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Evaluation of transnitrosating ability of *N*-nitrosoguanidines to alkyl thiols and thiol amino acids

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ABSTRACT

The transfer of the nitroso group from 1-nitroso-1-methyl-3-tolylsulfonylguanidine (NOTSG) and 1-nitroso-1-methyl-3-benzoylguanidine (NOBMG) to some thiols, including the amino acid cysteine, was studied in a pH range between 7 and 12.

The measured apparent bimolecular rate constant of transnitrosation (k_{tr}^{app}) revealed a bell-shaped pH dependence that clearly indicates that both nitrosoguanidines react through the corresponding neutral form, and the nucleophiles in the thiolate anion form to give the corresponding *S*-nitrosothiol. Regarding cysteine, the existence of three macroscopic acidity constants influenced the kinetic behavior of the transnitrosation reaction. Transnitrosation rates (k_{tr}) of the two possible nucleophilic species were obtained and it was found that NOBMG has lower thiol transnitrosation capacity due to the lower electron-withdrawing effect of benzoyl group and to the possible stabilization of the anionic structure as a consequence of the establishment of the intramolecular hydrogen bond. The k_{tr} values of the studied nucleophiles were calculated and a Brønsted-type plot was established giving unexpected negatives β_{nuc}(β_{nuc}(NOBMG)=−0,17 and β_{nuc}(NOTSG)=−0,11). The atypical β_{nuc} values were attributed to the need for previous desolvation of the nucleophile.

1. Introduction

The discovery of physiological properties of nitric oxide (NO), particularly those of vasodilation and inhibition of platelet aggregation,[1–3] is actually responsible for the major interest in the chemistry of nitrosothiols (RSNOs).

Several RSNOs like *S*-nitrosocysteine, *S*-nitrosoglutathione, *S*-nitrosoalbumins and *S*-nitrosohemoglobin, have been detected in vivo[4] and are believed to be responsible for the NO transport and release around the body.[5–7]

RSNOs are very readily generated in solution by nitrosation of the corresponding thiols using conventional nitrosation methods[8] or transferring the nitroso group from a nitrosocompound to thiolate ions (transnitrosation).[9–11]

From all RSNOs with biological relevance, *S*-nitrosocysteine is probably one of the most important and therefore this *S*-nitrosothiol has been the aim of several mechanistic studies concerning its decomposition with simultaneous NO generation.[4,8,11–13]

However, there is little information about *S*-nitrosation using transnitrosating ability of nitrosocompounds in the presence of sulfur nucleophiles such as cysteine (Cys). Only two mechanistic studies about MNTS reactivity in the presence of cysteine, at several pH values, were found in the literature that showed the *S*-nitrosocysteine obtention by a transnitrosation process.[9,14]

It is known that *N*-nitrosocompounds, like MNTS (Fig. 1), have a high cytotoxic activity due the possible formation of electrophilic species by a deamination process[15] and the possible transfer of the nitroso group to amines increases the risk of carcinogenicity of these compounds,[16] however, in the presence of sulfur nucleophiles

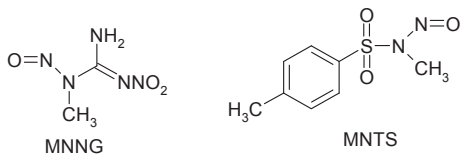

Fig. 1. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and *N*-methyl-*N*-nitroso-*p*-toluenosulfonamide (MNTS) structures.

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usually more reactive than amines, these compounds can assume a new and interesting role as NO donors.

Due to this duality, our group has been interested in the chemistry and reactivity of some *N*-nitrosocompounds, particularly nitrososulfonamides, nitrosothioureas and nitrosoguanidines. After confirming the ability of these compounds to transnitrosate to amines[15,17–19] and based on some studies realized with MNTS[9,14] and MNNG[20] (Fig. 1) involving nitric oxide and *S*-nitrosocysteine formation, it was our goal to study the nitroso group transfer from two *N*-nitrosoguanidines containing electro-withdraw groups (NOTSG and NOBMG) to thiolate ions including cysteine.

To accomplish our purpose, the transnitrosation ability of NOBMG and NOTSG to sulfur nucleophiles was kinetically evaluated, first with thioles containing only the thiol function with ionization capacity, and then with cysteine an amino acid that in addition to the thiol function has two other ionizable functions: α-carboxilate and α-amino.

The thioles used (Table 1) were chosen based on their basicity. The complete kinetic study was accomplished using the nucleophile 2-mercaptoethanol (ME).

Table 1
pK_a values of studied sulfur nucleophiles (RSH)

RSH	Trivial name	pK_a^{RSH}
$CH_3O_2CCH_2SH$	Methylhtioglycolate (MTG)	7,81[21]
$CH_3O_2CCH_2CH_2SH$	Methyl 3-mercaptopropionate (MMP)	9,33[22]
$OHCH_2CH_2SH$	2-mercaptoethanol (ME)	9,60[22,23]
CH_3CH_2SH	Ethanothiol (EtSH)	10,6[21]

2. Results and discussion

2.1. Preliminary analysis of the transnitrosation products

The transnitrosating ability of *N*-nitrosoguanidines (Fig. 2) to some thioles at different pH values was previously studied under similar conditions of those described for the kinetics studies developed in this work.

