Nitrosation of amino acids as a precursor of alkylation mechanisms. Reactivity of lactones*

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1. Introduction

N-nitroso compounds are today chemicals of interdisciplinary interest.

In his excellent monograph "*Chemistry and biology of N-nitroso compounds"* Lijinsky writes: «*N*-Nitroso compounds occupy a small an obscure corner of organic chemistry and would probably have remained there if it were not for the discovery that they are carcinogenic. They occur in the environment, sometimes at concentrations which might create apprehension even if they were not so biologically active. They are unique among carcinogenic agents in being active in all species and they have unparalleled spectrum of target cells and organs in which they can induce cancer.

Among the *N*-nitroso compounds are many of the most potent carcinogens we know, active in inducing tumors in animals at concentrations close to those to which humans may be exposed. There are even *N*-nitroso compounds that are synthesized by plants, but most are formed incidentally by nitrosation of amines.

Their versatility as carcinogens has fascinated many investigators, including this author, and they have provided useful models for investigating mechanisms of carcinogenesis. While we do not understand the mechanism by which any carcinogen causes cancer, *N*-nitroso compounds are providing clues more readily than do other carcinogens» [1].

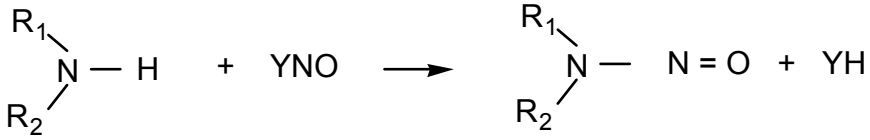
Since the *US Environmental Protection Agency* (EPA) stated in its *Cancer Principles* that "The majority of human cancers are caused by avoidable exposure to carcinogens" [2], the interest in the chemistry of *N*-nitroso compounds has increased. We also know that they are mutagenic and teratogenic agents.

Humans are exposed to nitroso compounds through two main routes, on one hand because they are already formed in the environment and are ingested or inhaled and, on the other hand, because they may be synthesized in *vivo* through simultaneous ingestion of nitrosating agents and nitrosable substances.

The interest in gaining knowledge of the mechanisms of formation of different nitroso compounds is therefore evident. Many reactions are now well understood, and in several cases the nature of the effective nitrosating species has been established, often by kinetic procedures. Features such as acid catalysis, base catalysis, nucleo-

philic catalysis, diffusion-controlled processes, rate-limiting proton transfers and intramolecular rearrangements, have all been established in nitrosation reactions under various experimental conditions [3, 4].

In this lecture, I should like to comment-on some of the mechanisms by which a reaction as apparently simple as this may occur



Here you can see the four types of nitrosation reactions we have worked on and to which I am going to refer now very briefly with the aim of exemplifying some of the very diverse routes of nitrosation reactions.

1. *Diffusion-controlled processes*. Dinitrogen trioxide as a nitrosating agent.
2. *Orbital-controlled processes*. Alkyl nitrites as a nitrosating agent.
3. *Intramolecular rearrangements*. Nitrosyl carboxylate as an internal nitrosating agent.
4. *Nitrosation reactions as precursor of alkylation mechanisms.*

2. *Diffusion-controlled processes*: Dinitrogen trioxide as a nitrosating agent

The early literature about nitrosation reactions by dinitrogen trioxide was confusing until some years ago [3, 4].

Whereas some authors for high-*pK* amines reported a constancy in the values of the rate-constants which seemed hard to explain, others described a degree of correlation between k and the pK_a of the substrate.

To gain further understanding of the various findings, we carried out a study of the kinetics of nitrosation of some secondary amines under conditions in which the effective nitrosating agent was dinitrogen trioxide.

The rate of nitrosation was followed using a differential spectrophotometric technique measuring the absorbance of each nitroso compound. All experiments were carried out with concentrations of nitrite low enough to avoid problems caused by spontaneous decomposition of nitrous acid.

The experimental rate equation was:

$$v_o = \frac{\alpha[nitrite]_o^2[amine]_o[H^+]}{(\beta + [H^+])^2} \qquad (1.1)$$

On the basis of the experimental findings [5] and bearing in mind the results of previous work [6-8], the following mechanism was therefore proposed:

$$NO_2^- + H^+ \overset{K_1}{\rightleftharpoons} HNO_2$$

$$R_2NH + H^+ \overset{K_2}{\rightleftharpoons} R_2NH_2^+$$

$$HNO_2 + H^+ \overset{K_3}{\rightleftharpoons} H_2NO_2^+$$

$$H_2NO_2^+ + NO_2^- \overset{K_4}{\rightleftharpoons} N_2O_3 + H_2O$$

$$R_2NH + N_2O_3 \xrightarrow{k} R_2NNO + NO_2H$$

that gives us the following rate equation:

$$v_o = \frac{\left(kK_3K_4 / K_1K_2\right)\left[nitrite\right]_o^2\left[amine\right]_o\left[H^+\right]}{\left(1/K_1 + \left[H^+\right]\right)^2} \qquad (1.2)$$

Here we should mention something that was really a matter of luck but which was very useful for us.

As you can see, in the proposed mechanism the value: $K=K_3K_4/K_1$ participates, which is precisely the equilibrium constant $2NO_2H \overset{K}{\rightleftharpoons} N_2O_3 + H_2O$.

The scatter of the values proposed for K in the literature ranged so widely that the measurements of some authors differ by as much as 70 times of the values proposed by others. However, a few years before our work, Markovits and coworkers proposed a new value for $K = 3.03 \cdot 10^{-3}$ M^{-1} [9]. Table 1 shows the results of our calculations of k for the amines studied by ourselves together with values of a number of amines studied by other authors which were recalculated using Markovits' K.

As you can see the values of k are of the order of 10^7-10^8. Both the values and their constancy supported the diffusion control hypothesis.

This hypothesis was further tested by calculating the enthalpies of activation for N_2O_3 attacking the free amine (step 5 of the mechanism). The values of $\Delta H^{\#}$ are shown in Table 2, and can be seen to lie within the generally permitted range for diffusion controlled processes.

These results and conclusions rendered irrelevant former discussions observed in the literature (including our own published in [6]) attempting to explain this mechanism of nitrosation.

Table 1 Values of k for various substrates in water at 25 ºC (except where stated).

Substrate	pK_a	k, $M^{-1}s^{-1}$
4-Trimethylammonium aniline (0 ºC)	2.51	$4.7 \cdot 10^6$
2-Chloroaniline	2.63	$1.4 \cdot 10^7$
3-Chloroaniline	3.46	$9.6 \cdot 10^7$
4-Chloroaniline	3.92	$2.8 \cdot 10^8$
2-Methylaniline	4.39	$4.2 \cdot 10^8$
Aniline	4.60	$7.5 \cdot 10^8$
3-Methylaniline	4.69	$8.2 \cdot 10^8$
N-Methylaniline	4.85	$4.0 \cdot 10^8$
4-Methylaniline	5.07	$1.9 \cdot 10^9$
4-Methoxyaniline (0 ºC)	5.29	$1.8 \cdot 10^8$
Piperazine	5.55	$1.3 \cdot 10^8$
Hydroxylamine	5.90	$2.0 \cdot 10^8$
Mononitrosopiperazine	6.80	$7.5 \cdot 10^7$
Morpholine	8.70	$2.2 \cdot 10^8$
Methylbenzylamine	9.54	$1.8 \cdot 10^8$
Ethylbenzylamine	9.68	$3.1 \cdot 10^8$
N-Methylglycine	10.20	$1.5 \cdot 10^8$
2-Butylamine	10.56	$1.6 \cdot 10^8$
Propylamine	10.67	$9.4 \cdot 10^7$
Methylamine	10.70	$1.6 \cdot 10^8$
Diisobutylamine	10.82	$1.3 \cdot 10^8$
Dimethylamine	10.87	$1.2 \cdot 10^8$

Table 2 Values of $\Delta H^{\#}$ for the reactions of N_2O_3 with various substrates.

