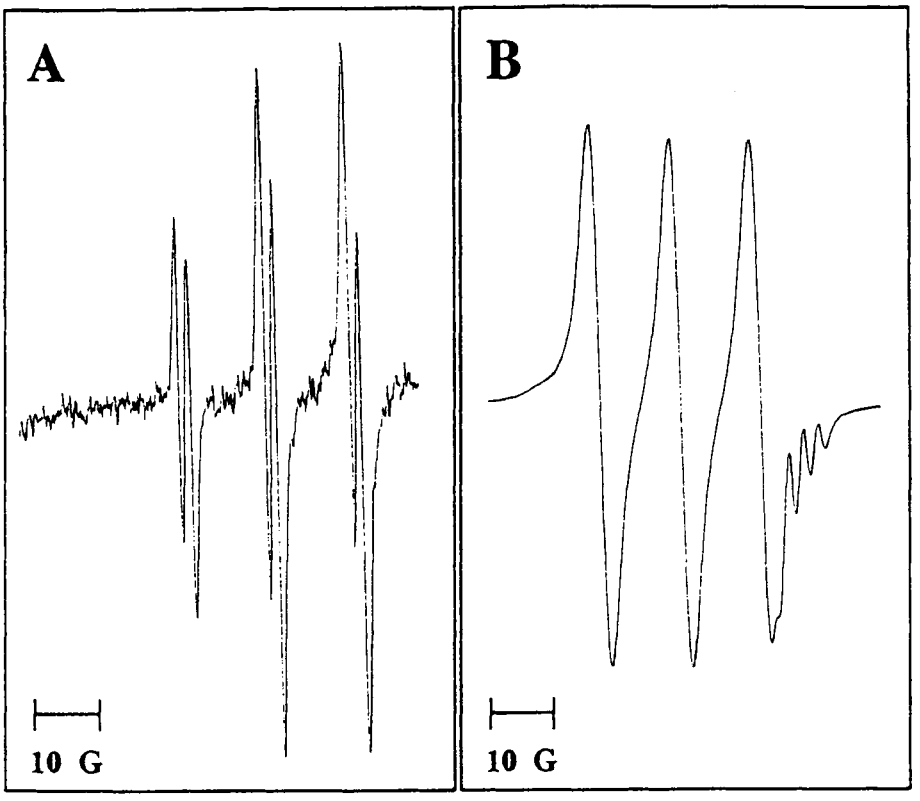

FIGURE 2 A. EPR spectrum recorded at room temperature in toluene of BPN-S-C_6H_5 adduct during decomposition of **1b**. **B**. EPR spectrum of NO$^{\bullet}$ trapped by [Fe(II)$(MGD)_2$] in dimethylformamide / water during decomposition of **3b** at room temperature (for details, see experimental section).

cell, and can act as a source of toxic hydroxyl radicals and nitrating species, especially in the presence of transition metal ions such as Cu^{2+} or Fe^{3+}.

The thionitrite **4b** possesses the di-*tert*-butylphenol moiety responsible for the therapeutic properties of probucol, a drug that lowers both serum low density lipoprotein (LDL) and high density lipoprotein (HDL) cholesterol levels, that contribute to the development of accelerated atherosclerosis and coronary artery

$$NO^{\bullet} + O_2^{\bullet -} \longrightarrow ONOO^- \xrightarrow{H^+} ONOOH \begin{cases} \longrightarrow NO_3^- + H^+ \\ \longrightarrow NO_2^{\bullet} + HO^{\bullet} \end{cases} \quad (2)$$

disease. Moreover, it has been shown that probucol, like the lipophilic antioxidant BHT (3,5-di-*tert*-butyl-4-hydroxytoluene) and related compounds, would have the capability of scavenging free radicals, including superoxide radical anion $O_2^{\bullet -}$, and thus would avoid the subsequent formation of peroxynitrite anion $ONOO^-$. We hypothesized that it might be advantageous to combine the properties of this agent to those of a nitric oxide releasing agent. The two properties will exhibit complementary actions and effects: when nitric oxide is generated to reach its biological target, the peroxynitrite ion concentration will be limited by the presence of the antioxidant moiety of the NO-carrier.

