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Reactivity of Amino Acids in Nitrosation Reactions and Its Relation to the Alkylating Potential of Their Products

M. del Pilar García-Santos, Samuel González-Mancebo, Jesús Hernández-Benito, Emilio Calle, and Julio Casado*

Contribution from the Departamento de Química Física, Universidad de Salamanca, E-37008 Salamanca, Spain

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Abstract: Nitrosation reactions of amino acids with an $-NH_2$ group [namely, six α-amino acids (glycine, alanine, α-aminobutyric acid, α-aminoisobutyric acid, valine, and norvaline); two β-amino acids (β-alanine and β-aminobutyric acid), and one γ-amino acid (γ-aminobutyric acid)] were studied. Nitrosation was carried out in aqueous acid media, mimicking the conditions of the stomach lumen. The rate equation was $r = k_{3\,exp}$[amino acid][nitrite]^2, with a maximum $k_{3\,exp}$ value in the 2.3−2.7 pH range. The existence of an isokinetic relationship supports the argument that all the reactions share a common mechanism. A nitrosation mechanism is proposed, and the following conclusions are drawn: (i) Nitrosation reactions of amino acids with a primary amino group in acid media occur with dinitrogen trioxide as the main nitrosating agent. The finding that the nitrosation rate is proportional to the square of the nitrite concentration suggests that the yield of nitrosation products in the stomach would increase sharply with higher nitrate/nitrite intakes. (ii) Stomach hypochlorhydria could be a potential enhancer of in vivo amino acid nitrosation. (iii) The reactivity ($k_{3\,exp}$) [α-amino acids > β-amino acids > γ-amino acids] is the same as that found in a previous work for the alkylating potential of lactones formed from nitrosation products of the same amino acids. This implies that the nitrosation reactions of the most common natural amino acids are the most efficient precursors of the most powerful alkylating agents. (iv) The order of magnitude (10^7-10^8 M^{-1} s^{-1}) of the bimolecular rate constants of nitrosation shows that such reactions occur through an encounter process.

Introduction

N-Nitroso compounds are unique among carcinogenic agents in being active in all species and in displaying a broad spectrum of target cells and organs in which they are able to induce cancer. The formation of nitroso compounds can occur in food (when preserved with nitrite), in the environment, and in the digestive tract (especially in the stomach). Some *N*-nitroso compounds are even synthesized by plants, although most of these are formed incidentally through the nitrosation of amines.[1−4]

Biologists have mainly been interested in the pathogenic mechanisms in which such species are involved,[1,3] whereas chemists have been more interested in their formation mechanisms[5−8] and hence in ways to block or inhibit them.[9−13]

The nitrosation of amino acids is particularly interesting. In the case of amino acids with a secondary amino group, besides direct nitrosation by NO^+ and N_2O_3, a nitrosation mechanism through the initial formation of a nitrosyl carboxylate followed by a slow intramolecular rearrangement has been reported.[14−16]

The nitrosation of amino acids with an $-NH_2$ group has received little attention because they afford unstable products. Due to this instability, our research was carried out in two stages: (i) study of the nitrosation of amino acids with $-NH_2$ group and (ii) identification of alkylating agents resulting from nitrosation and study of their alkylating potential.

In previous work,[17] our attention focused on the second of the above stages; the present study addresses the first aim.

* To whom correspondence should be addressed. E-mail: jucali@usal.es.

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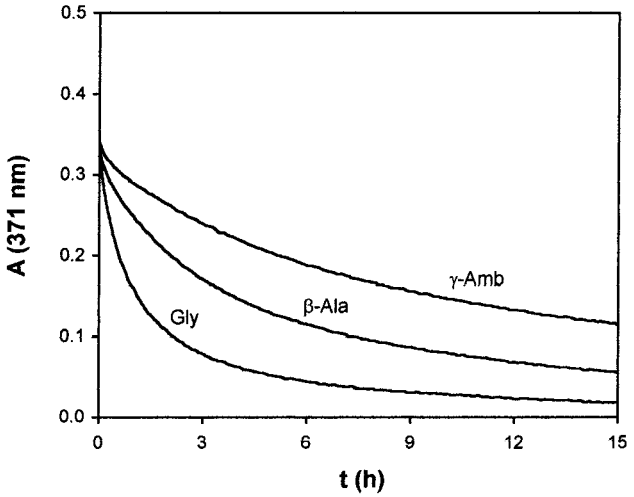