Substrate	$\Delta H^{\#}$, kJ mol^{-1}	***Substrate***	$\Delta H^{\#}$, kJ mol^{-1}
Dimethylamine	1	Dibuthylamine	12
Dipropylamine	12	Morpholine	7
Di-isopropylamine	7	2-Buthylamine	19

3. *Orbital-controlled processes*: Alkyl nitrites as a nitrosating agent

Since in 1978 Oae *et al.* studied the hydrolysis and aminolysis of alkyl nitrites [10, 11], there have been two main limitations-to research on nitrosation by nitrite esters: i) the role of pH had not been sufficiently analyzed, and ii) nitrous esters had been prepared *in situ*, without their isolation, with the consequent risk of the simultaneous participation of other nitrosating agents.

In 1992 we studied the kinetics of the nitrosation of sixteen secondary amines by propyl nitrite and butyl nitrite in a strongly alkaline medium. Six of the nitrosable species were alkylamines and ten were cyclic [12].

For the measurement of the reaction, alkyl nitrites were used as the control

species.

According to the results found in the literature and our own, we used the following reaction mechanism:

$$R_2NH_2^+ \rightleftharpoons R_2NH + H^+$$

$$R_2NH + R'ONO \xrightarrow[\text{slow}]{k_2} R_2NNO + R'OH$$

Scheme 1 Nitrosation by alkyl nitrites.

which allows the following rate-equation: $v = k_2\,[amine][nitrite]/(1+[H^+]/K_a)$ which, in strong basic media, coincides with the experimental $v = k_{2obs}\,[amine][nitrite]$.

Table 3 shows the k_2 values found by nitrosation by propyl nitrite (PrONO) and butyl nitrite (BuONO) for each of the substrates studied.

The negative slope of the Figure 1 shows a nucleophilic attack on alkyl nitrites by the amines.

Figure 2 shows a plot of log k_2 values against pK_a for different nucleophiles.

It is interesting to note that the non-existence of a linear dependence supports the idea of the reactions being mainly orbital-controlled. A number of results support this hypothesis:

1. Linearity is observed between log k_2 and the pK_a values (Fig. 3) in the case of the six-membered cyclic amines.

Indeed, as is known, orbital control should reflect a linear dependence between the log k_2 and the vertical ionization potential of the nucleophiles.

Unfortunately, the values of these vIP are not known. But, since the pK_a values of the structurally secondary amines are directly proportional to those of the vIP, the linearity of log k_2/pK_a can be seen in this particular case as a confirmation of that existing between log k_2 and those of the respective vIP.

2. We do have, however, the vIP values of five of the cyclic amines studied. As we can see in Figure 4, a study of their nitrosation by PrONO and BuONO reveals a tendency for the reactivity (k_2) to increase on decreasing the vIP.

3. It is known that in cycloalkanes the ***s*** character of the carbon-hydrogen bond increases with decreasing ring-size. The values of the nuclear-spin coupling constant of the respective cycloalkanes are reported in Table 3. Specially note the great similar-

ity between the *J* constant values in cyclopropane and in benzene (*J* = 159 Hz). So, the explanation of the facts noted is found in the overall hybridization of the nitrogen atom of the cycle, that is a change in the lone-pair from ***sp*²** hybrid in methylaziridine to ***sp*³** in larger cycles.

Table 3 Rate constants of the nitrosation reaction of secondary amines by PrONO, k_{2Pr} and BuONO, k_{2Bu} at 25 °C. [OH] = 0.1 M and I = 0.25 M; range of initial concentrations, $[PrONO]_o$ = (3.6-8.4)·10^{-3} M, $[BuONO]_o$ = (3.6-6.5)·10^{-3} M, $[nucleophile]_o$ = (3.6-42.0)·10^{-3} M.

Nucleophile	$10^{-3} \cdot k_{2Pr}$ $M^{-1}\ s^{-1}$	$10^{-3} \cdot k_{2Bu}$ $M^{-1}\ s^{-1}$	***Nucleophile*** pK_a	E_i(v),eV	$J(^{13}C\text{-}H)$ Hz[j]
1 Dimethylamine, DMA	66.4 ± 0.7		10.73[a]		
2 Diethylamine, DEA	12.5 ± 0.3		10.93[a]		
3 Dipropylamine, DnPA	13.6 ± 0.2		11.00[a]		
4 Diisopropylamine, DiPA	1.47 ± 0.02		11.20[a]		
5 Dibutylamine, DnBA	21.9 ± 0.2		11.25[a]		
6 Diisobutylamine, DiBA	15.2 ± 0.3		10.59[b]		
7 2-Methyl-aziridine, 2MAZIR	*i*	*i*		9.57[e]	161[f,g]
8 Azetidine, AZET	21.3 ± 0.3	18.2 ± 0.3	11.29[c]	9.04[e]	134[g]
9 Pyrrolidine, PYRR	175 ± 3	166 ± 4	11.35[b]	8.77[e]	128[f,g]
10 Piperidine, PIPER	26.0 ± 0.4	31.9 ± 0.4	11.12[b]	8.64[e]	123[f], 124[g]
11 2-Methylpiperidine, 2MPIPER	6.40 ± 0.08	5.59 ±0.09	10.93[d]		
12 Homopiperidine, HOMO	314 ± 7	281 ± *3*	10.89[b]	8.41[e]	123[g]
13 Heptamethyleneimine, HEPT	1080 ± 20	8990 ± 10	10.78[b]		122[g]
14 Piperazine, PIP	2.54 ±0.03	3.06 ± 0.03	10.20[b]		
15 1-Methyl-piperazine, 1MPIP	0.280 ±0.005[h]	0.171 ± 0.003[h]	9.16[d]		
16 Morpholine, MOR	0.0957±0.0016[h]	0.0740 ±0.0018[h]	8.45[d]		

[a]Ref [13]; [b]Ref [14]; [c]Ref [15]; [d]Ref [16]; [e]Ref [17]; [f]Ref [18]; [g]Ref [19]; [h]Corrected value, taking into account the hydrolysis of the alkyl nitrite. [i]No reaction observed; [j]For the corresponding cycloalkane.

As can be seen in Table 4, the rate constant falls with the polarity of the medium, suggesting that the two neutral reagents combine to form a polar transition-state complex.

Table 5 shows the values of the activation parameters in aqueous and water-organic media.

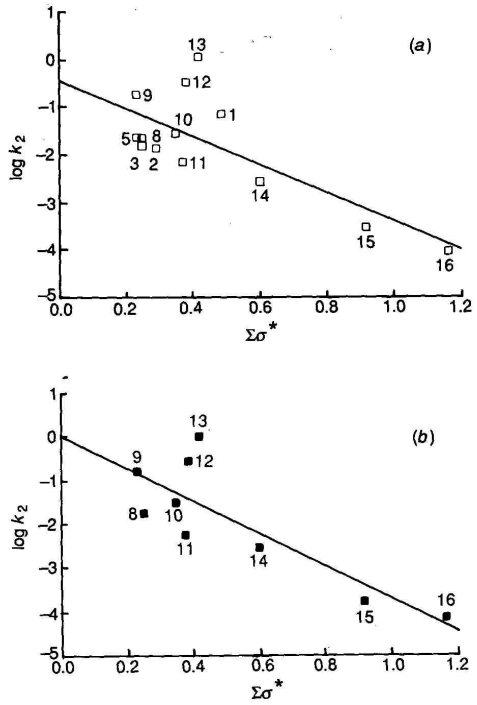


Fig. 1 log k_2 plotted against $\Sigma\sigma^*$ for the reaction of secondary amines with (*a*) PrONO and (*b*) BuONO at 25 ºC. [OH^-] = 0.1 M I = 0.25 M. Amines are numbered as shown in Table 3.

