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Peroxynitrite and NO^+ donors form colored nitrite adducts with sinapinic acid: potential applications

Shirin Akhter,[a] James R. Green,[a] Paul Root,[a] Gregory J. Thatcher,[b] and Bulent Mutus[a,*]

[a] *Department of Chemistry and Biochemistry, University of Windsor, 401 Sunset Avenue, Windsor, Ont., Canada N9B 3P4*
[b] *Department of Medicinal Chemistry and Pharmacognosy, The University of Illinois at Chicago, Chicago, IL 60612, USA*

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Abstract

Sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, SA) reacted with peroxynitrous acid at neutral pH with a second-order rate constant of $812\,M^{-1}\,s^{-1}$, to yield a red product (λ_{max}, 532 nm). The identical colored product could be formed with acidified decomposed peroxynitrous acid solutions or nitrite at slower rates (0.1 M HCl, $8.32\,M^{-1}\,s^{-1}$; 10% acetic acid, $0.0004\,M^{-1}\,s^{-1}$). The red compound is thought to be *O*-nitrososinapinic acid (3,5-dimethoxy-4-nitrosooxycinnamic acid) which can be formed by reaction with either peroxynitrous acid or nitrous acid. The extinction coefficient of *O*-nitrososinapinic acid (ONSA) was estimated to be $8419\,M^{-1}\,cm^{-1}$ at 510 nm in 10% acetic acid and 90% acetonitrile. ONSA was also formed via NO^+ transfer from *S*-nitrosoglutathione (GSNO). ONSA in turn can S-nitrosate low molecular weight thiols and protein thiols. SA was also shown to act as a peroxynitrite sink as it effectively prevented the oxidation of dihydrorhodamine under physiological conditions. The fact that *O*-nitrososinapinic acid is stable and can be used to S-nitrosate thiol containing amino acids, peptides, and proteins makes it a potentially useful reagent in the study of *S*-nitrosothiol biochemistry and physiology. In addition, the relatively high extinction coefficient of *O*-nitrososinapinic acid means that it could be utilized as an analyte for the spectroscopic detection of peroxynitrite or NO^+-donors in the submicromolar range.

Keywords: *O*-Nitrososinapinic acid; Sinapinic acid; Peroxynitrite; NO^+ donors; S-Nitrosation; SIN-1

The term peroxynitrite is commonly used to describe the equilibrium mixture of oxoperoxonitrate ($ONOO^-$) and its conjugate acid, hydrogen oxoperoxonitrate (peroxynitrous acid, ONOOH). Peroxynitrite is a strong oxidant formed in the diffusion-controlled reaction of superoxide ($O_2^{\cdot-}$) and nitric oxide ($k = 3.9$–$6.7 \times 10^9\,M^{-1}\,s^{-1}$) [1,2]. Peroxynitrite formation and reactions are proposed to contribute to pathogenesis of a series of diseases including stroke [3], heart disease [4], and atherosclerosis [5]. There is growing evidence that peroxynitrite mediates its destructing power via oxidizing protein and non-protein thiols [6], DNA [7], low-density lipoproteins [8], or membrane phospholipids [9]. Peroxynitrite homolysis product-mediated oxidation of dihydrorhodamine 123 to rhodamine 123 is used as a probe of peroxynitrite production [10]. SIN-1[1] is commonly used to generate peroxynitrite in situ [11]. In solution, SIN-1 decomposes to release $NO^{\cdot}$ and $O_2^{\cdot-}$ simultaneously in a 1:1 stoichiometry in the presence of oxygen [12].

Nitrosonium cation (NO^+) is the active electrophile in the nitrosation of phenols [13] and anilines. It is also an effective oxidant in electron transfer from other electron rich compounds [14]. NO^+ is rapidly hydrolyzed in aqueous solution to give nitrous acid. The equilibrium constant for this reaction is $10^{-6.5}$ at 25 °C [13] and the lifetime of NO^+ in water is about 3×10^{-10} s. NO^+ is only found in aqueous solution at very high acidity. Nitrosation is an important process

* Corresponding author. Fax: +519-973-7098.
E-mail address: mutusb@uwindsor.ca (B. Mutus).

[1] *Abbreviations used:* SIN, 1,3-morpholinosydnonimine *N*-ethylcarbamide; SA, 3,5-dimethoxy-4-hydroxycinnamic acid; GSNO, *S*-nitrosoglutathione; DHR, dihydrorhodamine; RH, rhodamine; ONSA, *O*-nitrosoSA.