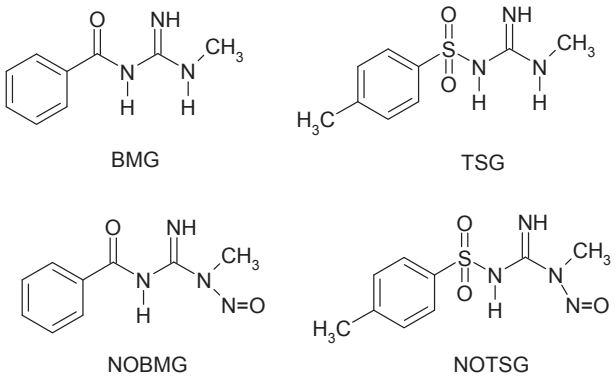


Fig. 2. Free guanidines structures: 1-methyl-3-benzoylguanidine (BMG) and 1-methyl-3-tolylsulfonylguanidine (TSG) and their correspondent *N*-nitrosated derivatives: 1-nitroso-1-methyl-3-benzoylguanidine (NOBMG) and 1-nitroso-1-methyl-3-tolylsulfonylguanidine NOTSG).

The obtained reaction mixtures were treated as described in the experimental section and analyzed by TLC that showed the denitrosated guanidines BMG and TSG (Fig. 2), respectively, as the principal final products of these reactions.

In the reactions performed at pH≥11 it was also detected the presence of benzoic acid, or *p*-toluenesulfonic acid if the nitrosating agent was NOBMG or NOTSG.

The presence of free guanidines BMG and TSG is consistent with the possible direct transfer of nitroso group to the thioles, and the acid benzoic (or *p*-toluenesulfonic acid) anions formation at pH≥11 is in agreement with the competition from basic hydrolysis of nitrosoguanidines.[24]

2.2. Transnitrosation to simple thioles (RSH type)

The influence of ME concentration on the observed rate constants, k_{obs}, of its reaction with the *N*-nitrosoguanidines was studied at different pH values. Figs. 3 and 4 illustrate the good linear

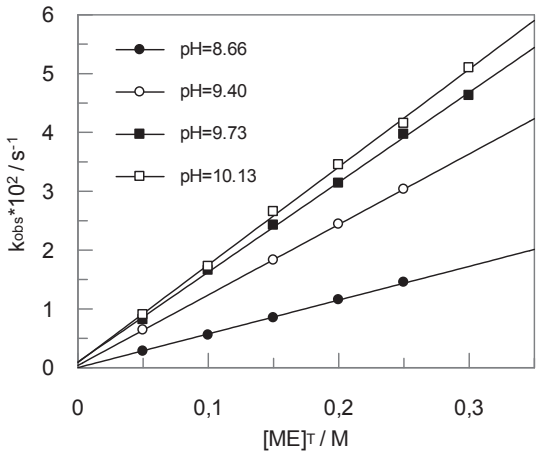


Fig. 3. Influence of 2-mercaptoethanol total concentration on k_{obs} in transnitrosation reaction with NOBMG at 25 °C.

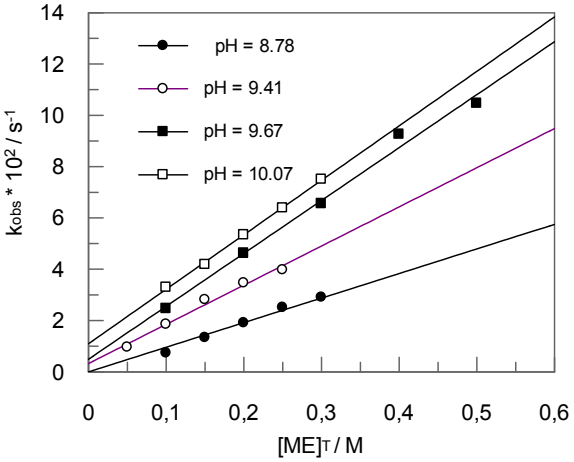


Fig. 4. Influence of 2-mercaptoethanol total concentration on k_{obs} in transnitrosation reaction with NOTSG at 25 °C.

relationship with a zero intercept between k_{obs} and the total thiol concentration, $[ME]_T$, indicating the absence of NOBMG (or NOTSG) basic hydrolysis competition.[9]

It is also evident, for both *N*-nitrosoguanidines, the slope increases with the increasing pH, which is consistent with the Eq. 1, where k_{tr}^{app} is an apparent bimolecular rate constant since it includes the different acid-base equilibrium constants of the nucleophile and, $[RSH]_T$ represents in this case the total concentration of ME.

$$k_{obs} = k_{tr}^{app}[RSH]_T \quad (1)$$

Only when pH was higher than 10, interceptions between k_{obs} and $[ME]_T$ were different than zero, indicating that basic hydrolysis of *N*-nitrosoguanidines takes place competing with the transnitrosation process, k_{obs} should be expressed as:

$$k_{obs} = k_{OH^-}\left[OH^-\right] + k_{tr}^{app}[RSH]_T \quad (2)$$

where $k_{\bar{OH}}$ is the bimolecular rate constant for the *N*-nitrosoguanidines hydrolysis catalyzed by the hydroxide ion existent in solution. However, basic hydrolysis studies of these *N*-nitrosoguanidines[24] showed, for similar pH ranges, k_{OH^-} values between 10^{-5}and10^{-4} $M^{-1}s^{-1}$, which are not considered significant when compared with the obtained k_{tr}^{app} rate constants. For this reason, in order to express k_{obs} we considered only Eq. 1.