Figure 1. Variation in the nitrite absorbance with the reaction time in the nitrosation of amino acids. $[Gly]_o = 0.300$ M, $[\beta\text{-Ala}]_o = 0.300$ M, $[\gamma\text{-Amb}]_o = 0.350$ M; $[Nit]_o = 0.0100$ M, $[NaH_2PO_4] = 0.50$ M, $I = 1.00$ M, pH 3.0, $T = 298$ K.

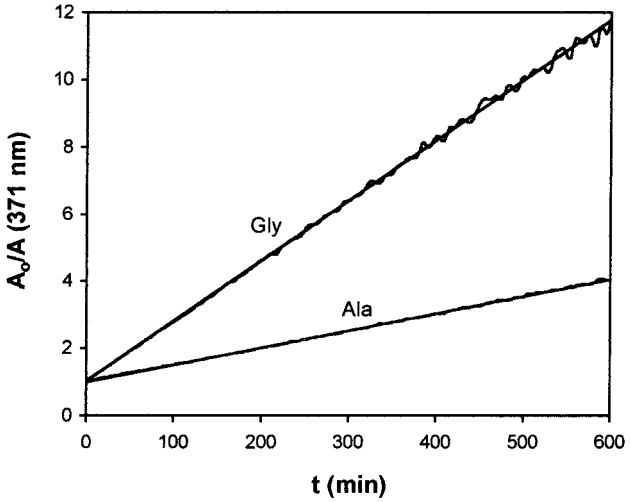
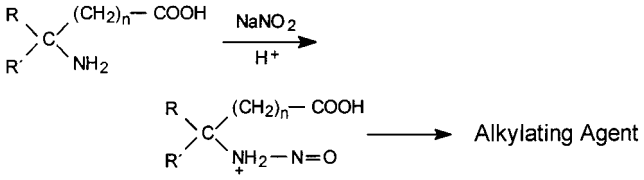


Figure 2. Integrated form of the second order rate equation (eq 1) for the nitrosation of glycine and alanine. $[Gly]_o = 0.300$ M, $[Ala]_o = 0.210$ M; $[Nit]_o = 0.0100$ M, $[NaH_2PO_4] = 0.50$ M, $I = 1.00$ M, pH 3.0, $T = 298$ K.

We investigated the nitrosation of six α-amino acids with $NaNO_2$ in acidic media [glycine (Gly), DL-alanine (Ala), DL-α-aminobutyric acid (α-Amb), α-aminoisobutyric acid (α-Amib), valine (Val), and norvaline (nor-Val)], two β-amino acids [β-alanine (β-Ala) and DL-β-aminobutyric acid (β-Amb)], and one γ-amino acid [γ-aminobutyric acid (γ-Amb)].



These nitrosatable substrates were chosen with two criteria: (i) structural, to analyze the influence of the relative position of the amino and carboxy groups in the nitrosation rate, and (ii) their presence in nature (more α-amino acids were chosen because these are the most common species found).

Experimental Section

Amino acid solutions were made up by weight from Merck 99% glycine and valine; Aldrich 99% alanine, α-aminobutyric, α-aminoisobutyric acid, norvaline, and β-alanine, and 97% β-aminobutyric acid and γ-aminobutyric acid.

The reagents $NaNO_2$, $NaClO_4$ (ionic strength, I, controller), and $HClO_4$ were purchased from Merck, and NaH_2PO_4 was purchased from Panreac.

$NaNO_2$ solutions were made up by weight, after desiccation for 2 h at 110 °C.

Since the HNO_2/NO_2^- system (henceforth nitrite, Nit) shows maximum absorption at $\lambda = 371$ nm, nitrite was used as the control species for monitoring the nitrosation reactions.

UV-absorption spectra and spectrophotometric measurements were carried out on a Shimadzu 2101PC double-beam spectrophotometer with a thermoelectric six-cell holder temperature control system (± 0.1 °C).

pH was measured with a Crison 2000 pH-meter, equipped with a combined glass electrode (Crison 52−02).

