S-Nitrosothiol and Disulfide Formation through Peroxynitrite-Promoted Oxidation of Thiols

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Peroxynitrite reacts with thiols **1** at acidic pH to give the corresponding *S*-nitrosothiols **2** and disulfides **3**. The formation of nitrosothiols **2**, the yield of which is strongly pH-dependent, can be rationalized in terms of acid-catalyzed decomposition of the undissociated HPN, probably through the intermediacy of the protonated form H_2PN^+, leading to a species, X–NO, capable of nitrosating the thiol function. In contrast, the formation of disulfides **3** occurs in a manner independent of the pH, without the intermediacy of sulfanyl radicals. Under basic conditions, the peroxynitrite anion (PN) oxidizes the thiolate ion to sulfanyl radicals, eventually leading to disulfide **3**, or undergoes a thiol-catalyzed decomposition. The former is the exclusive reaction exhibited by peroxynitrite at pH > 13.

Introduction

The peroxynitrite ion $ONOO^-$ (PN) is a relatively stable species capable of oxidizing biological targets according to either a one- or two-electron mechanism.[1]

At physiological to acidic pH,[1,2] PN is in equilibrium with its conjugate acid ONOOH (HPN) ($pK_a = 6.8$).[1b] The latter rapidly undergoes O−O bond scission ($\tau_{1/2} < 1$ s)[3] with formation of an "in cage" radical pair, which can act as a source of hydroxyl radicals and NO_2 (30%) or rearrange to nitric acid (70%).[2,4] However, it has been reported that HPN can also oxidize suitable substrates, either according to a direct one- or two-electron transfer mechanism or by an indirect one-electron mechanism.[1b,5]

Most notably, peroxynitrite[6] has been shown to be involved in the in vitro biological oxidation of the thiol function. In 1991, Radi et al.[7] reported that cysteine was oxidized to cystine: the authors proposed the involvement of a two-electron transfer process between peroxynitrite and the undissociated form of the thiol. Accordingly, the second-order rate constant was found to be pH-dependent with a maximum at 6.8, notwithstanding that the yield of disulfide increased with pH up to 10.

Many intermediates have been proposed for the formation of disulfides in the peroxynitrite-promoted thiol oxidation, e.g. sulfenic acids,[1a] sulfanyl radicals,[8] thionitrates, and sulfenyl nitrites.[1a] It has also been reported that at physiological pH peroxynitrite can give rise to the formation of small amounts of *S*-nitrosothiols, which can behave as slow NO carriers.[9] However, the mechanism of thiol nitrosation remains somewhat unclear, even though a direct electrophilic nitrosation has been suggested.[10]

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It would seem that the mechanism of peroxynitrite-promoted thiol oxidation is far from being fully understood. Except for the case of the aforementioned work by Radi,[7] who studied the oxidation of cysteine and bovine serum albumin in the pH range 6.5−10, all the available data in the literature concern thiol oxidations carried out at physiological pH (i.e. 7.4). At this value, both PN and HPN forms are present in equilibrium, hence it is not easy to ascertain the actual species responsible for the thiol oxidation. In our opinion, in order to achieve a better understanding of the reaction mechanism, we first need to study the behavior exhibited by peroxynitrite under conditions of acidic and basic pH, at which the undissociated HPN and the dissociated PN forms, respectively, largely predominate. To this end, we have explored the reaction of simple thiols **1** with peroxynitrite carried out under conditions of acidic (0−6) and basic (10.5−13.5) pH.