The influence of pH on k_{tr}^{app} rate constants (Fig. 5) reveal a bell-shape curve with a progressive increase of k_{tr}^{app} values until pH is

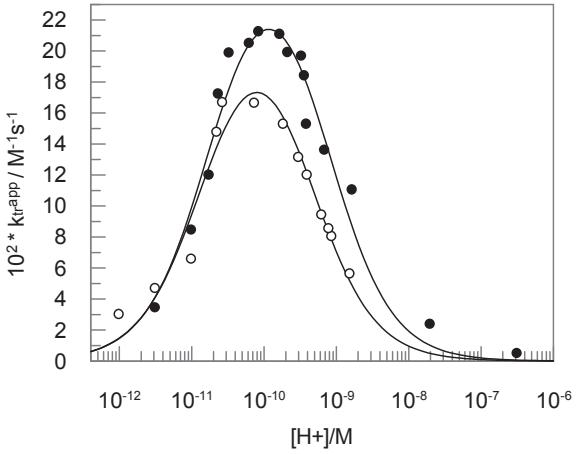


Fig. 5. Influence of pH on k_{tr}^{app} rate constants for the transfer of the nitroso group from *N*-Nitrosoguanidines to 2-mercaptoethanol at 25 °C.

near 10. After this point, apparent bimolecular rate constants decrease with the continuous increase of alkalinity.

Considering the acid-base equilibrium of ME (see pK_a^{RSH} on Table 1) the concentration of thiolate in solution is increasing with the increasing pH, reaching a maximum value when pH ≈ 10.6. This fact justifies the k_{tr}^{app} increase for both reactions until pH is near 10 however, if the thiolate concentration was the unique determining rate factor, k_{tr}^{app} values should stabilize when pH>10 but instead we observed decreasing values leading us to consider the following acid-base equilibrium (Scheme 1) for both *N*-nitrosoguanidines and the corresponding pK_a values:[24] pK_a(NOTSG)=10.6±0.1 and pK_a(NOBMG)=11.5±0.1.

For NOTSG, when pH is about 10, the anionic form is predominant in solution, however for NOBMG, at the same pH values, concentration of the neutral and the anionic forms are similar. When pH increases until ≈12.5 the equilibrium shifts towards the anionic form formation, which becomes predominant after that point. Thus, it seems that the appearance in solution of the anionic forms of the *N*-nitrosoguanidines explains the decrease of k_{tr}^{app}.

Experimental results can be quantitatively explained by a reaction mechanism involving nitroso group transfer from the neutral forms of *N*-nitrosoguanidines to the thiolate ion of ME (Scheme 2).

From Scheme 2, the following expressions (Eqs. 3 and 4) for the apparent bimolecular rate constant k_{tr}^{app} were obtained:

$$k_{tr}^{app} = \frac{k_{obs}}{[ME]_T} = \left(\frac{k \times [H^+] \times K_a^{RSH}}{\left(K_a^{XNO} \times K_a^{RSH}\right) + \left(K_a^{XNO} + K_a^{RSH}\right) \times [H^+] + [H^+]^2}\right) \quad (3)$$

$$\frac{[H^+]}{k_{tr}^{app}} = \frac{K_a^{XNO}}{k} + \frac{K_a^{XNO} + K_a^{RSH}}{k \times K_a^{RSH}} \times [H^+] + \frac{1}{k \times K_a^{RSH}} \times [H^+]^2 \quad (4)$$

where the equilibrium constants (K_a^{RSH} and K_a^{XNO}) refer to the thiol and *N*-nitrosoguanidines acid-base dissociations previously described (Table 1 and Scheme 1) and k is the rate constant corresponding to the transnitrosation reaction shown in Scheme 2.

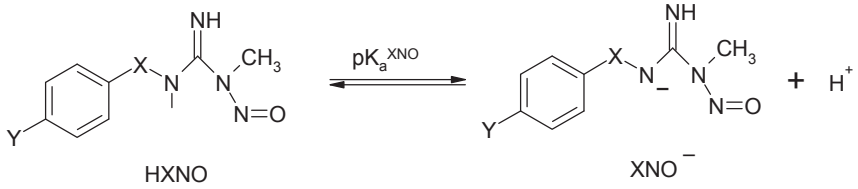
The experimental results (Fig. 5) fitted very well to Eq. 3 as shown by the curve plotted and the second-degree polynomial equation expressed by Eq. 4 is also in good agreement with the observed kinetic behavior as we can see in Fig. 6.

Transnitrosation velocity constants (k) and macroscopic equilibrium acidity constants (pK_a(NOTSG); pk_a(NOBMG) and pK_a(RSH)) were obtained from the fit of the kinetic results to Eqs. 3 and 4 (Table 2).

All estimated pK_a values for both *N*-Nitrosoguanidines compared well with those reported in the literature[24] with the exception of the NOBMG pK_a value obtained by fitting the results to Eq. 3.