All kinetic runs were followed to at least 70% completion and were performed in triplicate.

Results and Discussion

Experiments designed to investigate the influence of the nitrite concentration revealed the reaction to be second order with respect to this reagent

$$\text{rate} = k_{2\,\text{exp}}[\text{Nit}]^2 \qquad (1)$$

Figure 1 shows typical kinetic runs.

Figure 2 represents the integrated form of eq 1 in terms of absorbance A, A_o being the initial absorbance. The good linear fitting of the A_o/A (371 nm) values against those of t reveals second order with respect to the nitrite concentration, involving dinitrogen trioxide as the main nitrosating agent.[18]

Figure 3 shows the results obtained on working with different initial concentrations of each amino acid $[\text{amino acid}]_o$, first order being observed with respect to the amino acid concentration.

The above two sets of experiments imply the following rate equation:

$$\text{rate} = k_{3\,\text{exp}}[\text{amino acid}][\text{Nit}]^2 \qquad (2)$$

Comparison of eqs 1 and 2 yields

$$k_{2\,\text{exp}} = k_{3\,\text{exp}}[\text{amino acid}] \qquad (3)$$

allowing $k_{3\,\text{exp}}$ to be calculated. Figure 4 shows an example of the linear correlation between the values of $k_{2\,\text{exp}}$ and those of $[\text{amino acid}]_o$ (since $[\text{amino acid}] \cong [\text{amino acid}]_o$ because $[\text{amino acid}]_o \gg [\text{Nit}]_o$) with the intercept not significantly different from zero.

The results obtained (Table 1) show the following general sequence of $k_{3\,\text{exp}}$: α-amino acid > β-amino acid > γ-amino acid.

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Table 1. Dependence of the Nitrosation Rate of Amino Acids on the Acidity of the Medium

amino acid	pH	$10^2 k_{3\,exp}$ ($M^{-2}\,s^{-1}$)	pH	$10^2 k_{3\,exp}$ ($M^{-2}\,s^{-1}$)	pH	$10^2 k_{3\,exp}$ ($M^{-2}\,s^{-1}$)
glycine	2.53	16.6 ± 0.3	3.15	8.8 ± 0.2	3.51	4.1 ± 0.1
alanine	2.53	5.41 ± 0.06	3.17	3.34 ± 0.08	3.50	1.56 ± 0.05
α-aminobutyric acid	2.52	7.4 ± 0.4	3.16	4.31 ± 0.09	3.55	1.7 ± 0.1
α-aminoisobutyric acid	2.53	1.22 ± 0.07	3.18	0.69 ± 0.03	3.52	0.33 ± 0.01
valine	2.50	11.5 ± 0.2	3.18	6.1 ± 0.1	3.54	3.00 ± 0.03
norvaline	2.52	7.2 ± 0.2	3.12	4.2 ± 0.1	3.55	2.05 ± 0.07
β-alanine	2.65	2.9 ± 0.1	3.15	2.44 ± 0.07	3.50	1.58 ± 0.05
β-aminobutyric acid	2.66	1.60 ± 0.05	3.13	1.29 ± 0.02	3.50	0.80 ± 0.01
γ-aminobutyric acid	2.75	1.02 ± 0.01	3.12	0.95 ± 0.03	3.48	0.620 ± 0.006