Results and Discussion

Reactions under acidic conditions were carried out at 5−10 °C by adding 2 mL of a 0.5 M aqueous solution of peroxynitrite (pH = 13.5) to a 0.1 M acetonitrile solution (10 mL) of the appropriate thiol **1a−d** containing 0.15 mL of 12 M hydrochloric acid. Under these conditions, all the solutions immediately became colored due to the formation of the corresponding *S*-nitrosothiol **2a−d**.[11]

When benzylthiol **1a** was used, the solution became bright red. The yield of *S*-nitrosothiol **2a** increased over a period of 20 min, as monitored by spectrophotometric analysis at $\lambda = 550$ nm.[12] The reaction mixture was subsequently extracted with diethyl ether and the organic layer was separated and analyzed by ^{1}H NMR. The spectrum obtained showed the formation of the *S*-nitrosothiol **2a** and the disulfide **3a** in a 70:30 ratio in > 90% overall yield. Subsequent column chromatography allowed the isolation of **2a** in 55% yield, which rapidly decomposed upon

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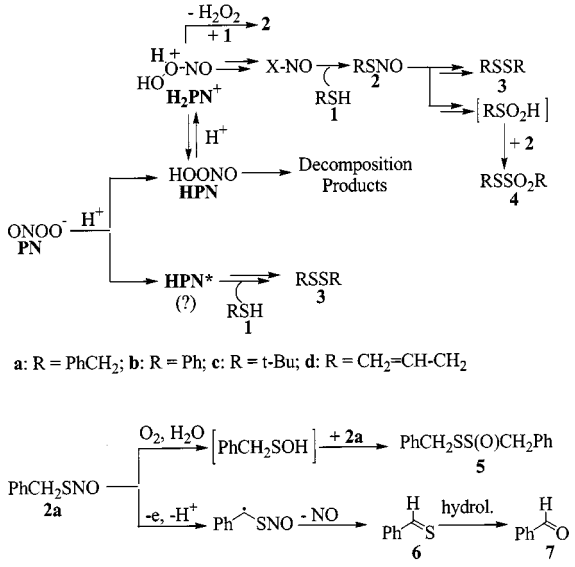
standing at room temperature in air to give a ca. 1:1 mixture of the disulfide **3a** and the benzyl thiosulfinate **5**. The formation of the thiosulfinate **5** would suggest the intermediacy of the sulfenic acid, $PhCH_2SOH$,[13] possibly originating from **2a** through air oxidation followed by hydrolysis due to the presence of moisture (see Scheme 1).



Scheme 1

From repeated experiments, we found that the reaction mixture slowly decomposed upon standing at room temperature over a period of 72 h to give mainly the disulfide **3a** together with trace amounts of the benzyl thiosulfonate **4a** and benzaldehyde **7**. When the reaction mixture was refluxed, decomposition of **2a** was found to be complete within 20 min. Thereafter, GC/MS and ^1H NMR analyses showed the presence of the disulfide **3a**, benzaldehyde **7**, and dibenzyl sulfone ($PhCH_2SO_2CH_2Ph$) in a 6:2:1 ratio. The formation of **4a** and **7** can be rationalized in terms of further oxidation of the *S*-nitrosothiol **2a**. Specifically, oxidation of **2a** might lead to thioaldehyde **6**, and then to aldehyde **7**, through an oxidation/deprotonation process followed by nitric oxide loss, whereas the formation of **4a** might be accounted for in terms of the intermediacy of the sulfinic acid, $PhCH_2SO_2H$,[13] as depicted in Scheme 1. At present, the formation of dibenzyl sulfone remains unclear.

As for the disulfide **3a**, at first sight this product might be viewed as being derived from decomposition of **2a** which, in all cases examined, afforded **3a** as the main product together with minor amounts of other compounds, the nature of which was found to depend on the mode of decomposition, as outlined above. However, we found *S*-nitrosothiol **2a** to be rather stable under the reaction conditions; on the other hand, we found that the disulfide **3a** was formed in 30% yield after just 20 min. On this basis, it seems likely that disulfide **3a** was formed, at least in part, from peroxynitrite-promoted reaction of **1a** in competition with the formation of *S*-nitrosothiol **2a**.