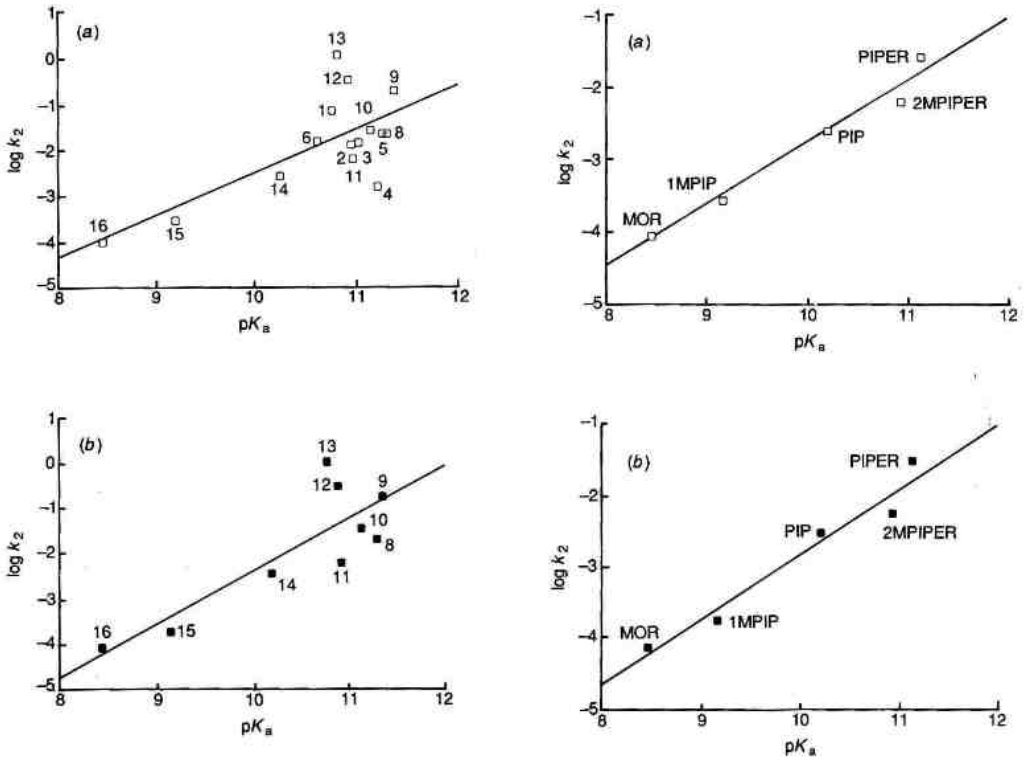


Fig. 2 log k_2 plotted against pK_a of secondary amines with (*a*) PrONO and (*b*) BuONO at 25 ºC. [OH^-] = 0.1 M amines with I = 0.25 M. Amines are numbered as shown in Table 3.

Fig. 3 Plot of log k_2 against pK_a for six- membered cyclic (*a*) PrONO and (*b*) BuONO.

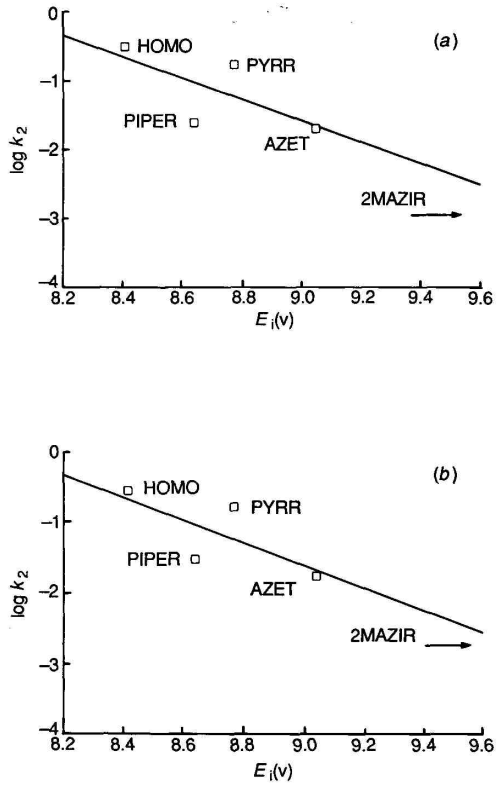


Fig. 4 Plot of log k_2 against E_i (v) in the nitrosation of secondary amines with (*a*) PrONO and (*b*) BuONO.

Table 4 Rate constants of the nitrosation reactions of secondary amines by PrONO in aqueous and water/THF media, at 25 ºC. $[OH^-] = 0.1$ M; I = 0.25 M; $[\text{nucleophile}]_o = 1.40 \cdot 10^{-2}$ M; $[\text{PrONO}]_o = 7.0 \cdot 10^{-3}$ M.

Nucleophile	k_2, $M^{-1} s^{-1}$		
	H_2O	0.5 M THF	1.00 M THF
AZET	21.3 ± 0.3	13.1 ± 0.2	12.5 ± 0.1
PYRR	175 ± 3	134 ± 7	124 ± 4
PIPER	26.0 ± 0.4	22.0 ± 0.9	20.4 ± 0.2
HOMO	314 ± 7	230 ± 9	202 ± 5
HEPT	1080 ± 20	470 ± 8	437 ± 3

Table 5 Activation parameters for the nitrosation reactions of secondary cyclic amines by PrONO in aqueous and water/THF media; temperature range 8-25 ºC ($\Delta H^{\#}$ in kJ mol^{-1}; $\Delta S^{\#}$ in J mol^{-1} K^{-1}).

Nucleophile	H_2O		0.5 M THF		1.00 M THF	
	$\Delta H^{\#}$	$-\Delta S^{\#}$	$\Delta H^{\#}$	$-\Delta S^{\#}$	$\Delta H^{\#}$	$-\Delta S^{\#}$
AZET	31.4 ± 1.4	171 ± 32	33.3 ± 1.4	169 ± 21	34.6 ± 1.8	165 ± 30
PYRR	39.2 ± 1.5	128 ± 10	28.9 ± 1.2	164 ± 21	22.7 ± 1.0	186 ± 30
PIPER	43.2 ± 0.0	130 ± 6	38.9 ± 1.9	146 ± 18	41.1 ± 1	139 ± 8
HOMO	37.2 + 1.7	130 ± 11	27.7 ± 0.9	164 ± 15	29.3 ± 0.8	160 ± 12
HEPT	30.3 ± 1.7	143 ± 16	26.9 ± 0.6	161 ± 18	24.7 ± 0.6	169 ± 11

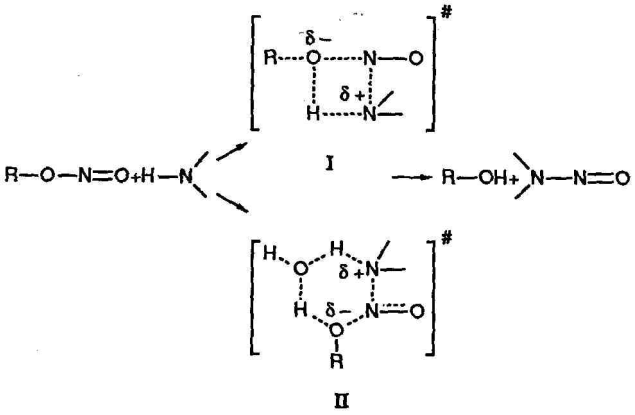
The high negative values of the activation entropy show that this complex must have a high degree of organization.

The determination of an isotope effect on the nitrosation reaction (Table 6) supports the hypothesis of a cyclic structure for the highly ordered intermediates. The decrease observed in the k_{2H}/k_{2D} ratio in the presence of THF in the reaction medium can be viewed as a test of the effect of proton transfer on the mechanism studied.

Table 6 Values of k_{2H}/k_{2D} in the nitrosation of piperidine by PrONO in aqueous and water/THF media, at 25 ºC. $[OH^-] = 0.1$ M; I = 0.25 M, $[PIPER]_o = 1.49 \cdot 10^{-2}$ M; $[PrONO]_o = 7.45 \cdot 10^{-3}$ M.

Reaction medium	k_{2H}/k_{2D}
Water	1.7
Water/THF (1.00 M)	1.3

This would mean that the readily solvated reagents (the amine in particular) would favor proton transfer in the actual formation of the nucleophile/electrophile bond. If this were so, the k_2 values would be higher in water than in water/THF media as, in fact, has been observed. In this context, structures such as **II (**once proposed by Oae *et al.* [11]) would exist in aqueous medium, while structures like **I** would appear in water-organic media. Since structure **I** is more rigid than **II**, the absolute values of $\Delta S^{\#}$ should change when the amount of THF if the reaction medium changes. As we have seen the results (Table 5) are consistent with such a hypothesis.



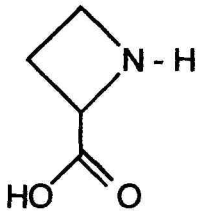
Scheme 2 Transition state in nitrosation by alkyl nitrites.