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in nitric oxide (NO) biochemistry where a significant portion of biosynthesized-NO reacts with molecular oxygen to produce N_2O_3. N_2O_3 can then nitrosate biologically relevant thiols like the Cys residues of serum albumin and glutathione to form *S*-nitrosothiols (RSNOs). RSNOs are involved in signaling pathways, immune responses, and in the actions of nitrovasodilating compounds [15–18]. More recently, we have directly demonstrated the involvement of cell surface protein–disulfide isomerase in the transfer of NO^+ from extracellular RSNOs to the cytosolic thiols [19].

S-Nitrosothiols are central players in nitric oxide physiology and pathophysiology. They are formed by the reaction of nitrosyl (NO^+) donors with free thiols or in transnitrosation reactions between *S*-nitrosothiols and free thiol containing amino acids, peptides, and proteins. Currently there are no reagents for the direct colorimetric detection of NO^+ in the biological system or in vitro.

Peroxynitrite is the major cytotoxic agent produced in many pathological conditions. Therefore, compounds that can selectively and rapidly react with peroxynitrite are in demand as they could potentially be used in the biological milieu to protect against peroxynitrite-induced toxicity.

This study was initiated by the keen observation of Dr. Don T. Krajcarski (ret., National Research Council of Canada) who mixed our peroxynitrite treated protein samples with SA in preparation for MALDI-TOF MS. He reported to us that the samples were transiently turning bright red. Therefore, we set out to identify the reactant(s) yielding the red compound and upon finding that it could be formed by the reaction of SA with nitrous acid or peroxynitrous acid, to determine its usefulness as a probe to study NO_x chemistry and biochemistry.

Materials and methods

Materials

Glutathione, SA, 4-hydroxycinnamic acid, 3,4,5-trimethoxycinnamic acid, 3,4,5-trimethoxyhydrocinnamic acid, and sodium nitrite were purchased from Sigma–Aldrich (St. Louis MO). DHR-6G was purchased from Molecular Probes (Eugene, OR).

Methods and instruments

Synthesis of peroxynitrite

Peroxynitrite was synthesized in quenched-flow reactor as described in [20]. Solutions of: (i) 0.6 M $NaNO_2$ and (ii) 0.6 M HCl/0.7 M H_2O_2 were pumped at 26 ml/min into a tee-junction and mixed in a 3-mm diameter by 2.5-cm glass tube. The acid catalyzed reaction of nitrous acid with H_2O_2 to form peroxynitrous acid was quenched by pumping 1.5 M NaOH at the same rate into a second tee-junction at the end of the glass tubing. Excess H_2O_2 was removed by passage of peroxynitrite solution over MnO_2 powder. The solution was frozen at −20 °C for as long as a week. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$) [21].

Synthesis of dihydroSA

A slurry of Raney Nickel in ethanol was added to a solution of SA (80.4 mg) in ethanol (15 ml) saturated with H_2 gas. The mixture was stirred for 24 h under an H_2 atmosphere. The ethanol was removed under reduced pressure and 3 M HCl was added. After stirring for 2 h, the mixture was subjected to repeated extraction with diethylether. Concentration of the combined diethyl ether layers, followed by filtration through Celite and re-concentration under reduced pressure gave 3-(3,5-dimethoxy-4-hydroxyphenyl)propionic acid (dihydroSA, 80.9 mg, 100%) of sufficient purity for further use. Recrystallization from Et_2O–petroleum ether gave colorless crystals: mp 103–104 °C (lit 90–91 °C (MeOH–$CHCl_3$) [22].

Kinetic studies of the peroxynitrite reactions

SA was dissolved in acetonitrile and diluted in 0.1 M sodium phosphate buffer, pH 7.4 to get a final concentration of 10 μM (final conc. of acetonitrile was 1%) in a cuvette. The reaction was started by the addition of 100 μM peroxynitrite. Time dependent spectra were monitored immediately after the addition of peroxynitrite on an Agilent 8453 UV–Vis spectrometer.

Reaction of SA in acidified nitrite

SA was dissolved in 90% acetonitrile 0.1 M HCl/10% acetic acid to a final concentration of 2.67×10^{-4} M. SA solution in 60% ACN and 10% acetic acid was prepared the same way to a final concentration of 5.35×10^{-4} M. The reaction was initiated by the addition of equimolar sodium nitrite. The UV–Vis spectrum of the solution was monitored as a function of time on an Agilent 8453 UV–Vis spectrometer.