This hypothesis is strongly supported by results obtained with *tert*-butylthiol **1c**. In this case, the reaction mixture immediately became reddish-green.[11] ^1H NMR analysis of the crude residue showed the formation of the disulfide **3c** and the *S*-nitrosothiol **2c** in a 40:60 ratio as the only detectable products. *S*-Nitrosothiol **2c** was found to be stable in acetonitrile solution for several days; as a consequence, we can infer that the disulfide **3c** did not derive from decomposition of **2c**, but was rather directly formed from thiol **1c** in competition with the formation of the *S*-nitrosothiol **2c**.

The reaction mixture obtained with thiophenol **1b** immediately became deep brown-red;[11] TLC analysis showed the formation of the disulfide **3b** and *S*-nitrosothiol **2b** as the exclusive products. On standing at 10 °C for 15 min, the color faded; TLC and GC/MS analysis of the resulting mixture showed the formation of the disulfide **3b** and the thiosulfonate **4b** as the only detectable products. Subsequent column chromatography allowed the separation of these products in yields of 45% and 15%, respectively.

Similarly to **1b**, the reaction of allylthiol **1d** gave the *S*-nitrosothiol **2d**,[11] which was found to be unstable under the reaction conditions. The bright-red color faded within 30−45 min. Subsequent ^1H NMR analysis of the reaction mixture showed the formation of the disulfide **3d** as the major product (60%), together with minor amounts of other unidentified compounds.

The overall findings indicate that the peroxynitrite-promoted reaction of thiols **1** under acidic conditions generally gives *S*-nitrosothiols **2** and disulfides **3** as the exclusive reaction products. However, the finding that the yields of products **2a** and **2c** increased over a period of 20 min (vide supra) clearly indicates that HPN itself ($\tau_{1/2} < 1$ s)[3] is not the actual species responsible for the nitrosation of the thiol function.

The reaction of thiol **1a** with HPN was repeated at different pH values in the range 0−6. In each case, *S*-nitrosothiol **2a** and disulfide **3a** were found to be the only reaction products, as detected by ^1H NMR analysis. We observed that the yield of the disulfide **3a** (30%, based on starting thiol) was essentially independent of the pH in the aforementioned range.

In contrast, the yield of the *S*-nitrosothiol **2a** rapidly decreased at pH > 1 (Figure 1). We suggest that at acidic pH the undissociated HPN might undergo, in competition with the fast decomposition pathway to nitrate ion,[2−4] an acid-catalyzed decomposition, leading to some species, X−NO, capable of behaving as the actual nitrosating agent. This acid-catalyzed decomposition, which probably occurs through the intermediacy of the protonated form, H_2PN^+, would then prevail over the decomposition to nitrate ion under very acidic conditions (pH < 1). On the other hand, we cannot exclude that the protonated form itself could be in part responsible for the formation of the *S*-nitrosothiol **2a**, through direct nucleophilic attack at the nitrogen atom by thiol **1** with displacement of hydrogen peroxide (Scheme 1).

The finding that the formation of the disulfide **3a** shows no pH dependence was somewhat surprising. At first sight, we might suppose that HPN can either afford the nitrosating species and then *S*-nitrosothiol **2a**, through the intermediacy of the protonated form H_2PN^+, or decompose lead-

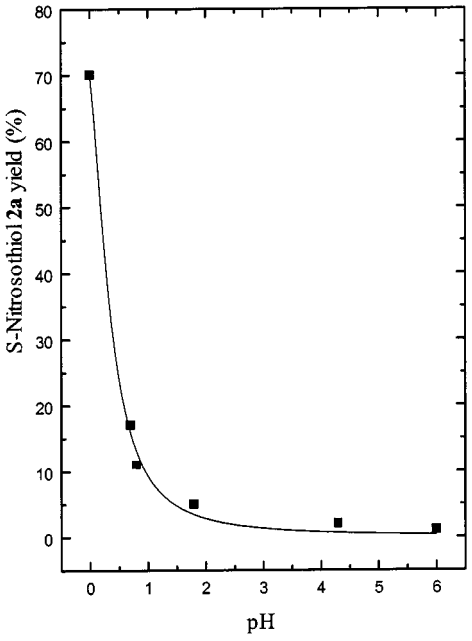


Figure 1. Influence of pH on the yield of *S*-nitrosothiol **2a** in the reaction of peroxynitrite with thiol **1a** under acidic conditions