4. *Intramolecular rearrangements*: Nitrosyl carboxylate as an internal nitrosating agent

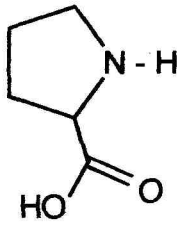
Although studies of the mechanisms of *N*-nitrosation have been numerous, the nitrosation of amino acids (AA) has received relatively little attention, in spite of their

chemical and biochemical relevance.

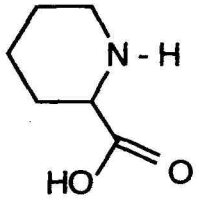
We carried out a systematic kinetic study of the nitrosation of five imino acids and of the ethyl esters of three of them [20-22]. Some of these acids are of interest in the biomedical field or Food Science.



azetidine-2-carboxylic acid

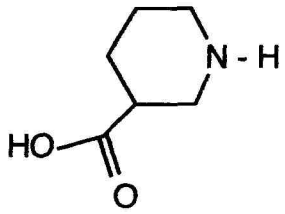


pyrrolidine-2-carboxylic acid



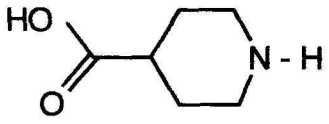
piperidine-2-carboxylic acid (pipecolic acid)

β-amino acid :



piperidine-3-carboxylic acid (nipecotic acid)

γ-amino acid :



piperidine-4-carboxylic acid (isonipecotic acid)

Scheme 3 Nitrosatable amino acids with secondary amino group.

Nitrosation kinetics were obtained by following the formation of nitroso compound by UV spectrophotometry.

Different sets of experiments imply the experimental rate equation:

$$r_o = \left(b[nitrite]_o + c[nitrite]_o^2\right)[\mathrm{AA}]_o \qquad (3.1)$$

Although this equation is the same as the rate equation found for the nitrosation of secondary amines in general, which is thought to involve direct diffusion-controlled attack on the amino nitrogen by N_2O_3 and NO^+, the pH-dependence of the ratio *b* to *c* is not as predicted by this mechanism (Table 7).

Table 7 Experimental values of the ratio *b*/*c* for azetidine-carboxylic acid at various acidities, and theoretical values calculated assuming only direct *N*-nitrosation by N_2O_3 and NO^+.

pH	$10^4 \cdot (b/c)$, **M**	
	Theoretical	Observed
1.50	5.98	1.22
1.78	3.43	1.31
1.95	2.34	1.50
2.05	1.89	1.67
2.10	1.71	1.37
2.35	1.03	1.56
2.40	0.93	1.42
2.80	0.48	1.89
2.85	0.43	2.52
2.95	0.36	2.64
3.05	0.33	3.34
3.20	0.27	4.60
3.35	0.24	5.90

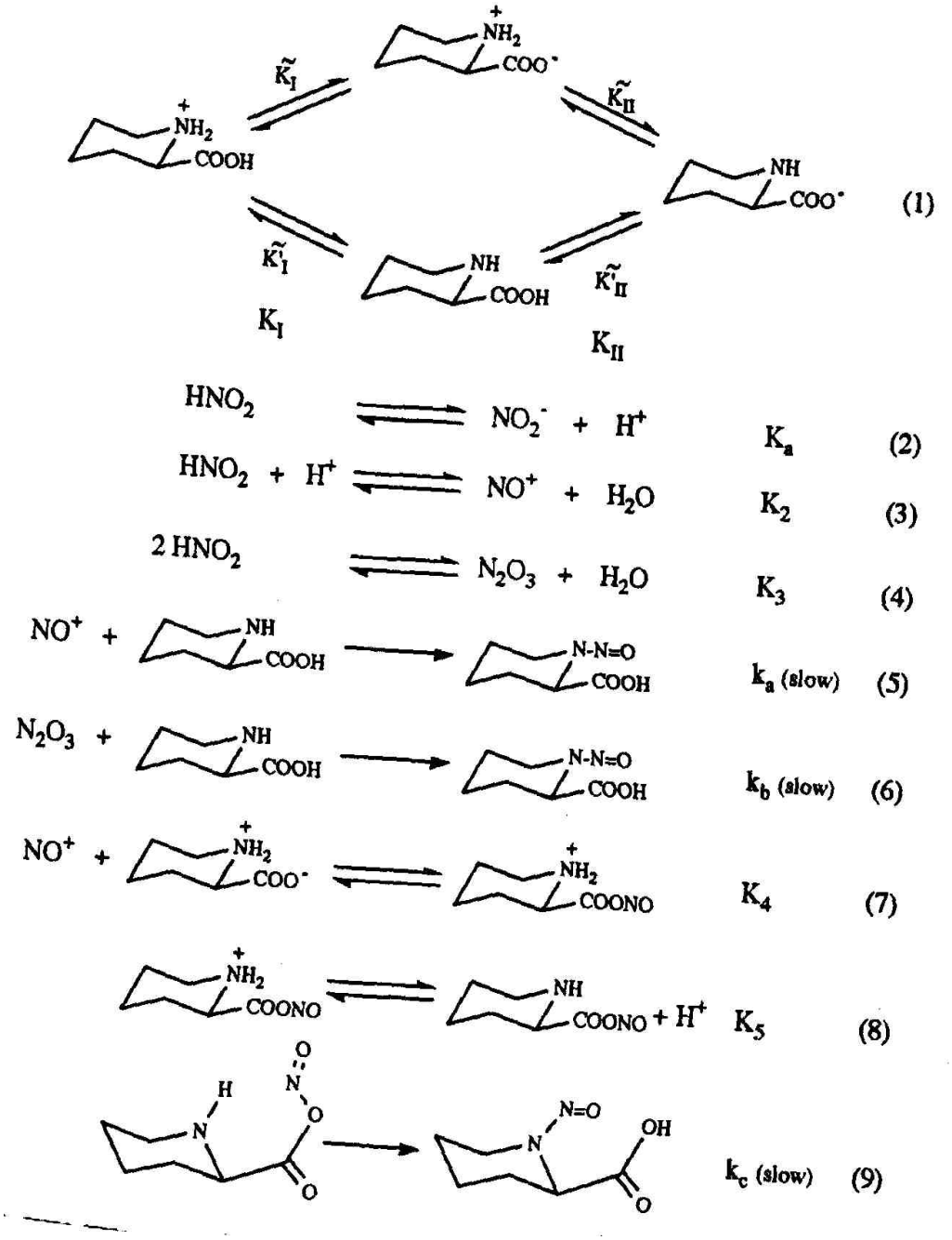
So we put forward an extended mechanism including an intramolecular pathway: formation of a nitrosyl carboxylate followed by the loss of a proton and subsequent intramolecular migration of the -*N*=*O* group.

The application of this mechanism to the pipecolic acid is shown in the Scheme 4 in which the first step represents the protonation equilibria of the amino acid, and steps 7-9 represent the nitrosyl carboxylate route.

This scheme implies the theoretical rate equation 3.2.

$$r_o = \varphi \frac{[\mathrm{Pip}]_o [nitrite]_o^2 [\mathrm{H}^+]_o^2}{\left(K_1 + [\mathrm{H}^+]_o\right)\left(K_a + [\mathrm{H}^+]_o\right)^2} + \eta \frac{[\mathrm{Pip}]_o [nitrite]_o [\mathrm{H}^+]_o^2}{\left(K_1 + [\mathrm{H}^+]_o\right)\left(K_a + [\mathrm{H}^+]_o\right)} + \xi \frac{[\mathrm{Pip}]_o [nitrite]_o [\mathrm{H}^+]_o}{\left(K_1 + [\mathrm{H}^+]_o\right)\left(K_a + [\mathrm{H}^+]_o\right)} \qquad (3.2)$$

in which the first term represents attack by N_2O_3 , the second attack by NO^+, and the third the nitrosyl carboxylate route.



Scheme 4 Intramolecular rearrangement in the nitrosation of amino acids with secondary amino group [21].

Table 8 lists the parameters of that rate equation as calculated by applying Marquardt's non-linear optimization method to all the data.

The *pH*-dependence of the ratio *b/c* calculated using the optimized values of φ, η, and ξ agrees well with the observed pattern (Fig. 5).

Table 8 Optimized values of the parameters φ, η, ξ, and K_I of eq. 3.2.