Assay of SIN-1 mediated oxidation of dihydrorhodamine

Stock solutions were freshly prepared (SIN-1 in phosphate buffer; SA in acetonitrile; dihydrorhodamine (DHR) 6G, and rhodamine 6G in DMF), and diluted using phosphate buffer (100 mM, pH 7.4) to the desired

concentrations and a total volume of 200 μl. Concentrations of SIN-1 and DHR-6G were fixed at 20 and 5 μM, respectively. Readings were taken in a multiplate fluorescence reader (Spectra Max Gemini XS Multiplate Reader Spectrophotometer, Molecular Devices) in triplicate and averaged. Data were normalized by setting the value of flourescence intensity of rhodamine-6G to 100%, and that of DHR-6G at time zero to 0%. Excitation and emission wavelengths were set at 528 and 554 nm, respectively. Reactions were carried out in covered air equilibrated solutions. The chemicals were added in the order of DHR, phosphate buffer, SA, and lastly SIN-1 to initiate reaction. Percentage of all the organic solvents in the final mixture was in the range of 0.3–0.1% (for acetonitrile it was less than 0.1% and for DMF it was less than 0.2%).

O-NitrosoSA formation from GSNO and SA

GSNO solutions (0.3–1.5 μM) were incubated with 0.5 mM SA for 15 min. The absorption spectra of the mixtures were recorded at 25 °C within the wavelength range of 450–600 nm after the incubation period.

Isolation of the red product

SA (0.2 g) was dissolved in 15 ml methanol and diluted in 125 ml of 0.1 M acetic acid pH 2.0. Sodium nitrite (0.11 g) was added to the SA solution followed by the addition of 4.8 ml of 2 M HCl. The reaction was instantaneous. The pH of the reaction mixture was adjusted back to 7.4 with 2 M NaOH after the completion of the reaction and the reaction mixture was freeze dried immediately and stored at −80 °C.

Spectroscopic measurement of BSA-SNO

Three hundred and seventy five μM BSA solution was prepared in phosphate-buffered saline, pH 7.4. Freeze dried neutral red product (*O*-nitrosoSA) was dissolved in minimal volume of methanol and added in equimolar amounts to the BSA solution. The reaction mixture was incubated at room temperature for 35 min under stirring. At the end of the incubation period the product was passed through Sephadex G-25 column preequilibrated with the same buffer.

Results

Reaction of peroxynitrite with SA

Reaction of SA (10 μM) with 10-fold molar excess of peroxynitrite in 0.1 M phosphate buffer at pH 7.4 (Fig. 1, □) resulted in the formation of a red compound (λ_{max}, 532 nm; Fig. 1 inset). Nitrite did not react with SA under these conditions (Fig. 1, ○). The second-order rate constant extracted from the data performed under pseudo-first-order experimental conditions was estimated to be 812 $M^{-1} s^{-1}$. The extinction coefficient of the red product was found to be 2074.5 $M^{-1} cm^{-1}$ at 532 nm under these experimental conditions. Decomposed peroxynitrite, when added to SA (10 μM) in 10% acetic acid, also resulted in a red product, but the rate of formation was ~300-fold lower.

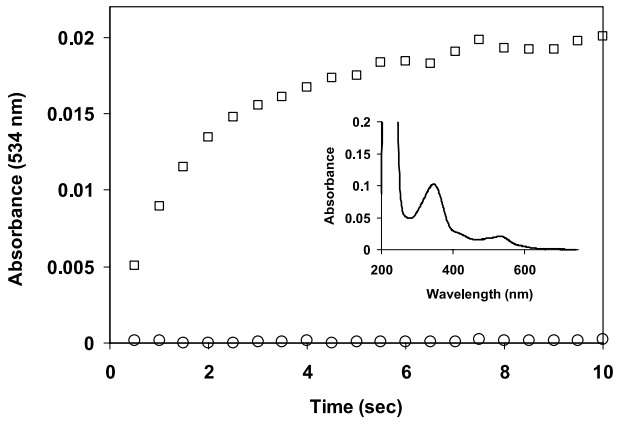

Fig. 1. Assay of peroxynitrite with SA. Either 100 μM peroxynitrite (□) or 100 μM sodium nitrite (○) solution was added to 10 μM SA in 0.1 M sodium phosphate buffer, pH 7.4. The absorbance (535 nm) was monitored as a function of time. Inset: The spectrum of the peroxynitrite plus SA solution after 10 s.