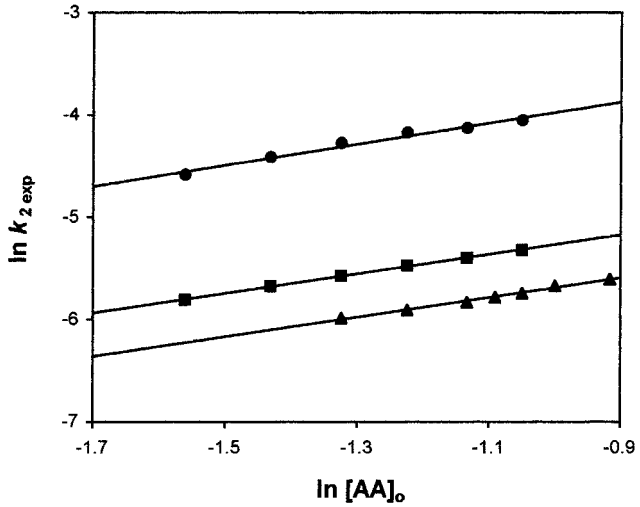


Figure 3. Kinetic order in amino acid in the nitrosation reactions. $[Nit]_o = 0.0100$ M, $[NaH_2PO_4] = 0.50$ M, $I = 1.00$ M, $T = 298$ K: (●) norvaline, pH 3.03; (■) β-aminobutyric acid, pH 3.00; (▲) γ-aminobutyric acid, pH 3.14.

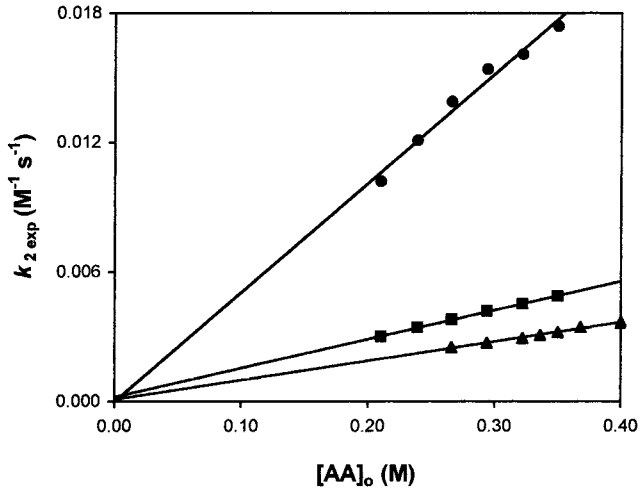


Figure 4. Determination of the rate constant $k_{3\,exp}$ in the nitrosation of amino acids (eq 3). $[Nit]_o = 0.0100$ M, $[NaH_2PO_4] = 0.50$ M, $I = 1.00$ M, $T = 298$ K: (●) norvaline, pH 3.03; (■) β-aminobutyric, pH 3.00; (▲) γ-aminobutyric, pH 3.14.

No influence of the ionic strength on the rate constant $k_{3\,exp}$ was observed (Figure 5).

Figure 6 shows the dependence of $k_{3\,exp}$ on the pH of the medium.

To approach in vivo conditions, nitrosation was carried out in aqueous acid media, mimicking the conditions of the stomach lumen. All reactions showed analogous profiles, with a maximum in the 2.3−2.5 pH range for α-amino acids and centered at pH 2.7 for β- and γ-amino acids.

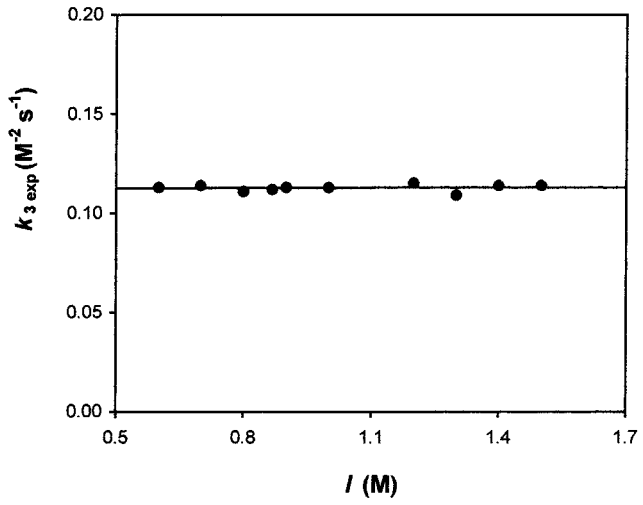


Figure 5. No influence of the ionic strength on the nitrosation rate constant $k_{3\,exp}$ (eq 2). $[Gly]_o = 0.300$ M, $[Nit]_o = 0.0100$ M, $[NaH_2PO_4] = 0.50$ M, pH 3.00, $T = 298$ K.