	$10^4 \cdot \varphi$, $M^{-1}s^{-1}$	$10^6 \cdot \eta$, $M^{-1}s^{-1}$	$10^8 \cdot \xi$, s^{-1}	$10^3 \cdot K_I$, M	pK_I
α-Amino acids					
Azetidine-2-carboxylic acid, Aza	18.3 ± 0.6	NS	26 ± 2	13.0 ± 0.6	1.89
Pyrrolidine-2-carboxylic acid, Pro	17.5 ± 0.9	2.3 ± 0.3	8.7 ± 0.3	9.7 0.7	2.01
Piperidine-2-carboxylic acid, Pip	5.7 ± 0.3	NS	11 ± 3	5.9 ± 0.5	2.23
β-Amino acid					
Piperidine-3-carboxylic acid, Npa	3.0 ± 0.2	7 ± 3	2.4 ± 0.8	7.0 ± 0.8	2.16
γ-Amino acid					
Piperidine-4-carboxylic acid, Ipa	1.42 ± 0.08	NS	NS	8.8 ± 0.8	2.06

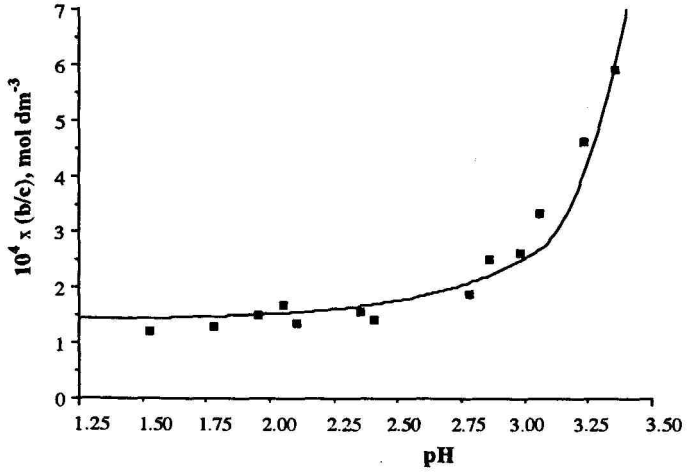


Fig. 5 *pH*-Dependence of the ratio *b*/*c* (eq. 3.1) for azetidine-2-carboxylic acid, as predicted by eq. 3.2 (continuous curve) and found experimentally (▪).

The good agreement between the pK_I calculated and those obtained by non-kinetic methods strongly supports the proposed mechanism.

Further support derives from the close compliance with the Arrhenius law shown by the values of ξ.

When working with the amino acid ethyl esters, instead of amino acids, there can be no nitrosyl carboxylate pathway and so these reactions must involve the standard mechanism for secondary amines which leads to the theoretical rate equation (3.3).

$$r_o = \delta \frac{[AA]_o[nitrite]_o[H^+]_o}{(K_a + [H^+]_o)} + \theta \frac{[AA]_o[nitrite]_o^2[H^+]_o}{(K_a + [H^+]_o)^2} \qquad (3.3)$$

This equation is in keeping with the experimental rate equation, and the optimized values of δ, θ and K_a obtained by fitting it to the experimental data are listed in Table 9.

Table 9 Optimized values for the parameters δ and θ of eq. (3.3).

Ester	$10^4 \cdot \theta$, $M^{-1} s^{-1}$	$10^6 \cdot \delta$, $M^{-1} s^{-1}$	$10^3 \cdot K_a$
EPip	5.3 ± 0.2	NS	0.87 ± 0.03
ENpa	1.48 ± 0.04	2.5 ± 0.4	1.10 ± 0.01
EIpa	0.26 ± 0.01	0.5 ± 0.1	0.84 ±0.04

The good agreement between the value obtained for K_a and the commonly accepted supports the validity of equation 6 and the mechanism from which it was derived.

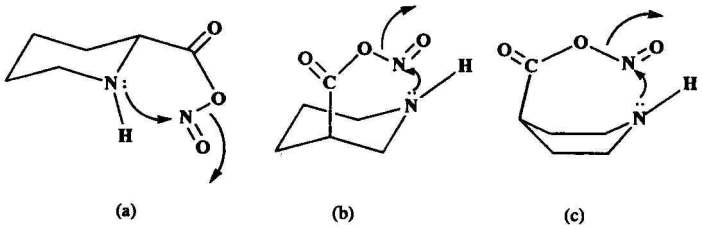
The relative values of φ, η and ξ (Table 8) imply that nitrosation by the intramolecular nitrosyl carboxylate route (represented by ξ) is a minor process in comparison with direct *N*-nitrosation by NO^+ or N_2O_3.

The differences between the various amino acids in this respect are nevertheless interesting, the α-amino acids having ξ values about 5-10 times greater than the β-amino acid while the value of ξ for the γ-amino acid is not significantly different from zero.

These differences can be explained in terms of the stereochemistry of the reaction (Scheme 5). The five-numbered ring formed during the intramolecular transfer of the -*NO* group in the α-amino nitrosylcarboxylates is sterically favored. For the β-amino compound the formation of the necessary six-membered ring involves prior rearrangement of an equatorial conformation to an axial orientation of higher energy. For the γ-amino compound formation of seven-numbered cyclic intermediate requires not only the change in orientation from equatorial to axial, but also the conversion of the piperidine ring from a chair form to a boat conformation with an energy some 20 kJ mol^{-1} higher.

Quantitative support of this explanation is forthcoming if the values of ξ are used in Eyring's equation to calculate the corresponding values of $\Delta G^{\#}$. And hence, by subtraction, the difference in $\Delta G^{\#}$ can be attributed to the transition from the equatorial to the axial orientation of the C_{ring}-$C_{carboxyl}$ bond; the result, 3.8 kJ mol^{-1}, agrees

well with accepted values for this kind of conformational change.



Scheme 5 Intermediates involved in the *N*-nitrosation of Pip (a), Npa (b) and Ipa (c) by the intramolecular *–NO* transfer route [21].

Furthermore, Eyring's equation, together with the estimated 20 kJ mol^{-1} increase in energy associated with conversion from the chair to the boat conformation of piperidine, also shows that the value of ξ for the γ-amino acids must be negligible in comparison with its value for the β-amino acids. Once more this agrees with the experimental observations.

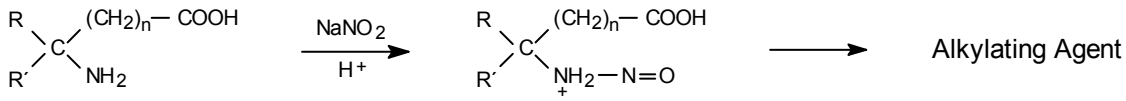
5. *Nitrosation reactions as precursor of alkylation mechanisms*

Regarding the nitrosation of amino acids with -NH_2 group, some results had suggested that mutagenic products arose primarily from nitrosation of the primary amine rather than the amide or indole group [23, 24]. It thus became of interest to identify the alkylating agent as well as its alkylating potential.

This research was carried out in two stages [25-27]: i) Kinetic study of the nitrosation of amino acids; ii) identification of alkylating agents, and investigation of their alkylating potential through their reactions with 4-(*p*-nitrobenzyl)pyridine (NBP) (introduced to trap alkylating agents [28]; NBP has nucleophilic characteristics similar to DNA bases [29]; *vide infra*).

Nitrosation reactions were carried out in aqueous acid conditions (mimicking the conditions of the stomach lumen) while the alkylating potential of the nitrosation products was investigated at neutral *pH*, as in the stomach lining cells into which such products can diffuse.

5.1. ***Nitrosation reactions***. We investigated the nitrosation of six α-amino acids with $NaNO_2$ in acidic media: glycine (Gly), DL-alanine (Ala), DL-α-amino butyric 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 attending 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).

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

$$r = k_{2\,\mathrm{exp}}\,[nitrite]^2 \tag{4.1}$$

Figure 6 shows typical kinetic runs.