Acidified nitrite also forms a red compound with SA

UV–Vis spectra were recorded following the mixing of equimolar amounts of SA (5.35×10^{-4} M) and sodium nitrite in 60% acetonitrile and 10% acetic acid as a function of time. The spectra were characterized by time dependent increase of 490 and 520 nm absorbance peak and a concomitant ~40 nm red shift and decrease of the SA absorbance peak at 324 nm (Fig. 2). The formation

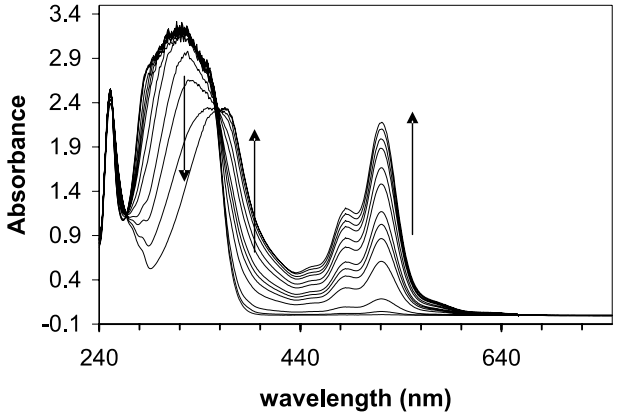

Fig. 2. Time course of *O*-nitrosoSA formation in the presence of acetic acid. Reaction of 5.35×10^{-4} M SA with equimolar $NaNO_2$ in 60% acetonitrile and 10% acetic acid. Spectra were taken at 7, 60, 240, 900, 1500, 2400, 3000, 4000, 4600, 5400, and 6600 s.

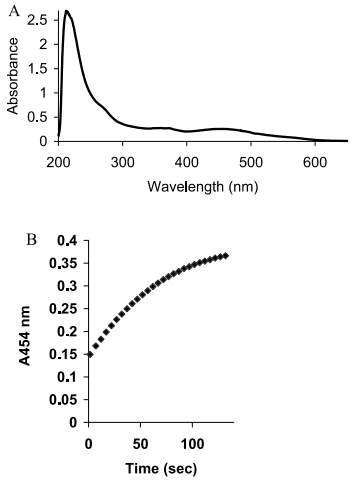

Fig. 3. (A) The spectrum of the red product after 300 s (at completion) of reaction between 2.67×10^{-4} M SA in 90% acetonitrile 0.1 M HCl and equimolar sodium nitrite. (B) Time dependent increase of *O*-nitrosoSA formation from 2.67×10^{-4} M SA in 90% acetonitrile 0.1 M HCl upon addition of equimolar sodium nitrite.

of the 520 nm peak was well fitted to a first-order process. The rate constant of the reaction was determined to be $0.0004\,s^{-1}$. The extinction coefficient of the 520 nm peak was estimated to be $8419\,M^{-1}\,cm^{-1}$ (in 90% ACN and 10% acetic acid).

In order to further examine the reactivity of sodium nitrite towards SA, the reaction was carried out in 0.1 M HCl instead of 10% acetic acid. Reaction of SA under these conditions with equimolar nitrite led to the formation of a red product as evidenced by the growth of a peak at 454 nm along with a decrease of SA peak at 323 nm as a function of time (Fig. 3). The rate constant of this process was determined to be $8.32\,M^{-1}\,s^{-1}$.

There is a possibility that the minor shift observed for the absorption maximum from 532 nm (0.1 M Na_2HPO_4, pH 7.4) to 520 nm (10% acetic acid and 60% acetonitrile) to 454 nm (0.1 M HCl) could be due to the differences in the solvent system. To check for this possibility, an aliquot (50 μl) of the product formed through the reaction of SA with acidified nitrite (10% acetic acid or 0.1 M HCl) was immediately transferred into two different cuvettes containing 0.1 M Na_2HPO_4 buffer, pH 7.4. As expected, both products exhibited peaks at ~532 nm.

Characterization of the red compound

There have been several suggestions in the literature that the reaction of aryloxide ion with alkyl nitrites always occurs through the oxygen atom to yield an unstable *O*-nitroso compound [23,24]. This intermediate is likely to undergo an internal rearrangement of the NO group to give corresponding C-nitrosoproduct, competing with homolysis of the O–NO bond to yield nitric oxide. Phenols are ambient neucleophiles and their reaction with the nitroso group can also take place at the oxygen atom. Challis and Higgins [23] have suggested that nitrosation of the 2-naptholate ion at pH < 5 occurs by reversible formation of an unstable aryl nitrite. This same species has been proposed as an intermediate in the nitrous acid catalyzed nitration of phenols [24].

In the case of SA the nitrosation product is stable. In order to explore the structural features of SA that yield this stability, the reaction of acidified nitrite was studied with several structurally similar compounds. 4-Hydroxycinnamic acid, or 3,4,5-trimethoxycinnamic acid, or 3,4,5-trimethoxyhydrocinnamic acid, or dihydroSA solutions were reacted with eqimolar nitrite in 60% acetonitrile/10% acetic acid (see Fig. 4 for structures). Among the SA analogues used, only dihydroSA gave rise to a red product with a λ_{max}, 520 nm. However the estimated extinction coefficient for this product was ~10-fold less than that obtained with SA.