In fact, considering the value near 11.5, the lack of experimental results close the ionization pH range may be the reason for this discrepant value. The fitted values show less acidity behavior for NOBMG, which is consistent with fact that amides are indeed more basic than sulfonamides due to the smaller electron-withdrawing effect of the benzoyl group.

For ME pK_a values are in agreement with the literature value (Table 1) except the estimated value of 8.3 obtained from the kinetic results fitted to Eq. 4 that describes a second-degree polynomial behavior ($y=a+bx+cx^2$). The fitting process with this

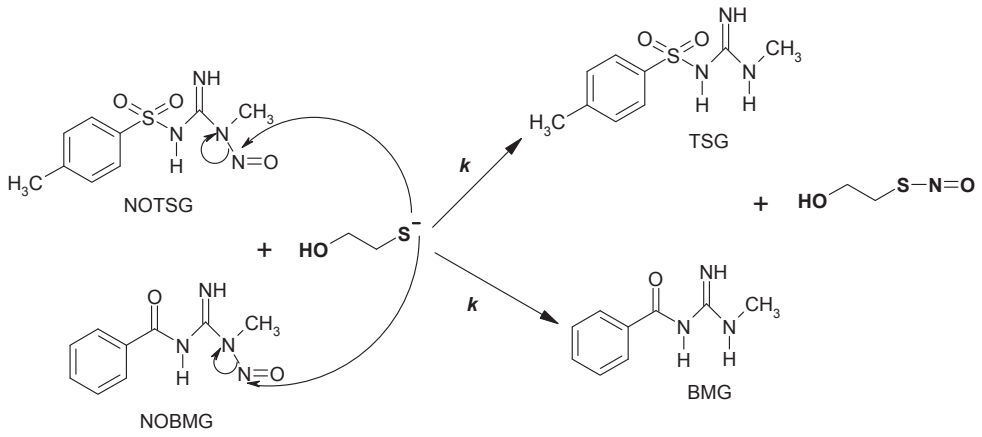


HXNO XNO⁻

NOBMG: X=CO; Y=H

NOTSG: X=SO_2;Y=CH_3

Scheme 1. Acid-base equilibrium of *N*-nitrosoguanidines.



Scheme 2. Reaction mechanism involving nitroso group transfer from the neutral forms of *N*-nitrosoguanidines to the thiolate ion of cysteine.

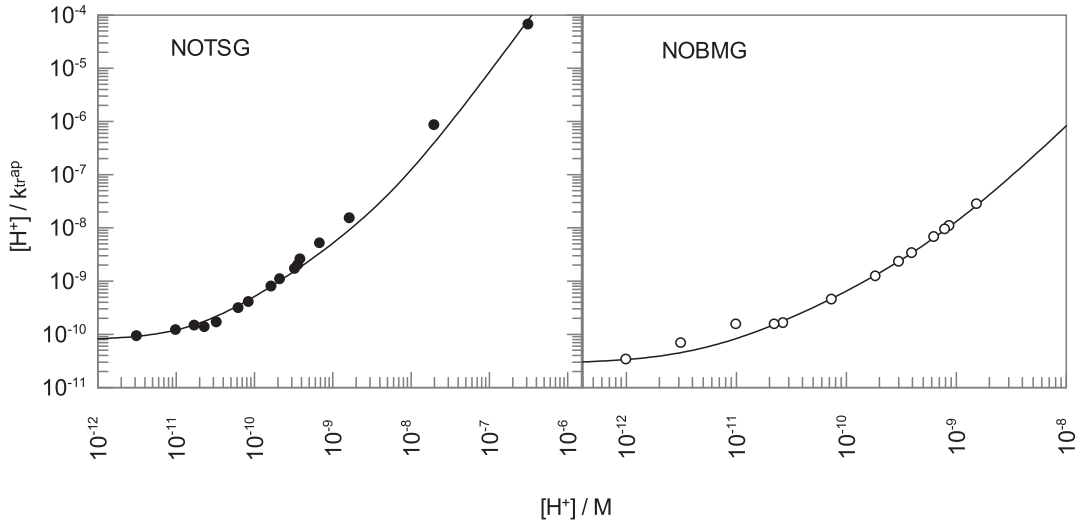


Fig. 6. Influence of pH on the quotient $[H^+]/k_{tr}^{app}$.

Table 2
Estimated transnitrosation velocity constants (k) and equilibrium constants for the studied thiol and *N*-nitrosoguanidines

XNO	Fit to Eq. 3			Fit to Eq. 4		
	pK_a	K/M^{-1}s^{-1}	pK_a^{RSH}	pK_a	K/M^{-1}s^{-1}	pK_a^{RSH}
NOTSG	10.73	2.88×10^{-1}	9.14	10.73	2.33×10^{-1}	8.30
NOBMG	10.81	2.48×10^{-1}	9.39	11.29	1.84×10^{-1}	9.16

equation was only possible considering constant the parameter a previously obtained by a linear fit of the results with a linear behavior. This assumption and the mathematic calculations needed to get the final values may be the cause of the observed deviation.

Analysing the transnitrosation rate constants we can see that both fitting results gave values in good agreement and comparing transnitrosating velocities of NOTSG with NOBMG we can see that there is a very little difference between obtained values: $k_{NOTSG}/k_{NOBMG} \approx 1.2$.