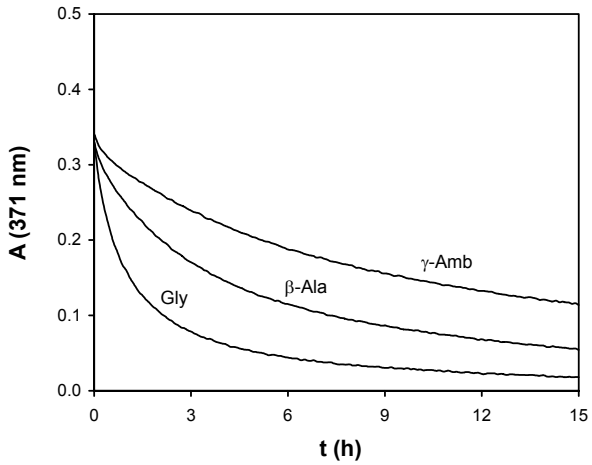


Fig. 6 Variation in the nitrite absorbance with the reaction time in the nitrosation of amino acids.$[\mathrm{Gly}]_o$ = 0.300 M, $[\beta\text{-Ala}]_o$ = 0.300 M and, $[\gamma\text{-Amb}]_o$ = 0.350 M; $[nitrite]_o = 10^{-2}$ M, $[NaH_2PO_4]$ = 0.50 M, I = 1.00 M, *pH* = 3.0, *T* = 298 K.

Figure 7 represents the integrated form of equation (4.1) in terms of absorbance A, A_o being the initial absorbance. The good linear fitting of the A_o/A (λ = 371 nm) values against those of ***t*** reveals second-order with respect to the nitrite concentration, involving dinitrogen trioxide as the main nitrosating agent [8].

Results obtained on working with different initial concentrations of each amino acid showed first-order with respect to the amino acid concentration.

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

$$r = k_{3\,\mathrm{exp}}\,[\text{amino acid}]\,[nitrite]^2 \tag{4.2}$$

Comparison of equations (4.1) and (4.2) yields

$$k_{2\,\mathrm{exp}} = k_{3\,\mathrm{exp}}\,[\text{amino acid}] \tag{4.3}$$

allowing $k_{3\,\mathrm{exp}}$ to be calculated.

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

Figure 8 shows the dependence of $k_{3\ exp}$ on the *pH* of the medium.

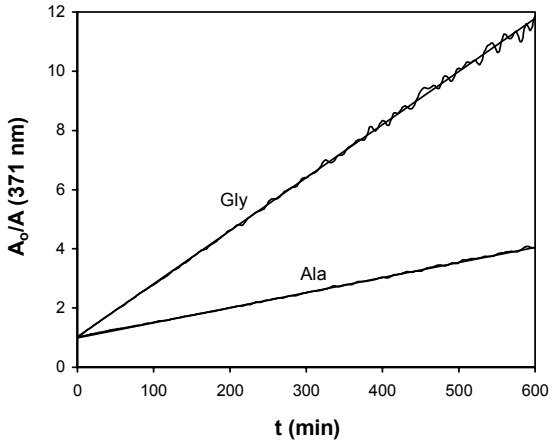


Fig. 7 Integrated form of the second-order rate equation (eq 4.1) for the nitrosation of glycine and alanine.$[Gly]_o$ = 0.300 M, $[Ala]_o$ = 0.210 M; $[nitrite]_o = 10^{-2}$ M, $[NaH_2PO_4]$ = 0.50 M, I = 1.00 M, *pH* = 3.0, *T* = 298 K.

To approach in vivo conditions, nitrosation was carried out in aqueous acid media. 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.

To rationalize the results obtained, the mechanism shown in Scheme 6 was proposed.

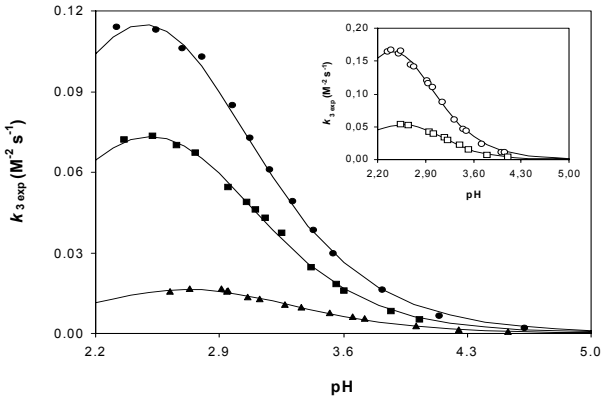
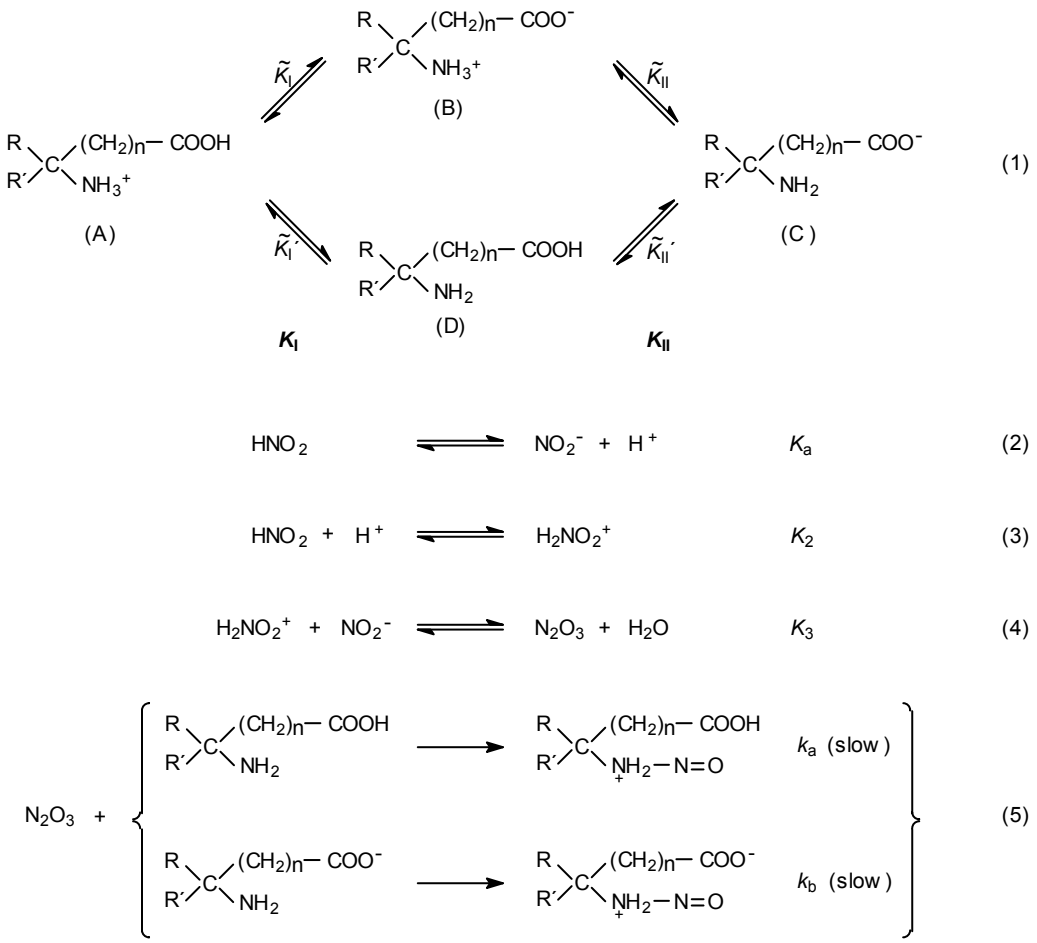


Fig. 8 Fitting of the experimental nitrosation rate constant to the theoretical rate equation.



Scheme 6 Nitrosation mechanism [26].

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 approximately equal to the acidity constant of an ester of the amino acid K_e [30, 31]. 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.

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

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

The reaction rate of nitrosation can be written as:

$$r = k_a\,[D][N_2O_3] + k_b\,[C][N_2O_3] \tag{4.4}$$

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, besides N_2O_3 as a nitrosating agent NO^+ should also be included in the mechanism [20-22]. 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 [*nitrite*] = [HNO_2] + [NO_2^-], it is easy to deduce that:

$$r = k_a K_M K_e \frac{[AA][nitrite]^2[H^+]^2}{(K_I + [H^+])(K_a + [H^+])^2} + k_b K_I K_{II} K_M \frac{[AA][nitrite]^2[H^+]}{(K_I + [H^+])(K_a + [H^+])^2} \tag{4.5}$$

where $K_M = K_a\,K_2\,K_3 = 3.03\cdot10^{-3}$ M^{-1} [9].