Evidence for involvement of NO^+ in the O-nitrososinapinic acid formation

In order to determine the reacting species derived from peroxynitrite or acidified nitrite, SA was mixed with NO (aq) or NO_2^- solutions in PBS (200 mM, pH 7.4) or an aqueous solution of $NOBF_4$ (a NO^+ donor). The spectrum with identical absorption properties (λ_{max}, 532 nm) as the red product formed with $ONOO^-$, was obtained only in the presence of $NOBF_4$ (data not shown). The reaction rate of $NOBF_4$ with SA was also comparable to the reaction rate of peroxynitrite with SA. These results suggest the involvement of NO^+ in the reaction with SA.

SA protects DHR from SIN-1 mediated oxidation

Dihydrorhodamine has been frequently used for assaying the formation of cell and tissue derived peroxynitrite. The two-electron oxidation of dihydrorhodamine to rhodamine (RH; $\lambda_{ex} = 500$ nm, $\lambda_{em} = 536$ nm) results in the formation of highly fluorescent products [10]. This method allows for the detection of submicromolar levels (e.g., 50 nM) of peroxynitrite. A favored method to generate peroxynitrite in situ is to use SIN-1 [11].

In the present study, SIN-1 (20 μM) was mixed with DHR (5 μM). The fluorescence at 554 nm increased with time and reached a plateau in 100 min. The addition of SA (0.01–7 μM) was able to quench DHR oxidation by SIN-1 (Fig. 5).

O-NitrosoSA formation upon incubation with GSNO

GSNO is well known to be able to S-nitrosate thiols by NO^+ transfer in biological systems [25]. Since our

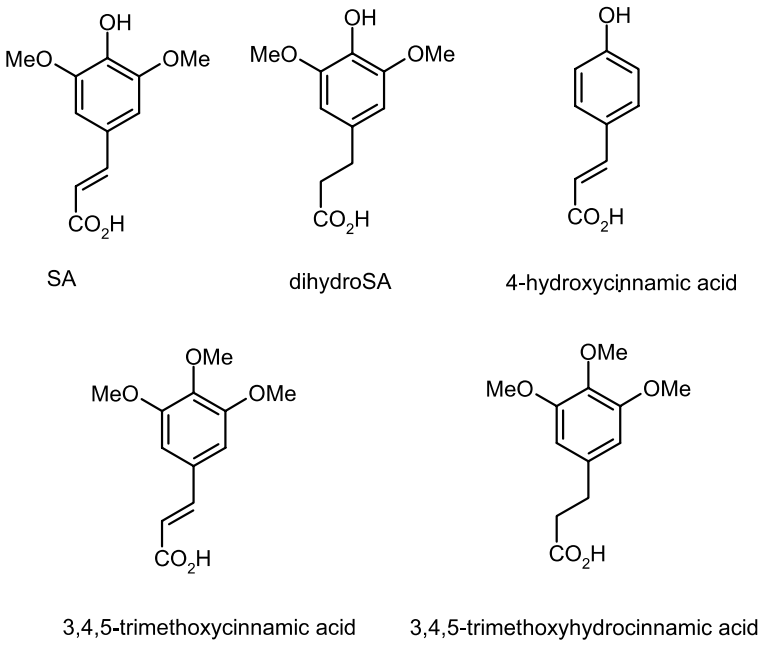

Fig. 4. Structures of SA and related compounds.

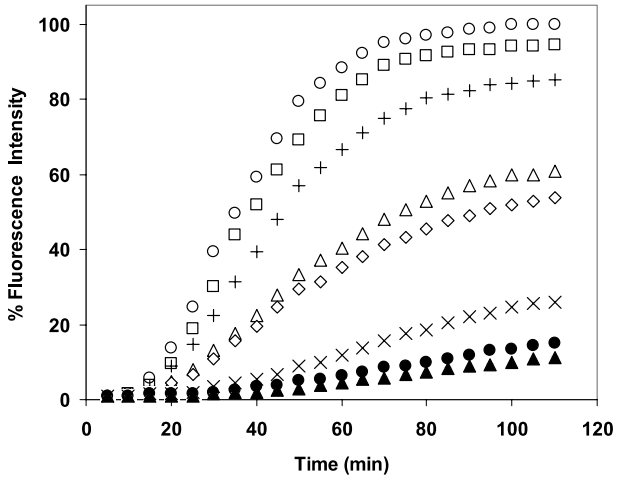

Fig. 5. Inhibition of SIN-1-mediated dihydrorhodamine oxidation by SA. SIN-1 (20 μM) was added to 5 μM dihydrorhodamine (DHR) in the presence of increasing SA concentration at 37 °C and pH 7.4: no SA (○); 0.05 μM SA (□); 0.1 μM SA (+); 0.3 μM SA (△); 0.7 μM SA (◇); 1.0 μM SA (×); 3.0 μM SA (●); 7.0 μM SA (▲). Data were normalized by setting the value of fluorescence intensity of rhodamine-6G to 100%, and that of DHR-6G at time zero to 0%. Excitation and emission wavelengths were set at 528 and 554 nm, respectively. Values are means ± SD, $n = 3$.