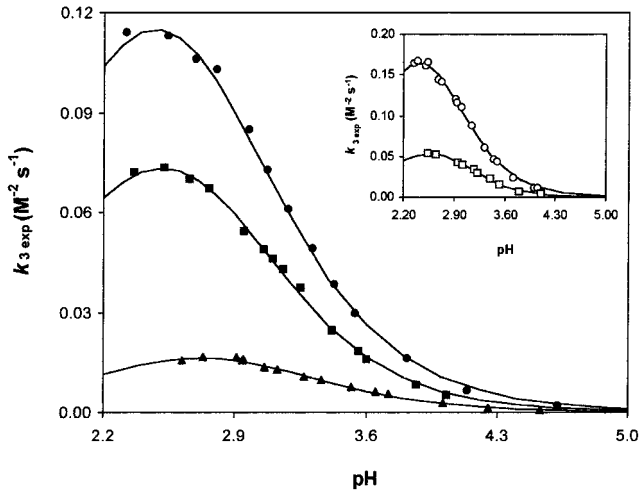


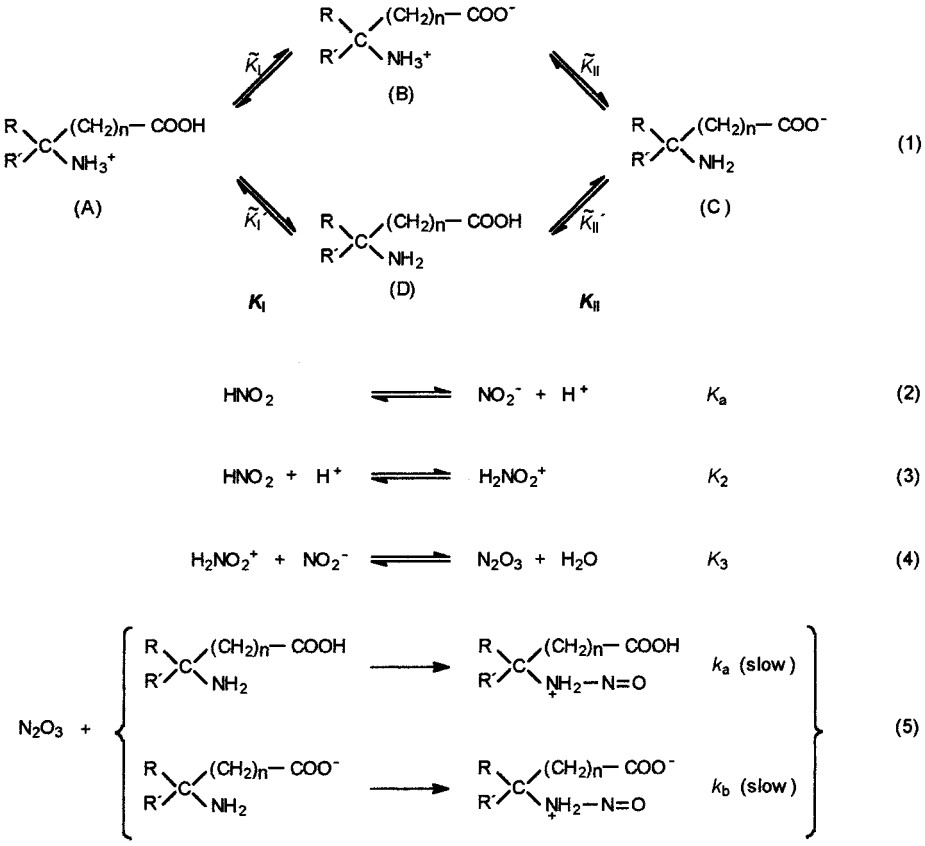
Figure 6. Fitting of the experimental nitrosation rate constant to the theoretical rate equation. $[Nit]_o = 0.010$ M, $[NaH_2PO_4] = 0.50$ M, $I = 1.00$ M, $T = 298$ K: (●) $[Val]_o = 0.300$ M, (■) $[\alpha\text{-Amb}]_o = 0.300$ M, (▲) $[\beta\text{-Amb}]_o = 0.300$ M, (○) $[Gly]_o = 0.300$ M, (□) $[Ala]_o = 0{,}300$ M. Dots are experimental values. Solid lines fit eq 7.