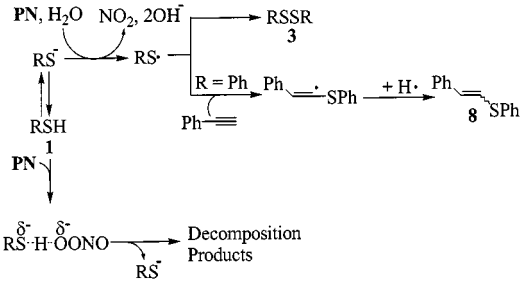
ing to some species[2,4] capable of oxidizing **1a** to disulfide **3a**. If this were to be the case, the decrease in the yield of **2a** on increasing the pH would be expected to be compensated by an increase in the yield of disulfide **3a**, which instead was found to be independent of the yield of **2a**. Thus, two different species are seemingly responsible for the formation of **2a** and **3a**.

In the mid 1990s, Pryor et al.[1b] suggested that, at acidic pH, the protonation of PN leads to two isomeric species, HPN and HPN*. On this basis, we might hypothesize that the HPN form could give the nitrosating species, X−NO, under very acidic conditions, or decompose,[2−4] whereas the HPN* isomer could be responsible for the oxidation of the thiol function, affording the disulfide **3** (Scheme 1). However, at the present time, many doubts exist concerning the identity and even the actual existence of this HPN* species and, therefore, the question of the formation of the disulfide **3** remains open.

In order to obtain evidence for the possible intermediacy of sulfanyl radicals in the formation of disulfides **3**, we carried out the reactions of thiols **1b** and **1c** at pH = 0 in the presence of a twofold excess of phenylacetylene. Phenylacetylene is known to be a good scavenger for benzenesulfanyl radicals, which add to the C−C triple bond to eventually give a thiol/alkyne adduct through vinyl radical intermediates.[14] We found that in neither case was a thiol/alkyne adduct formed, suggesting that no sulfanyl radicals are involved as intermediates. At present, the formation of disulfides **3** in the peroxynitrite-promoted reaction of thiols **1** under acidic conditions remains unclear.

Peroxynitrite-promoted oxidation of the thiol function under basic conditions was studied in the pH range 10.5−13.5, in which the dissociated PN form largely predominates.

At pH = 13.5, thiols **1a**,**b** were found to be totally consumed within 5 min, leading to the corresponding disulfides **3a**,**b** as the only detectable products in > 90% yield. When the reaction of **1b** was carried out in the presence of a twofold excess of phenylacetylene, it gave instead a mixture of the disulfide **3b** and the thiol/alkyne adduct **8** in an 80:20 ratio as the only detectable products, as determined by GC/MS and ^{1}H NMR analyses. The formation of the thiol/alkyne adduct **8** indicated the presence of sulfanyl radicals and suggests that these species are likely to be involved as intermediates in the formation of disulfides **3** under basic conditions (Scheme 2).



Scheme 2

The reaction with thiol **1a** was repeated at various pH values in the range 10.5−13. The desired pH was attained by adding appropriate amounts of 12 M hydrochloric acid to the peroxynitrite solution (pH = 13.5). Under these conditions, peroxynitrite proved to be fairly stable, but immediately decomposed (within 30 s) when thiol **1a** was added, as could be monitored by spectrophotometric analysis. However, the conversion of **1a** dramatically decreased on decreasing the pH, as shown in Figure 2. In all cases, the disulfide **3a** was found to be the exclusive reaction product (> 90% yield, based on reacted thiol).