EXPERIMENTAL

General

Nuclear magnetic resonance spectra were determined on a Bruker AC 250 instrument at 250 MHz. Electron spin resonance spectra were recorded on a Bruker ER 200 D spectrometer at 9.71 GHz. Ultraviolet spectra were recorded with an UVIKON 931 spectrometer (Kontron instruments), and Infra red spectra with a 1760X transform Fourier spectrophotometer (Perkin Elmer). Silica plates (60 Å—17 μm, SDS) were used for thin layer chromatography. *N-tert*-butyl-α-phenylnitrone (BPN) was purchased from Sigma. All other chemicals were obtained from Aldrich and used without further purification. Disulfides **1c–4c** generated after decomposition of corresponding thionitrites were compared to authentical samples purchased from Aldrich (**1c**) or obtained by oxidation of the corresponding thiols with hydrogen peroxide in acetonitrile (**2c–4c**). 4-hydroxy-3,5-di-*tert*-butylthiophenol (**1a**) was prepared in two steps by treating 2,6-di-*tert*-butylphenol with thiocyanogen followed by a reduction with $LiAlH_4$. *N*-methyl-D-glucamine dithiocarbamate (MGD) was synthesized from D-glucamine and carbon disulfide and purified by recrystallization in 10% acetone in water as previously published.[19]

General Procedure for Thionitrites Generation

To a magnetically stirred solution of thiol (1.5 mmol) in dichloromethane (20 ml) are added dropwise 180 μl of *tert*-butyl nitrite (1.5 mmol, 1 equivalent) at 0 °C. After 15 minutes, the solution is concentrated under reduced pressure to a volume of 5 ml, and kept at −20 °C under argon; thin layer chromatography on silica (eluent: cyclohexane / dichloromethane: 90/10): **1b**, R_f = 0.75; **2b**, not detectable; **3b**, R_f = 0.26; **4b**, R_f = 0.70.

Characterization of Thionitrites

NMR experiments

Thionitrites **1b** to **4b** are generated *in situ* in the NMR tubes: to a solution of thiol (0.2 M) in $CDCl_3$ (1 ml) are added 200 µl of a solution of *tert*-butyl nitrite in $CDCl_3$ (1.1 M, 1.1 equivalent) at room temperature; the spectra are then immediatly recorded; δ (ppm): **1b**, 7.20–7.23 (d, 2H), 7.53–7.58 (m, 3H); **2b**, 3.90 (s, 3H), 7.07 (s, 4H); **3b**, 7.43–7.46 (d, 2H), 8.38–8.41 (d, 2H); **4b**, 1.47 (s, 18H), 5.63 (s, 1H), 6.99 (s, 2H).

UV experiments and decomposition studies

To a solution (1 ml) of thiol (0.25 M) in dichloromethane are added 250 µl of a solution of *tert*-butyl nitrite (1.1 M) in dichloromethane. Then, 20 µl of this solution are added to 3 ml of dichloromethane in an UV cell, and the spectra are immediatly recorded from 300 to 700 nm; for λ_{max} and ε values: see Table.

The decomposition studies of the thionitrites are carried out by noting the disappearance of the absorbance at 374–380 nm or at 570–578 nm. Half-lives at 1.3 mM: to 1 ml of a solution of thiol (0.25 M) in dichloromethane are added 250 µl of a solution of *tert*-butyl nitrite (1.1 M) in the same solvent. 20 µl of this solution are then added to 3 ml of dichloromethane in an UV cell, and the spectra are recorded. Half-lives at 85 mM: to 1 ml of a solution of thiol (0.25 M) in dichloromethane are added 35 µl of neat *tert*-butyl nitrite, and to this solution are added 2 ml of dichloromethane, and the spectra are recorded.

IR experiments

To a solution of thiol (40–80 mM) in chloroform is added 1 equivalent of *tert*-butyl nitrite at room temperature, and the spectra are then recorded in a CaF_2 cell between 900 and 4000 cm^{-1}.

EPR experiments

Thiyl radicals trapping: 0.5 ml of a solution of thiol (0.1 M) in toluene with 1 equivalent of *tert*-butyl nitrite at 0 °C is added to a solution of BPN (0.1 M) in toluene (0.5 ml) and the spectra are immediatly recorded.

NO trapping: a solution of thiol (0.05 M) in DMF (880 µl) with 6 µl of neat *tert*-butyl nitrite (1 equivalent) is added to 120 µl of a fresh stock aqueous solution of $[Fe(II)(MGD)_2]$ (1 mM Fe^{2+}) in water at room temperature. The

EPR signals are then recorded. The ferrous complex [Fe(II)$(MGD)_2$] is prepared by addition of 5 equivalents of MGD to an aqueous solution of ammonium iron(II) sulfate hexahydrate[20] (final concentration: 1 mM Fe^{2+}).

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