Although there is a slight difference of reactivity between NOTSG and NOBMG, the first one shows more reactivity in the transnitrosation process. This result is compatible with the greater electron-withdrawing capacity given by the sulfonyl group that generates a positive charge towards the guanidine group leading to a fast nucleophilic attack of the thiolate ion.

The lower reactivity of NOBMG can also be explained by the possible stabilization of the anion resonant structure 2 (Scheme 3), due to the establishment of the intramolecular hydrogen bond[24] (Fig. 7, structure I).

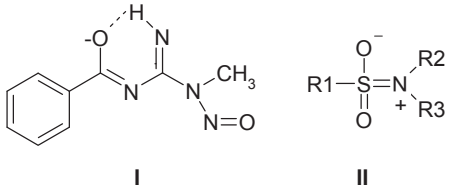
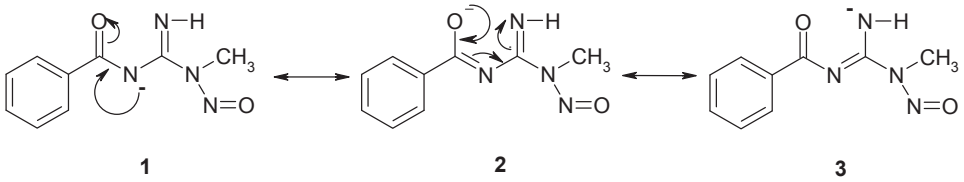


Fig. 7. NOBMG anion stabilization by the establishment of the intramolecular hydrogen bond (I); possible resonant structure for sulfonamides (II).

For NOTSG, the same stabilization is highly improbable because various studies carried out with sulfonamides indicate that structure II (Fig. 7) has no significant contribution in its stabilization.

2.3. Transnitrosation to cysteine

Since an identical transnitrosation kinetic profile was found for both *N*-nitrosoguanidines with only a slight difference in terms of



Scheme 3. Stabilization of the anion resonant structure of NOBMG.

reactivity, the kinetic study of transnitrosation reactions to the biological aminoacid cysteine (Cys) was performed using only NOTSG as NO donor.

As observed with ME, the influence of total Cys concentration, $[Cys]_T$, on the observed rate constants, k_{obs}, in its reaction with the NOTSG at different pH values (8.48<pH<11.11) show good linear relationships and visible slope increase with the increasing pH (Fig. 8). Only when pH is higher than 10.5, interceptions between k_{obs} and $[Cys]_T$ are different than zero indicating the basic hydrolysis of *N*-nitrosoguanidines competition but, as in the previous study, $k_{\bar{O}H}$ constants for similar pH values can be disregarded when compared with the obtained k_{tr}^{app} rate constants for the transnitrosation process leading us to consider Eq. 1 (in this case $[RSH]_T$ refers to $[Cys]_T$) in all studied pH range.

Fig. 9 a) illustrates the obtained pH-rate profile and as it is evident the pH influence on the apparent bimolecular rate constants (k_{tr}^{app}) also follows a bell-shape behavior but now with a less pronounced increase of k_{tr}^{app} with the increasing basicity. The wider bell-shape form probably may indicate the presence of more chemical species in the transnitrosation reaction.

As previously mentioned for NOTSG, due to the existence of an acidic proton when pH is between ≈9.6 and 11.6, two chemical species coexist in solution (see Scheme 1) but when pH>11.6 the anionic one becomes predominant.

With respect to cysteine, according to Scheme 4 and in the pH range studied, the presence of three possible chemical species in solution can be considered: (H_2Cys), $(HCys)^-$ and (Cys^{2-}).

Analyzing experimental results, k_{tr}^{app} increases with the increasing pH reaching a maximum value when pH is near 10.4. In what concerns cysteine, this basicity increase in solution means the gradual appearance of the anionic form $(HCys)^-$ that becomes predominant when pH≈9.3. If $(HCys)^-$ was the unique nucleophilic form of cysteine, and both neutral and anionic forms of NOTSG were equally reactive, from this point rate constants would be expected to become pH independent but instead we observe decreasing values with the increasing appearance of $(Cys)^{2-}$ and NOTSG anionic form.

The proposed mechanism (Scheme 5) involving nitroso transfer from neutral form of *N*-nitrosoguanidine to the anionic species of cysteine $(HCys)^-$ and $(Cys)^{2-}$ lead to the following expression for k_{tr}^{app} (Eq. 5):

$$k_{tr}^{app} = \frac{k_1 \times K_{a2} \times \left[H^+\right] + \times k_2 \times K_{a3} \times K_{a2}}{a \times \left[H^+\right]^3 + b \times \left[H^+\right]^2 + c \times \left[H^+\right] + \left(K_{a3} \times K_{a2} + K_{a2} \times K_a^{XNO}\right) + \frac{K_{a3} \times K_{a2} \times K_a^{XNO}}{\left[H^+\right]}} \quad (5)$$