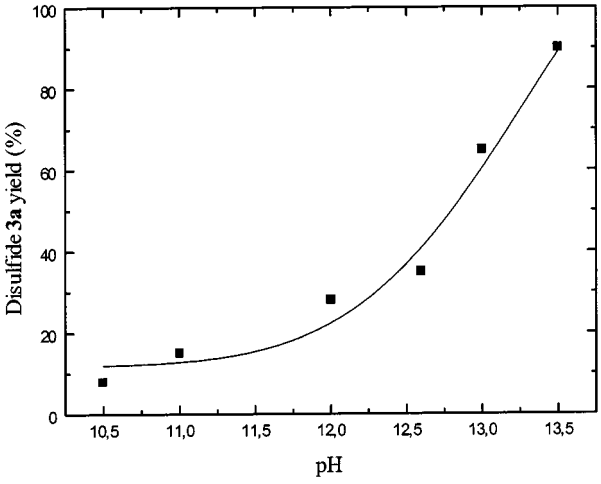


Figure 2. Influence of pH on the yield of disulfide **3a** in the reaction of peroxynitrite with thiol **1a** under basic conditions

These results indicate that two competing reactions are operative: a thiol-catalysed decomposition of PN and a PN-promoted oxidation of thiol to disulfide. The latter prevails under strongly basic conditions (pH > 13), where the thiolate ion is clearly prevalent over the undissociated thiol (Fig-

ure 2). This indicates that the oxidation occurs on the thiolate ion, rather than the thiol, leading first to the formation of sulfanyl radicals and then to the disulfide. In the pH range 10.5−13, in which both thiolate and thiol exist,[15] thiolate oxidation competes with the thiol-catalysed decomposition of PN, which possibly occurs through the formation of a hydrogen-bonded PN/thiol complex (Scheme 2).

Conclusions

Our results indicate that at very acidic pH the undissociated HPN undergoes acid-catalyzed decomposition, probably through the intermediacy of the protonated form H_2PN^+, leading to some species, X−NO, capable of nitrosating the thiol function. The latter reaction affords *S*-nitrosothiols **2** in yields that are strongly dependent on the pH (up to 70% at pH = 0) and represents a convenient synthetic route to these compounds, alternative to that reported in the literature.[13] In competition with the formation of nitrosothiols **2**, the formation of disulfides **3** occurs in a manner independent of the pH, without the intermediacy of sulfanyl radicals.

Under basic conditions, the PN form either oxidizes the thiolate ion to sulfanyl radicals or undergoes thiol-catalyzed decomposition. The former is the exclusive reaction exhibited by PN at pH > 13.

Experimental Section

General: NMR spectra were recorded with a Varian Gemini 200 instrument using Me_4Si as an internal standard. − GC-MS analyses were performed with a Carlo Erba QMD 1000 instrument. − UV/Vis spectra were recorded with a Perkin−Elmer Lambda 12 instrument.

Materials: Thiols **1a−d** and phenylacetylene were commercial products and were used as received. Peroxynitrite was synthesized following a procedure described in the literature.[16] According to this method, a solution of 30% H_2O_2 (10.0 mL) was diluted to a volume of 50 mL with water and chilled to 4 °C. To this solution, 5.0 M NaOH solution (18 mL) and a 0.04 M solution of diethylenetriaminepentaacetic acid (DTPA) in 0.05 M NaOH (5.0 mL) were added and the resulting mixture was diluted to a total volume of 100 mL with water (the final pH was ca. 13.5). This solution was then allowed to react with an equimolar amount of isoamyl nitrite (12.3 mL) under vigorous stirring for 3−4 h at ca. 15 °C. Thereafter, the aqueous phase was washed with chloroform (5 × 75 mL) to remove the contaminating isoamyl alcohol and the unchanged nitrite. The resulting yellow solution was treated with an excess of MnO_2 in order to destroy the unchanged H_2O_2, and then filtered. The concentration of peroxynitrite (usually in the range 0.45−0.50 M) was determined spectrophotometrically (ε_{302} = 1670 $M^{-1}cm^{-1}$).[17] Solutions kept at −18 °C showed little sign of decomposition over several weeks.