Equation (4.5) is consistent with the experimental reaction orders.

Setting $\alpha = k_a\,K_M\,K_e$, $\beta = K_a$, and $\gamma = k_b\,K_I\,K_{II}\,K_M$, equation (4.6) can be written:

$$r = \alpha \frac{[AA][nitrite]^2[H^+]^2}{(K_I + [H^+])(\beta + [H^+])^2} + \gamma \frac{[AA][nitrite]^2[H^+]}{(K_I + [H^+])(\beta + [H^+])^2} \tag{4.6}$$

Comparison of the experimental (eq 4.2) and theoretical (eq 4.6) rate equations gives:

$$k_{3\,exp} = \alpha \frac{[H^+]^2}{(K_I + [H^+])(\beta + [H^+])^2} + \gamma \frac{[H^+]}{(K_I + [H^+])(\beta + [H^+])^2} \tag{4.7}$$

Since for each *pH* the value of $k_{3\,exp}$ is experimentally known, α, β and γ can be calculated by a non-linear optimization algorithm [32].

As examples, Figure 8 shows the good fits of the experimental results to equation (4.7) for α-, β-, and γ-amino acids.

The results obtained showed the following:

i) 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 [21, 22] as well as for the nitrosation of very different amines [3, 4].

ii) The values of the rate coefficient k_a, follow the sequence of: 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 of the *–COOH* increasing the nucleophilicity of the $–NH_2$ group in the following order: α-amino acids < β-amino acids < γ-amino acids.

iii) 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 [33] (the possibility of using steric hindrance for blocking or inhibiting nitrosation by N_2O_3 has been discussed in the literature [34]).

iv) The observed fact that k_b values are always higher than those of k_a is consistent with the electron-donating (+I) effect 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 [35], the existence of an isokinetic relationship can serve as an argument -but not proof- that the reactions studied share a common feature.

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 [36].

The results shown in Figure 9 support the idea of a common mechanism.

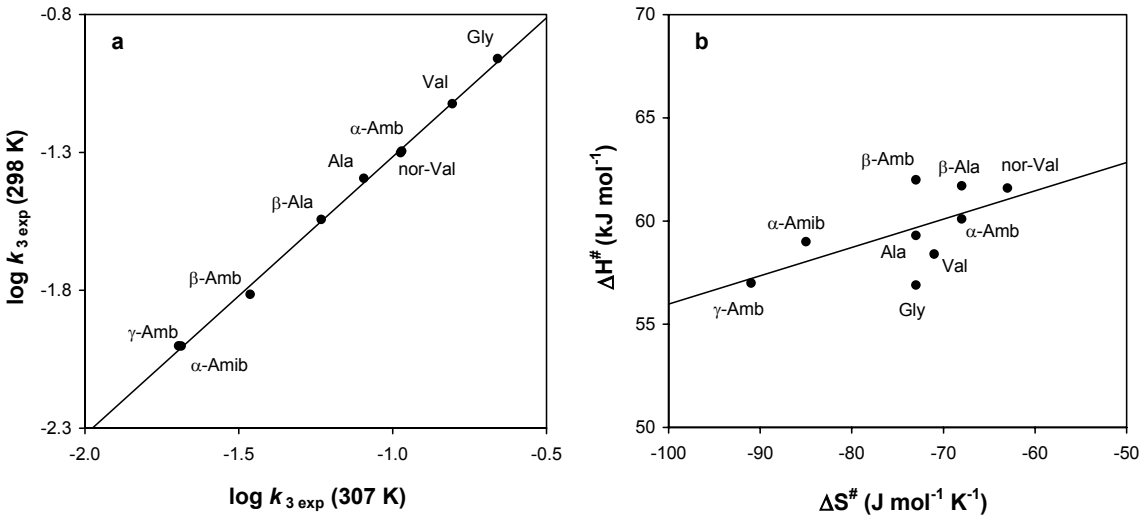


Fig. 9 Isokinetic relationship in the nitrosation of amino acids.
(a) Diagram log k (T_2) / log k (T_1); (b) Compensation effect $\Delta H^{\#}/\Delta S^{\#}$.

5.2. ***Alkylation reactions***. Formation of the adduct between NBP and the alkylating agent resulting from the nitrosation was studied as a function of the nitrosation time at different alkylation times [25, 26].

Table 10 shows the wavelength of maximum absorption for each adduct, and reveals important differences between the nitrosation and alkylation times that lead to the maximum concentration of the adduct. The nitrosation times required for the maximum adduct concentration to be reached follow the sequence: α-amino acids < β-amino acids < γ-amino acids, which is coherent with the k_{3exp} values found in the nitrosation reaction (Table 11).

Since the alkylation time necessary for the maximum adduct concentration to be reached is very different, depending on whether the precursor is α-, β- or γ-amino acid, a study was made of the alkylating potential of the nitrosation products of aspartic acid, which has two acid groups at positions α and β with respect to the amino group.

As may be seen from Table 10, the nitrosation and alkylation times corresponding to aspartic acid are similar to those obtained with the β-amino acids. This means that the carboxyl group located at the β position with respect to the amino group exerts a greater influence than that situated at the α position.

Table 10 Alkylating potential of the amino acid nitrosation products
Nitrosation reactions ($pH \approx 3.5$), $[\text{Amino acid}]_o = 0.060$ M, $[NaNO_2]_o = 0.060$ M, $[NaH_2PO_4] = 0.50$ M, I = 1.00 M ($NaClO_4$), T = 298 K. Alkylation reactions ($pH \approx 7$), $[NBP]_o = 0.116$ M, $T = 298$ K.

Amino acid	λ_{max}, nm	$t^*_{\text{nitrosation}}$, min	$t^*_{\text{alkylation}}$, min
Glycine	562	100	20
Alanine	521	60	10
α-Aminobutyric acid	525	60	7
α-Aminoisobutyric acid	550	very long	≈60
Valine	532	100	10
Norvaline	525	40	10
β-Alanine	587	150	125
β-Aminobutyric acid	588	long	very long
Aspartic acid	598	300	400
γ-Aminobutyric acid	585	long	very long

* Times for maximum adduct concentration to be reached.

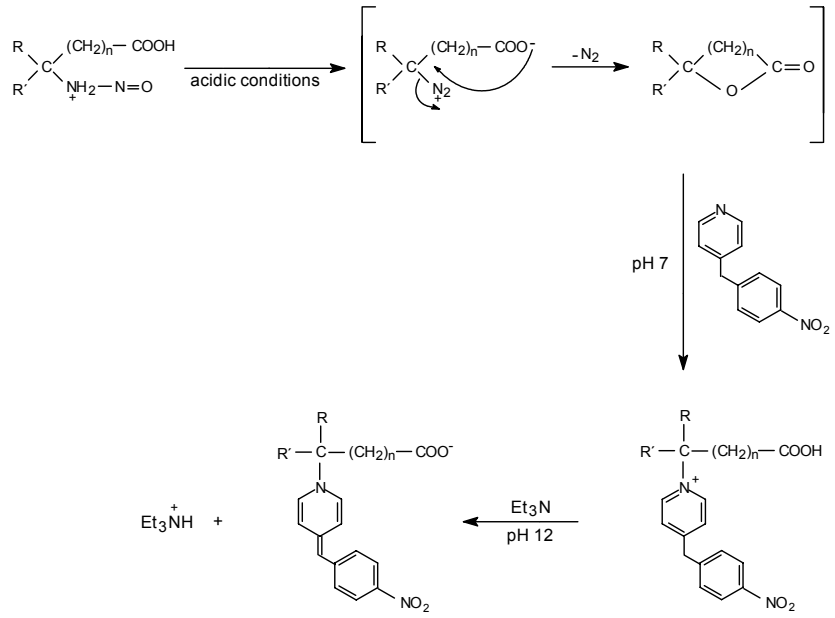
Table 11 Nitrosation rate of amino acids.