$$a = K_{a1}$$

$$b = K_{a1} \times K_a^{XNO} + 1$$

$$c = K_{a2} + K_a^{XNO}$$

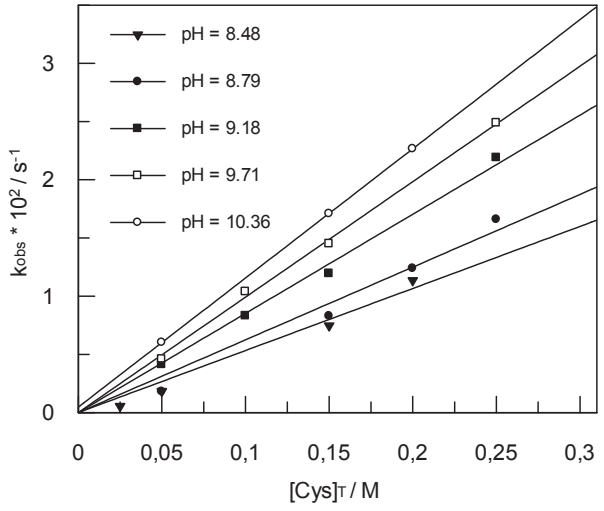


Fig. 8. Influence of cysteine total concentration on k_{obs} in transnitrosation reaction with NOTSG at 25 °C.

Experimental results fit very well to the deduced Eq. 5 as is made clear by the plotted curve in Fig. 9b but in this case, due to the complexity of the equation, macroscopic acidity constants of cysteine were kept constant for the fitting process: pK_{a2}=8.3 and pK_{a3}=10.2.

The kinetic data analysis yields a value for the acidity constant of NOTSG: pK_a=10.51, which compares well with the described value in the literature.[24] Transnitrosation velocity constants obtained, k_1=9.07×10^{-2} M^{-1} s^{-1} and k_2=2.36×10^{-1} M^{-1} s^{-1}, showed higher reactivity for the anion $(Cys)^{2-}$. Compatible results were obtained (k_1=9.87×10^{-2} M^{-1} s^{-1}; k_2=1.71×10^{-1} M^{-1} s^{-1}) when the same data was fitted to a rearranged form of Eq. 5 (Eq. 6) showing clear linear dependence between the product (k_{tr}^{app}×Eq. 5 denominator) and $[H^+]$ (Fig. 10).

$$\underbrace{k_{tr}^{app} \times (equation5denominator)}_{Y} = \underbrace{k_1 \times K_{a2}}_{m} \times \underbrace{\left[H^+\right]}_{x} + \underbrace{k_2 \times K_{a3} \times K_{a2}}_{b} \quad (6)$$

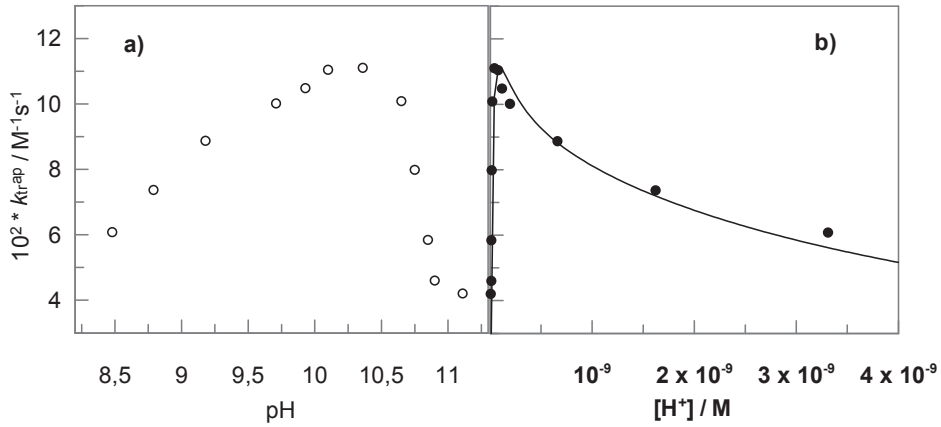
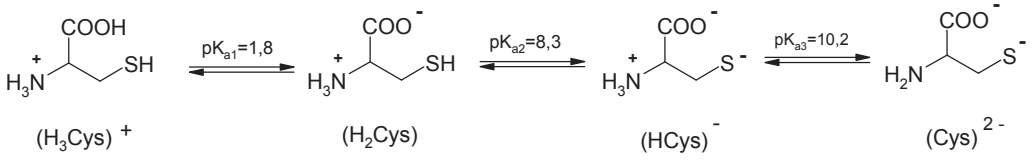
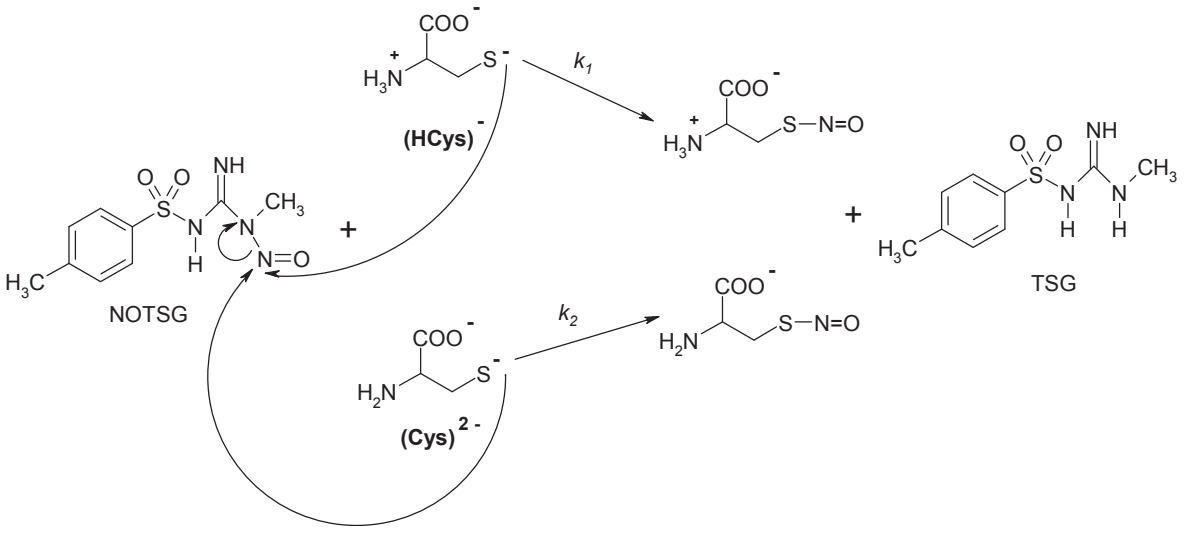