Reaction of Thiols 1a−d with Peroxynitrite under Acidic Conditions − General Procedure: To a 0.10 M acetonitrile solution of thiol **1a−d** (10.0 mL), 12.0 M hydrochloric acid (0.15 mL) was added at 5−10 °C. A 0.50 M aqueous solution of peroxynitrite (2.0 mL) was then added with stirring. The apparent final pH was 0. The reaction mixture immediately became colored due to the formation of the respective *S*-nitrosothiol **2a−d**.

Reaction with 1a: A bright-red coloration immediately developed[11] and slowly intensified over a period of 20 min, as determined by spectrophotometric measurements at 550 nm. After this time, the reaction mixture was extracted with diethyl ether, and the combined organic phases were washed with water and dried with Na_2SO_4. The solvent was removed under reduced pressure. ^{1}H NMR analysis of the residue showed the formation of *S*-nitrosothiol **2a**[12] and disulfide **3a** as the exclusive products in a 70:30 ratio. Subsequent column chromatography on silica gel gave, on elution with petroleum ether (b.p. 40−70 °C), the *S*-nitrosothiol **2a** (85 mg, 55%), identified by its UV/Vis spectrum[11] [^{1}H NMR (200 MHz): δ = 4.65 (br. s, 2 H), 7.11−7.42 (m, 5 H)] and dibenzyl disulfide **3a** (35 mg, 30%). *S*-Nitrosothiol **2a** decomposed within 2 h on standing in air to give a ca. 1:1 mixture of disulfide **3a** and benzyl phenylmethanethiosulfinate **5**,[18] as determined by ^{1}H NMR analysis. − In a repeated reaction, the reaction mixture was split into two portions. One of these was allowed to stand at room temperature. The bright-red coloration disappeared within 72 h. The resulting mixture was extracted with diethyl ether, the combined organic phases were washed with water, and the solvent was evaporated. The residue was found to consist mainly of the disulfide **3a**, contaminated with trace amounts of benzyl phenylmethanethiosulfonate **4a**[19] and benzaldehyde **7**, as determined by ^{1}H NMR and GC/MS analyses. The second portion of the reaction mixture was refluxed for 20 min and then worked up as described above. The residue was found to consist of the disulfide **3a**, dibenzyl sulfone ($PhCH_2SO_2CH_2Ph$), and benzaldehyde **7** in a 6:1:2 ratio, as determined by ^{1}H NMR and GC/MS analyses. − In independent experiments, aliquots of a 0.50 M aqueous solution of peroxynitrite (2.0 mL) were added at 5−10 °C under stirring to portions of a 0.10 M acetonitrile solution of thiol **1a** (10.0 mL) containing variable amounts of 12 M hydrochloric acid. The apparent final pH values were 0.0, 0.7, 0.8, 1.8, 4.3, and 6.0. The reaction mixtures were stirred for 20 min, and then the absorption at λ = 550 nm was measured. *S*-Nitrosothiol **2a** yields were determined on the basis of a 70% yield at pH = 0 (see Figure 1). Disulfide **3a** yields were determined by ^{1}H NMR analysis using an internal standard.

Reaction with 1b: A deep brown-red coloration immediately developed.[11] TLC analysis performed after 2 min showed the formation of the *S*-nitrosothiol **2b**[13] and the disulfide **3b** as the exclusive products. After stirring for a further 15 min, the color faded. The reaction mixture was then extracted with diethyl ether, the combined organic phases were washed with water, and the solvent was evaporated in vacuo. TLC analysis of the residue showed the presence of the disulfide **3b** and the thiosulfonate **4b** as the main reaction products. Subsequent silica gel column chromatography gave, on gradual elution with petroleum ether/diethyl ether, diphenyl disulfide **3b** (50 mg, 45%) and phenyl benzenethiosulfonate **4b** (20 mg, 15%). The reaction was then repeated, but with the addition of phenylacetylene (200 mg, 2.0 mmol) to the acetonitrile solution of **1b**. TLC and GC/MS analyses of the residue showed the presence of **3b** and **4b** as the only detectable products.