To rationalize the results obtained, the mechanism shown in Scheme 1 is proposed.

The first step represents the protonation equilibrium of the amino acid. K_I and K_{II} are the macroscopic constants for the loss, respectively, of the first and second protons. $\tilde{K}_I$, $\tilde{K}'_I$, $\tilde{K}_{II}$, and $\tilde{K}'_{II}$ are microscopic constants whose values are not accessible experimentally.

The values of the microscopic constants can be estimated from K_I and K_{II} by assuming the microscopic constant $\tilde{K}'_I$ to be

Scheme 1. Nitrosation Mechanism



approximately equal to the acidity constant of an ester of the amino acid K_e.[19,20] The concentrations of the species A, B, C, and D can then be expressed as a function of the acidity and the total concentration of the amino acid: [amino acid] = [AA] ≅ [A] + [B] in the working conditions.

$$[A] = \frac{[AA][H^+]}{(K_I + [H^+])}; [B] = \frac{K_I[AA]}{(K_I + [H^+])}$$

$$[C] = \frac{K_I K_{II}[AA]}{(K_I + [H^+])[H^+]}; [D] = \frac{K_e[AA]}{(K_I + [H^+])}$$

The reaction rate of nitrosation can be written as:

$$r = k_a[D][N_2O_3] + k_b[C][N_2O_3] \quad (4)$$

where the first and second terms, respectively, represent the attack of the neutral and basic forms of each amino acid by dinitrogen trioxide.

As in the nitrosation of amino acids with a secondary amino group,[14−16] besides N_2O_3 as a nitrosating agent NO^+ should also be included in the mechanism. Nevertheless, since the rate constant of the attack by NO^+ is negligible compared with that of N_2O_3, for simplicity we have not taken it into account here.

Since [Nit] = [HNO_2] + [NO_2^-], it is easy to deduce that:

$$r = k_a K_M K_e \frac{[AA][Nit]^2[H^+]^2}{(K_I + [H^+])(K_a + [H^+])^2} + k_b K_I K_{II} K_M \frac{[AA][Nit]^2[H^+]}{(K_I + [H^+])(K_a + [H^+])^2} \quad (5)$$

where $K_M = K_a K_2 K_3 = 3.03 \times 10^{-3}$ M^{-1}.[21]

Equation 5 is consistent with the experimental reaction orders.

Setting $\alpha = k_a K_M K_e$, $\beta = K_a$, and $\gamma = k_b K_I K_{II} K_M$, eq 6 can be written:

$$r = \alpha \frac{[AA][Nit]^2[H^+]^2}{(K_I + [H^+])(\beta + [H^+])^2} + \gamma \frac{[AA][Nit]^2[H^+]}{(K_I + [H^+])(\beta + [H^+])^2} \quad (6)$$

Comparison of the experimental (eq 2) and theoretical (eq 6) rate equations gives:

$$k_{3\,exp} = \alpha \frac{[H^+]^2}{(K_I + [H^+])(\beta + [H^+])^2} + \gamma \frac{[H^+]}{(K_I + [H^+])(\beta + [H^+])^2} \quad (7)$$

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Table 2. Parameters from the Eq 7 Calculated by a Nonlinear Optimization Algorithm[a]