Fig. 9. **a**) Influence of pH on k_{tr}^{app} rate constants for the transfer of the nitroso group from NOTSG to cysteine at 25 °C. **b**) Fitted results to Eq. 5.



Scheme 4. Acid–base equilibrium of cysteine: possible cysteine ions in solution.



Scheme 5. Proposed mechanism for nitroso transfer from neutral form of *N*-nitrosoguanidine to the anionic species of cysteine ($HCys^-$ and $(Cys)^{2-}$.

The higher reactivity of $(Cys)^{2-}$ wasnot a surprising result since it is expected a more basic character of $(Cys)^{2-}$ then $(HCys)^-$. Similar studies of cysteine transnitrosation using MNTS as NO donor accomplished by *C. Adam* et al.[9] also showed the same pattern of reactivity between $(Cys)^{2-}$ and $(HCys)^-$ however, in this last case, obtained transnitrosation velocity constants are 100 times higher. This huge difference compared to our study can only be explained by the highest electron-withdrawing effect towards the nitroso group in the MNTS (see Fig. 1) since it is directly bounded to the sulfonyl group. In the case of our NO donor, NOTSG we have the guanidine function between the sulfonyl and the nitroso group that attenuates the electron-withdrawing effect giving lower transnitrosation velocity constants.

Therefore, since NOBMG is structurally identical to NOTSG we predict a similar mechanistic behavior but less reactive since the electron-withdrawing effect of benzoyl group is lower when compared with the same effect of sulfonyl group.[24]

2.4. Structure-reactivity correlations

The influence of the nucleophilic reactivity of studied thiolate ions with their basicity was evaluated (Fig. 11) and as we can see, for both *N*-nitrosoguanidines obtained Brønsted exponents are negative (β_{nuc} (NOBMG)=−0.17 and β_{nuc} (NOTSG)=−0.11) suggesting a reactivity decrease with the increasing basicity strength,

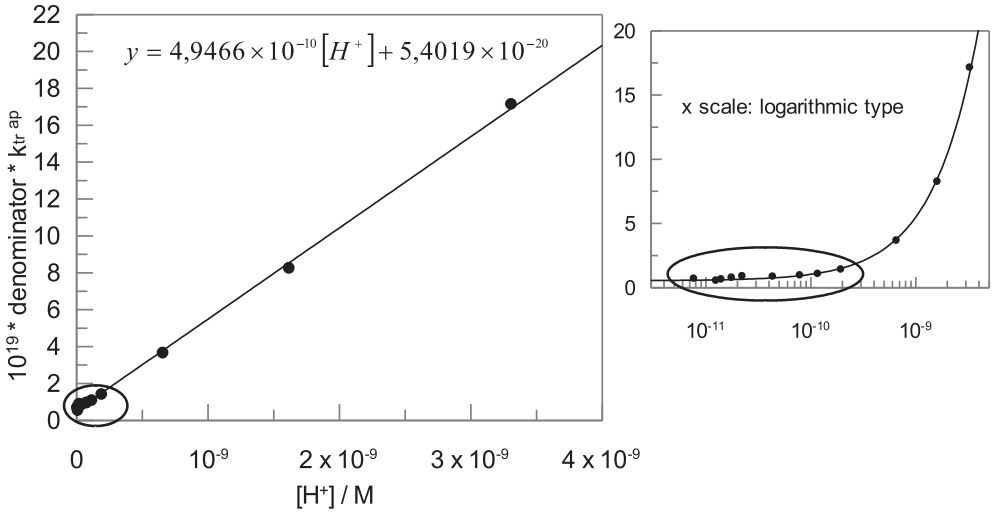


Fig. 10. Fitted results to Eq. 6.

which is an apparent contradiction when we are considering a typical nucleophilic attack mechanism.

In fact, for SN reactions, expected Brønsted exponents are positive, although, some described cases in the literature also show negative or near zero β_{nuc} values as a consequence of the desolvation effect on the reaction rate or on the reactivity-structure correlations.

The same behavior has been observed for some phosphoryl transfer reactions to amines[25] and for reactions of highly reactive carbocations with amines[26] and also for reactions of thiolate ions with Fisher carbene complexes.[27]

Likewise, Brønsted exponents near zero were found for reactions of diphenylketene with amines[28] and for transnitrosation reactions from MNTS[9] and *N*-nitrososulfonamides[18] to thiolate ions.