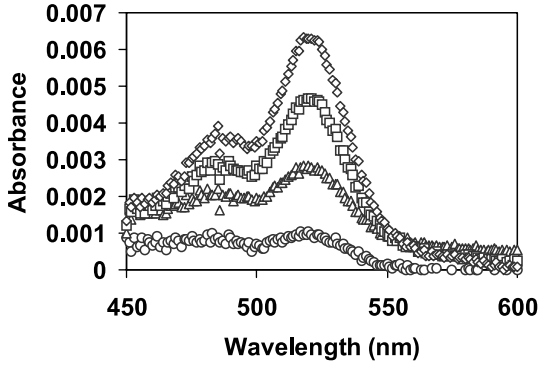

Fig. 6. *O*-NitrosoSA formation in the presence of: (○) 0.3 μM; (△) 0.6 μM; (◇) 1 μM; and (□) 1.5 μM GSNO in 60% acetonitrile and 10% acetic acid after 15 min incubation at 25 °C.

previous experiments suggested the involvement of NO^+ in the reaction with SA, we next examined whether GSNO could promote *O*-nitrosoSA formation by NO^+ transfer. These reactions were performed in 60% ACN with 10% acetic acid. The *O*-nitrosoSA formation was monitored spectrally at 520 nm as a function of [GSNO]. As can be seen from Fig. 6, the yield of *O*-nitrososinapinic acid was also increased as a function of [GSNO].

O-NitrosoSA as a potential S-nitrosating agent

Since *O*-nitrosoSA is a direct analog of *S*-nitroso compounds, it should be able to S-nitrosate thiols or amines. To test for this possibility, neutralized *O*-nitrosoSA was incubated with L-Trp, L-Tyr, D,L-Hcys, GSH, and bovine serum albumin. As can be seen from Table 1, only thiol containing amino acids and BSA resulted in rapid decrease in the 520 nm peak with comparable rate constants.

Evidence for S-nitrosation of BSA was obtained by the characteristic S–NO absorption peak (343 nm) of BSA subsequent to its reaction with *O*-nitrosoSA (Fig. 7).

Table 1
Rate constants for transfer of NO^+ from *O*-nitrosoSA

	Rate constant ($M^{-1} s^{-1}$)
GSH	23.6 ± 1.6
Homocysteine	15.2 ± 1.2
Tryptophan	0
Tyrosine	0

Decomposition of 0.25 mM ONSA solution was monitored at 510 nm as a function of time in the presence of 10-fold molar excess of GSH/homocysteine/Trp/tyrosine in phosphate-buffered saline. Data are means ± SD, $n = 3$.

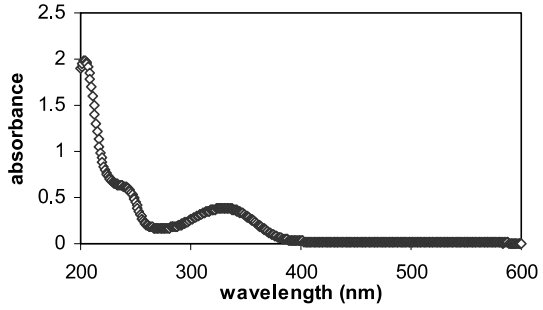

Fig. 7. UV–Vis spectra of BSA (375 μM) upon incubation with equimolar *O*-nitrosoSA in PBS at pH 7. Spectrum of the product was taken using same concentration of BSA as blank.

Discussion

Peroxynitrite has been demonstrated to oxidize a wide variety of biomolecules in vitro either by direct reactions or by secondary radicals ($CO_3^{\cdot-}$, $^{\cdot}NO_2$, and $^{\cdot}OH$) previously. In the present study, peroxynitrite was observed to react with SA leading to the formation of red colored compound (*O*-nitrosoSA) ($\lambda_{max} = 532$ nm) with an estimated second-order rate constant of $812\ M^{-1} s^{-1}$. We propose the mechanism in Fig. 8A for the reaction of peroxynitrous acid with SA. In this mechanism, ONOOH directly reacts with SA to donate NO^+. The product of this reaction is *O*-nitrosoSA and H_2O_2. van der Vliet et al. [26] has proposed a similar mechanism for the S-nitrosation of thiols by peroxynitrite.