amino acid		$\alpha \cdot 10^4$ ($M^{-1}\ s^{-1}$)	pK_a calc.* = [log (1/β)]	$\gamma \cdot 10^7$ (s^{-1})	$k_a \cdot 10^{-7}$ ($M^{-1}\ s^{-1}$)	$k_b \cdot 10^{-7}$ ($M^{-1}\ s^{-1}$)
glycine	H_2N-CH_2-COOH	24 ± 1	2.7 ± 0.2	30 ± 10	4.25	133
alanine	$CH_3-CH(NH_2)-COOH$	5.9 ± 0.2	3.1 ± 0.1	1.2 ± 0.6	1.23	6
α-aminobutyric acid	$CH_3-CH_2-CH(NH_2)-COOH$	9.3 ± 0.2	3.1 ± 0.1	2.4 ± 0.8	1.57	10
α-aminoisobutyric acid	$CH_3-C(CH_3)(NH_2)-COOH$	1.9 ± 0.1	2.7 ± 0.1	3 ± 2	0.79	37
valine	$(CH_3)_2CH-CH(NH_2)-COOH$	15.7 ± 0.5	2.9 ± 0.1	8 ± 2	1.60	27
norvaline	$CH_3-CH_2-CH_2-CH(NH_2)-COOH$	10.1 ± 0.5	2.8 ± 0.1	10 ± 5	1.67	44
β-alanine	$H_2N-CH_2-CH_2-COOH$	1.73 ± 0.05	2.9 ± 0,1	0.22 ± 0.03	7.70	44
β-aminobutyric acid	$CH_3-CH(NH_2)-CH_2-COOH$	1.2 ± 0.1	2.8 ± 0.1	0.3 ± 0.1	**	27
γ-aminobutyric acid	$H_2N-CH_2-CH_2-CH_2-COOH$	0.54 ± 0.03	2.9 ± 0.1	0.04 ± 0.01	9.14	51

[a] A single asterisk indicates the experimental value:[23] $pK_a = 3.006$. The double asterisk indicates that no value was calculated because the corresponding K_e value was not available in the literature.

Since for each pH the value of $k_{3\,exp}$ is experimentally known, α, β, and γ can be calculated by a nonlinear optimization algorithm.[22]

As examples, Figure 6 shows the good fits of the experimental results to eq 7 for α-, β-, and γ-amino acids.

Table 2 summarizes the values obtained for α, γ, and $pK_a = \log(1/\beta)$, as well as the calculated k_a and k_b values.

The results in Table 2 show the following:

(i) There is good agreement between the value obtained for $pK_a = \log(1/\beta)$ and the commonly accepted value ($pK_a = 3.006$ at ionic strength $I = 0.937$ M),[23] which supports the validity of eq 5 and the mechanism from which it was derived.

(ii) The order of magnitude (10^7-10^8 $M^{-1}s^{-1}$) of the bimolecular rate constants k_a and k_b is coherent with an encounter mechanism. This result agrees with that found for the nitrosation of amino acids with a secondary amino group[15,16] as well as for the nitrosation of very different amines.[24]

(iii) The values of the rate coefficient k_a follow the sequence α-amino acids < β-amino acids < γ-amino acids.

Even though the k_a values lie within the accepted range corresponding to an encounter process, the results appear to demonstrate the influence of the different nucleophilicities of the amino acids attacked by the electrophilic N_2O_3. This can be explained through the electron-withdrawing (−I) effect[25] of the −COOH increasing the nucleophilicity of the $-NH_2$ group in the following order: α-amino acids < β-amino acids < γ-amino acids.

(iv) In the α-amino acid series, the following sequence of k_a values is observed: α-Amib < Ala ≈ α-Amb ≈ Val ≈ norVal < Gly.

This sequence is understandable in terms of steric hindrance for the electrophilic attack of the $-NH_2$ by the voluminous dinitrogen trioxide. Hindrance is maximum for α-aminoisobutyric acid and minimum for glycine. This is consistent with the known fact that in peptides with glycine as the terminal amino acid, a glycine residue can adopt many conformations that are sterically hindered for other amino acids due to the small size of the hydrogen substituent[26] (the possibility of using steric hindrance for blocking or inhibiting nitrosation by N_2O_3 has been discussed in the literature[13]).

(v) The observed fact (Table 2) that k_b values are always higher than those of k_a is consistent with the electron-donating (+I) effect[25] of the COO^- group on the nucleophilic site of the amino acid. Nevertheless, a high dispersion in k_b values is seen; this is a consequence of the large deviations affecting the γ-values used to calculate those of k_b.

As is known,[27] the existence of an isokinetic relationship can serve as an argument, but not proof, that the reactions studied share a common feature.