According to Jencks,[25] these anomalous β_{nuc} values are a result of the requirement of nucleophile desolvation prior to the reaction, a pre-equilibrium occurring in a separated step as it is shown in Scheme 6:

Assuming this partial desolvation, the experimental value of the rate constant for the nucleophilic attack (transnitrosation) corresponds to the product $K_d k'$, where K_d is the equilibrium constant for the partial desolvation of the thiolate ion and observed β_{nuc} will be given by Eq. 7:

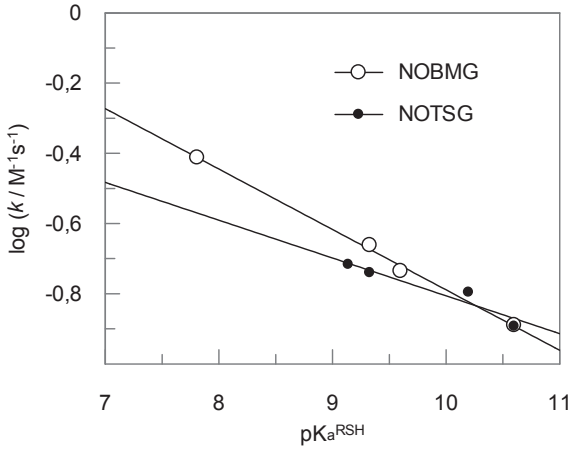
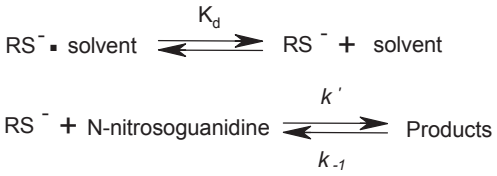


Fig. 11. Influence of the nucleophilic reactivity of studied thiolate ions with their basicity for transnitrosation reactions with NOBMG and NOTSG.



Scheme 6. Partial desolvation pre-equilibrium of the thiolate ion and *N*-nitrosoguanidine reaction with free thiolate ion.

$$\beta_{nuc} = \frac{d(\log k)}{d\left(pK_a^{RSH}\right)} = \frac{d(\log K_d k')}{d\left(pK_a^{RSH}\right)} = \frac{d(\log K_d)}{d\left(pK_a^{RSH}\right)} + \frac{d(\log k')}{d\left(pK_a^{RSH}\right)} = \beta_d + \beta'_{nuc} \quad (7)$$

Considering a more difficult desolvation with the higher basicity of thiolate ions (RS^-), $\beta_d<0$ it is expected. Thus if β'_{nuc} is low, indicating a transition state with little bond formation, observed β_{nuc} values may be dominated by β_d and be close to zero or even negative like those obtained in study.

3. Conclusion

Although *N*-nitrosocompounds may be potential carcinogens when they decompose by a deamination reaction due to their N-alkylating ability, it was found that *N*-nitrosoguanidines studied, *N*-nitroso-1-methyl-3-benzoylguanidine (NOBMG) and *N*-nitroso-1-methyl-3-tolylsulfonylguanidine (NOTSG), react faster with thiol type sulfur nucleophiles in neutral and basic media, transferring directly the nitroso group to the corresponding thiolate ion forming *S*-nitrosotiol. Thus, it can be considered that these *N*-nitrosoguanidines are potential nitric oxide donors.

4. Experimental

4.1. General

All reactants used in the synthesis were of the highest purity commercially available and used without further purification. All reagents used for the kinetics studies were analytical grade and deionized water was used throughout this study.

1-methyl-3-benzoylguanidine (BMG) was prepared as described in the literature for 1-methyl-3-tolylsulfonylguanidine (TSG)[29] and nitrosation was accomplished mixing the free guanidines with acidic aqueous solution of sodium nitrite as described for the nitrosation of clonidine.[30] The nitrosoguanidines obtained showed similar spectroscopic data by ^{1}H NMR analysis as described previously in the literature.[17]

Transnitrosation reactions at 25 °C were monitored following the formation of the correspondent *S*-nitrosothiol at 330 nm using a UV–vis spectrophotometer and a stopped-flow for faster reactions. All kinetic experiments were triggered using a small aliquot of acetonitrile solution of *N*-nitrosoguanidines. The final percentage of acetonitrile in the reaction mixtures was always 3, 33% (v/v) and the final *N*-nitrosoguanidines concentration was $(4–5)\times10^{-5}$ M.

In most reactions pH was controlled by using the nucleophile itself as buffering agent. When pH was between 6 and 8, reactions were carried out using H_2PO_4Na/HPO_4Na_2 buffer and for pH values higher than 10, 5 aqueous NaOH solution was used to fix pH.

At the end of all the studied reactions the presence of nitrite ion was qualitatively determined using a modification of Shinn's method.[31]

In order to carry out the reactions under pseudo-first order conditions, all the experiments were performed with a large excess of nucleophile relative to the *N*-nitrosoguanidines following every run to at least 90% of the reaction. In the experiments followed by stopped-flow each run was repeated at least five times and the average value of the pseudo-first order rate constant, k_{obs}, was obtained. In all cases, obtained k_{obs} values were reproducible with a standard error less than 3%.

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