SA was also shown to react with decomposed peroxynitrite solutions, only if they were acidified, again producing a red compound ($\lambda_{max} = 510$ nm) but with ~300-fold lower rates. These observations suggested that some specie derived from peroxynitrite decomposition could also react with SA in acidic conditions. Nitrite was likely the decomposition product in question since the reaction of equimolar SA (5.35×10^{-4} M) in 60% acetonitrile and 10% acetic acid with sodium nitrite also gave rise to a red product ($\lambda_{max} = 520$ nm) (Fig. 2). The growth of 520 nm peak appeared to be a first-order process with a rate constant of $0.0004\ s^{-1}$. This red product had an extinction coefficient (in 90% ACN and 10% acetic acid) that was ~495-fold larger than the 543 nm extinction coefficient reported for small molecular weight *S*-nitrosothiols such as *S*-nitrosoglutathione. When the reaction was carried out between equimolar sodium nitrite and SA, in 0.1 M HCl instead of 10% acetic acid, a red product was also formed (Fig. 3). However, the rate constant estimated in HCl was found to be 20,800-fold larger in comparison to that observed in the presence of 10% acetic acid. In addition the absorption maximum was shifted to 454 nm. This observed shift was due to solvent effects since the spectrum shifted to 533 nm when transferred to the 0.1 M phosphate buffer, pH 7.4.

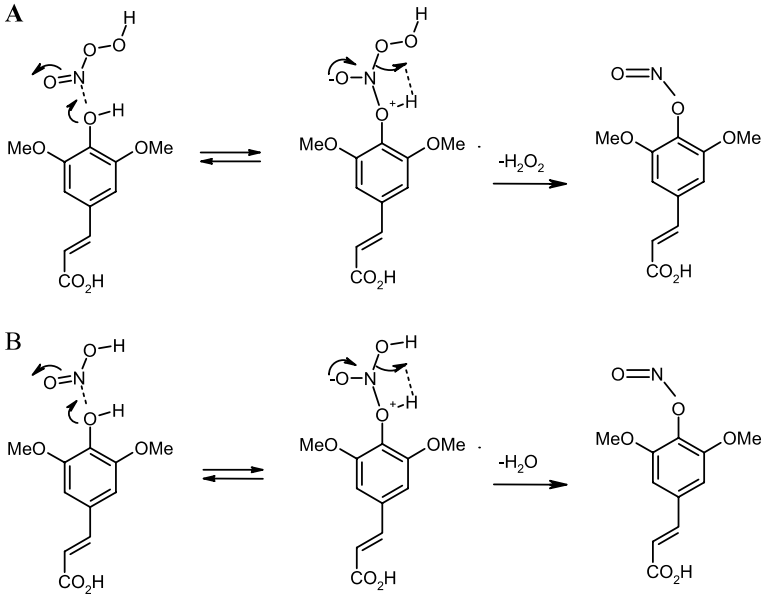

Fig. 8. Proposed mechanisms for the reaction of SA with: (A) peroxynitrite and (B) nitrite.

It is well established that in the presence of mineral acids sodium nitrite is converted to nitrous acid. In aqueous phase, direct protonation of nitrous acid occurs to form nitrosonim ion. SA can react with the newly formed NO^+ from nitrous acid leading to the formation of the red compound.

The apparent difference in reaction rate of SA toward sodium nitrite observed in acetic acid vs. HCl can therefore be attributed to the nature of the acid used. Acetic acid is a weak acid ($pK_a = 4.75$). As a result very low levels of HNO_2 will be formed ultimately leading to the slower production of NO^+ from HNO_2. Since NO^+ has a very short half-life ($t_{1/2}$ 3×10^{-10} s) in water, slow formation of NO^+ in the presence of acetic acid increases the percentage of NO^+ reacting with SA instead of being lost through the reaction with water. Thereby, slow formation of NO^+ promotes more efficient reaction between SA and nascent NO^+. Therefore, the extinction coefficient of the red product was determined to be higher in acetic acid even though the reaction rate was lower. The rate of the red product formation was much faster in the presence of HCl than in the presence of acetic acid. However, the yield of the red product was much lower. We suggest the following explanations for this observation. HCl dissociates readily to produce H^+. As a result, the formation of NO^+ from HNO_2 is fast. Although high concentrations of NO^+ are produced, only a low percentage of the newly formed NO^+will react with SA owing to its high reactivity with water. Therefore, the yield of the red product was lower even though the reaction rate was much faster in the presence of HCl.