Reaction with 1c: A reddish-green coloration immediately developed.[11] After 20 min, the reaction mixture was extracted with diethyl ether, and the combined organic phases were washed with water and dried with Na_2SO_4. The solvent was removed under reduced pressure. ^{1}H NMR analysis of the residue using an internal standard showed the formation of *S*-nitrosothiol **2c**[13] and disulfide **3c** in yields of 40% and 30%, respectively. The reaction was then repeated, but with the addition of phenylacetylene (200 mg,

2.0 mmol) to the acetonitrile solution of **1c**. ^{1}H NMR analysis of the residue showed the presence of **2c** and **3c** as the only detectable products in a 60:40 ratio.

Reaction with 1d: A bright-red coloration immediately developed[11] due to the formation of *S*-nitrosothiol **2d** [UV/Vis: λ_{max} = 550, 518, and 416 nm]. The color faded within 30−45 min. The reaction mixture was then extracted with diethyl ether. The combined organic phases were washed with water, dried with Na_2SO_4, and the solvent was removed under reduced pressure. ^{1}H NMR analysis using an internal standard showed the formation of the disulfide **3d** in 60% yield, accompanied by unidentified by-products.

Reaction of Thiols 1a,b with Peroxynitrite under Basic Conditions: To portions of a 0.10 M acetonitrile solution of thiol **1a**,**b** (10.0 mL), aliquots of a 0.50 M aqueous solution of peroxynitrite (2.0 mL) (pH = 13.5) were added with stirring at 5−10 °C. Spectrophotometric analysis showed complete consumption of the peroxynitrite within 5 min. After this time, the reaction mixtures were extracted with diethyl ether and the combined organic phases were washed with water. GC/MS analysis using an internal standard showed that the starting thiols **1a**,**b** had been consumed and that the corresponding disulfides **3a**,**b** had been formed in 90% yield. − The reaction with **1b** was then repeated in the presence of phenylacetylene (200 mg, 2.0 mmol). ^{1}H NMR and GC/MS analyses of the reaction mixture showed the formation of the disulfide **3b** and β-(phenylthio)styrene (**8**)[14] in an 80:20 ratio. − The reaction with thiol **1a** was repeated at various pH values by using peroxynitrite solutions at pH = 13.0, 12.6, 12.0, 11.0, and 10.5; these were obtained from the 0.5 M solution at pH = 13.5 by the addition of appropriate amounts of 12.0 M hydrochloric acid. In all cases, spectrophotometric analysis showed the consumption of peroxynitrite within 30 s. The reaction mixtures were worked up as described above and analyzed by GC/MS using an internal standard to determine the conversion of thiol **1a** (see Figure 2) and the yield of disulfide **3a** (90% in all cases).

Acknowledgments

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[11] A more convenient and reliable identification of *S*-nitrosothiols is provided by their characteristic visible spectra. The bright-red *S*-nitrosothiol **2a** shows λ_{max} at 550 nm (ref.[13]); the reddish-green *S*-nitrosothiol **2c** shows λ_{max} at 552, 562, 598, and 605 nm (ref.[14]); the brown-red *S*-nitrosothiol **2b** shows λ_{max} at 530 and 570 nm (ref.[14]); the bright-red *S*-nitrosothiol **2d** shows λ_{max} at 550 and 518 nm.

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[15] Actually, the pH range in which the thiolate and thiol forms coexist should be around 9−10 considering the pK_a value (9.3); in fact, we observed the presence of the thiol even at pH ≤ 13. This is most likely the result of a co-solvent effect, which determines a minor dissociation of both PN and the thiol.

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