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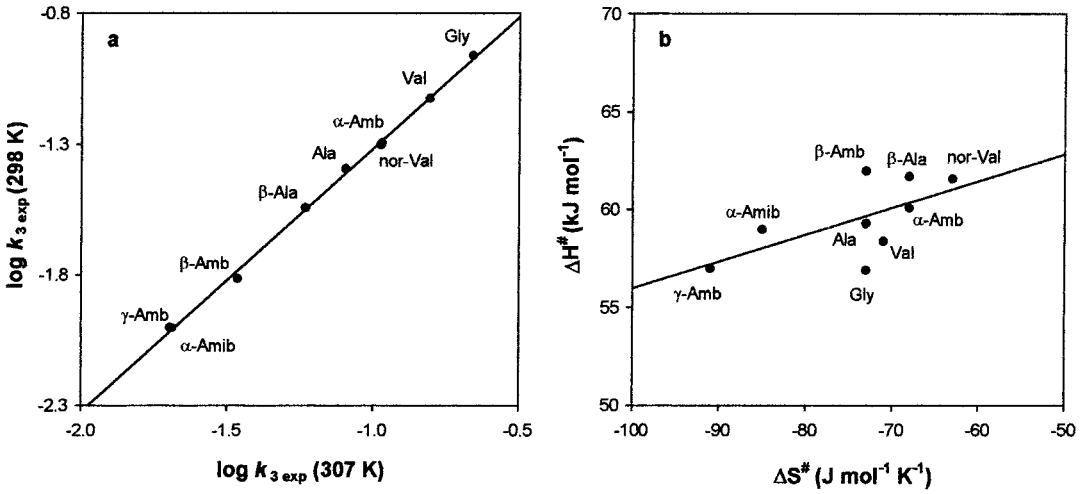

Figure 7. Isokinetic relationship in the nitrosation of amino acids. (a) Diagram of log k (T_2)/log k (T_1); (b) Compensation effect $\Delta H^{\#} - \Delta S^{\#}$.

A mathematical formulation of the isokinetic effect is the linear relationship between two series of log k values measured at two temperatures: T_1 and T_2. Thus

$$\log k(T_2) = a + b \log k(T_1)$$

The meaning of the isokinetic relationship is the existence of a compensation effect between the values of enthalpy, $\Delta H^{\#}$, and the entropy of activation, $\Delta S^{\#}$, such that the Gibbs' energy of activation, $\Delta G^{\#}$, is approximately constant.[28]

The results shown in Figure 7 support the idea of a common mechanism.

In the second stage of this investigation,[17] the kinetic evolution of the nitrosation products was studied. It was observed that after the nitrosation of amino acids, lactones are formed, whose alkylating potential was studied. The following sequence of alkylating power was found: α-lactones > β-lactones > γ-lactones, coming respectively from the nitrosation of α-, β-, and γ-amino acids. This implies that the nitrosation reactions of the most common natural amino acids are the most efficient precursors of the most powerful alkylating agents.

Conclusions

(i) Nitrosation reactions of amino acids with a primary amino group in acid media occur with dinitrogen trioxide as the main nitrosating agent.

The finding that the nitrosation rate is proportional to the square of the nitrite concentration suggests that the yield of nitrosation products in the stomach would increase sharply with higher nitrate/nitrite intakes.

The experimental nitrosation rate constant shows a maximum in the 2.3−2.5 pH range for α-amino acids and pH 2.7 for β- and γ-amino acids. As a consequence, attention should be paid to stomach hypochlorhydria as a potential enhancer of in vivo amino acid nitrosation reactions.

(ii) The reactivity (in terms of the experimental rate constant of nitrosation) α-amino acids > β-amino acids > γ-amino acids is the same as that found for the alkylating potential of lactones formed from nitrosation products. This implies that the nitrosation reactions of the most common natural amino acids are the most efficient precursors of the most powerful alkylating agents.

(iii) The experimental results suggest a mechanism for the nitrosation of amino acids, whose rate-limiting step is bimolecular attack by N_2O_3 on the free base form of the amino group of the neutral and basic forms of the amino acids.

Although the bimolecular rate constants lie within the range accepted for encounter processes, the results point to a certain influence of the different nucleophilicities of the amino acids attacked by the electrophilic N_2O_3 as well as the steric hindrance of this molecule when it approaches the nitrosation site of the nitrosatable substrates.

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