Involvement of an unstable intermediate, *O*-nitroso compound in the reaction of aryloxide ion with alkyl nitrites has been reported in the literature [23,24]. This intermediate was suggested to undergo an internal rearrangement of the NO group leading to the formation of corresponding C-nitrosoproduct. Phenols were also demonstrated to react through their oxygen atom by following a similar mechanism. Nitrosation of the 2-naptholate ion at $pH < 5$ had also been suggested to occur via reversible formation of an unstable aryl nitrite. In addition, nitrous acid catalyzed nitration of phenols had also been proposed to undergo through the intermediate formation of arylnitrite [24].

We suggest that NO^+ derived from peroxynitrite or acidified nitrite reacts with the –OH group of SA forming an aryl nitrite, 3,5-dimethoxy-4-nitrosooxycinnamic acid. Since both ortho- and para-positions are blocked in the case of SA, rearrangement of the NO group of arylnitrite is probably not feasible as in any other phenols. We demonstrated this by reacting structurally related compounds (Fig. 4) with nitrite under acidic conditions. The fact that only dihydroSA gave rise to an identical peak characteristic of the red compound, suggests that both a phenolic-OH and blocked ortho- and para-positions are essential for stable O-nitrosation. These findings also correlate well with the suggested formation of *O*-nitrosoSA from the reaction between SA and peroxynitrite or acidified nitrite. Even though dihydroSA gave a red product, the estimated extinction coefficient of this product was ~10-fold lower in comparison to that formed with SA. This difference in reactivity between SA and dihydroSA towards sodium nitrite could be attributed to the difference in conjugation system in these two compounds. DihydroSA has less extended conjugated system compared to SA due to the reduction of the side chain double bond. Extinction coefficient tends to increase as the extension of conjugation system increases.

Based on these observations we propose the mechanism in Fig. 8B for the reaction of nitrous acid with SA. Support for this mechanism comes for our results with the NO^+ donors $NOBF_4$ or GSNO which resulted in the formation of *O*-nitrosoSA. Neither NO (aq) nor NO_2^- (200 mM phosphate buffer, pH 7.4) resulted in the formation of the red product. These results provide yet other lines of evidence for the ability of SA to act as an NO^+ sink.

Dihydrorhodamine has been frequently used for assaying the formation of cell and tissue derived peroxynitrite. The two-electron oxidation of dihydrorhodamine to rhodamine (RH; $\lambda_{ex} = 500$ nm, $\lambda_{em} = 536$ nm) results in the formation of highly fluorescent products [10]. This method allows for the detection of submicromolar levels (e.g., 50 nM) of peroxynitrite. A favored method to generate peroxynitrite in situ is to use SIN-1 [11].

In the present study, SA (0.01–7 μM) successfully prevented the oxidation of DHR (5 μM) by SIN-1 (20 μM) as monitored by the quenching of hydrorhodamine fluorescence (Fig. 5). This observation indicates the potential use of SA as an inhibitor of peroxynitrite mediated damage.

Upon incubation of neutralized *O*-nitrosoSA with L-Trp, L-Tyr, DL-Hcys, GSH, and bovine serum albumin only thiol containing amino acids and BSA resulted in rapid decrease in the 510 nm peak (Table 1) with comparable rate constants. S-nitrosation of BSA was also demonstrated by the characteristic S–NO absorption peak (343 nm) on BSA subsequent to its reaction with *O*-nitrosoSA (Fig. 6). The fact that *O*-nitrososinapinic acid can promote S-nitrosation of thiols demonstrates that it holds the potential of being used as a S-nitrosating agent in vitro.

In conclusion, we have shown in this study that SA could be utilized as an analyte for the spectroscopic detection of peroxynitrite and NO^+ donors in submicromolar range in physiological conditions or in vitro. In addition, SA was very effective in preventing the oxidation of diydrorhodamine. The effectiveness of SA to serve as a peroxynitrite-sink in physiological conditions is currently being investigated. Furthermore, the

demonstrated ability of *O*-nitrosoSA to serve as a S-nitrosating agent for thiol containing amino acids, peptides, and proteins, makes it potentially very useful in the study of *S*-nitrosothiol biochemistry and physiology.

Apart from highlighting SA as an analyte the present study has revealed that peroxynitrite in addition to its previously demonstrated [25] ability to S-nitrosate thiols, can also O-nitrosate aromatic alcohols. We were also able to demonstrate that *O*-nitrosoSA could efficiently transfer its NO^+ to thiols but not to phenolic oxygen of Tyr. *S*-Nitrosothiols on the other hand were able O-nitrosate SA. This raises the possibility that in the biological milieu, thiols can transfer peroxynitrite-induced nitrosative damage far from the site of its generation.

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