Amino acid	*pH*	$10^2 \cdot k_{3\,\text{exp}}$, $M^{-2}\,s^{-1}$
Glycine	3.00	11.3 ± 0.3
Alanine	3.05	3.91 ± 0.09
α-Aminobutyric acid	3.05	4.8 ± 0.1
α-Aminoisobutyric acid	3.00	0.87 ± 0.04
Valine	3.05	7.6 ± 0.2
Norvaline	3.03	5.1 ± 0.4
β-Alanine	3.00	2.80 ± 0.08
β-Aminobutyric acid	3.05	1.34 ± 0.02
γ-Aminobutyric acid	3.14	0.89 ± 0.03

Scheme 7 shows the proposed mechanism. Following nitrosation, the corresponding diazocompound is formed, which rapidly loses N_2.

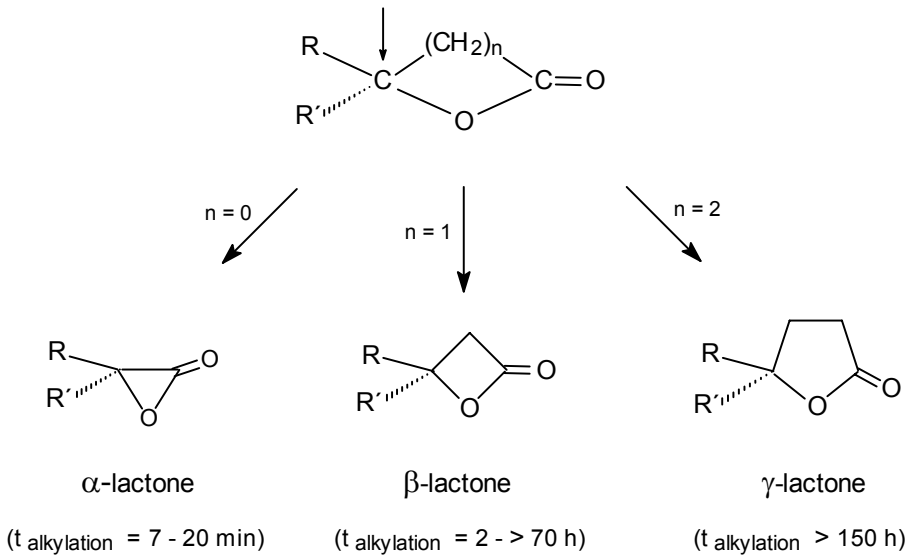
The times required for the maximum concentration of adduct to be reached (Table 10) rules out the hypothesis of a carbocation as the alkylating agent. We thought in terms of a product resulting from its cyclization, such as lactones.

On the basis of the tricentric, tetracentric and pentacentric structures of the lactones corresponding to the α-, β- and γ-amino acids (Scheme 8), it is to be expected that the reactivity of such lactones would decrease when their size increases. That is, the alkylation rate would decrease in the same order i.e., their alkylation time would increase, as was in fact observed (Table 10).



Scheme 7 Alkylation mechanism [25].

These results are consistent with those reported in biomedical research: β-propiolactone and four-membered ring lactones are generally, but not always, active carcinogens. β-Propiolactone (in our case resulting from the nitrosation of β-alanine) has been extensively investigated and has found to be a potent, though slow-acting, carcinogen [37]. Unlike the β-lactones, γ-lactones are not inherently reactive because of their lack of ring strain. This lack of reactivity of γ-butyrolactone is therefore in accordance with its inactivity as a carcinogen [37].



Scheme 8 Lactones resulting from nitrosation of α-, β- y γ-amino acids; arrow ↓ shows electrophilic center; $\mathbf{t}_{alkylation}$ corresponds to the maximum adduct concentration [25].

The times of alkylation by α-lactones are shorter than those corresponding to β- and γ-lactones (Scheme 8) owing to ring stability. All the lactones derived from α-amino acids have similar alkylating times, with the exception of the lactone deriving from the nitrosation of α-aminoisobutyric acid, with a slightly longer alkylation time. The cause of this must lie in the fact that in this case there are two methyl groups bound directly to the electrophilic carbon atom that provide charge density.

For the reasons mentioned above, the β-amino acids have longer alkylation times than the α-amino acids. Upon comparing the alkylation due to the β-lactones coming from β-alanine and β-aminobutyric acid, it may be seen that it is much slower in the case of the latter; the reason for this may lie in the presence of the methyl group adjacent to the electrophilic carbon, thus providing charge density.

In the case of aspartic acid, the alkylation time observed -similar to that of β-amino acids- is coherent with the formation of a lactone with the intervention of the carboxyl group located at β-position with respect to the amino group, less strained than the lactone formed with the α-carboxyl group.

Following its nitrosation, the γ-amino acid studied leads to a highly stable γ-lactone and, as a result, is only sparingly reactive in both chemical and biological terms.

With a view to checking experimentally that the lactones were indeed responsible for the alkylation, they were identified. Owing to their greater stability the β- and γ-lactones were chosen.

Amino acids and $NaNO_2$ were made to react in acid medium. After a sufficient nitrosation time for a significant concentration of alkylation reagent to be reached, extraction was carried out with $CHCl_3$ (β-lactones) or CH_2Cl_2 (γ-butyrolactone), separating the organic phase, which was then washed with a saturated solution of NaCl and dried with anhydrous Na_2SO_4. This was then filtered and the solvent evaporated off under reduced pressure.

Identification of β-propiolactone. The ^1H NMR spectrum of the nitrosation products of β-alanine revealed the following signals: (200 MHz; $CDCl_3$) δ (ppm): 4.28 (2H, t, J = 5.8 Hz), 3.55 (2H, t, J = 5.8 Hz), attributed to β-propiolactone owing to their agreement with those reported in the literature [38].

Identification of β-butyrolactone. The ^{1}H NMR spectrum of the nitrosation products of β-aminobutyric acid revealed the following signals: ^{1}H NMR (200 MHz; $CDCl_3$) δ (ppm): 4.70 (1H, m, H-4), 3.57 (1H, dd, *J* = 16.5 Hz, *J* = 6.0 Hz, H-3), 3.07 (1H, dd, *J* = 16.5 Hz, *J* = 3.6 Hz, H-3), 1.57 (3H, d, *J* = 6.0 Hz, CH_3), which are in agreement with the values found in the literature for β-butyrolactone [39, 40].

Identification of γ-butyrolactone. The IR spectrum of the nitrosation products of γ-aminobutyric acid was obtained; this featured a band centered at 1771 cm^{-1}.

The lactones with a pentagonal ring show a band corresponding to the *C=O* group at 1760-1780 cm^{-1}. In particular, γ-butyrolactone shows a band at 1770 cm^{-1} [38, 41] which can be identified with the one observed by us.

The ^{1}H NMR spectrum of the nitrosation products of γ-aminobutyric showed the following signals: (200 MHz; $CDCl_3$) δ (ppm): 4.34 (2H, t, *J* = 7 Hz), 2.49 (2H, t, *J* = 7.7 Hz), 2.26 (2H, q, *J* = 7, *J* = 7.7 Hz), in agreement with those found in the literature [38, 41].

Further proof in favor of the hypothesis that the lactones are indeed the alkylating agents was obtained when we reacted commercial β-butyrolactone with NBP, affording an analogous spectrum (λ_{max} = 587 nm) to that observed in the alkylating reaction of NBP with the nitrosation products of β-Amb (it should also be mentioned that a β−lactone was obtained by reaction of aspartame, L-aspartyl-L-phenylalanine methyl ester, with $NaNO_2$ in a biphasic reaction solution of aqueous HCl and dichloromethane [42]).

This study [25, 26] provided the following conclusions:

i) The alkylating species resulting from the nitrosation of amino acids with an $–NH_2$ group are the corresponding lactones.

ii) The sequence of alkylating potential is: α-lactones > β-lactones > γ-lactones coming, respectively, from the nitrosation of α-, β- and γ-amino acids, *i.e.* the most common natural amino acids are the precursors of the most powerful alkylating agents.

iii) In view of the existence of a correlation between the mutagenic index of nitrosation products and the alkylating potential index [29], the results obtained here could be useful in predicting the mutagenic effectiveness of the nitrosation products of amino acids since NBP has nucleophilic characteristics similar to those of DNA bases.

Since some lactones give alkylating reactions with any of a number of nucleophilic sites in tissues, a study was performed with two main objectives: i) To gain quantitative knowledge about the lactones' reactivity and to relate this to their carcinogenic activity; ii) to gain insight into the formation of γ-hydroxy butyric acid (GHB) in the hydrolysis of γ-butyrolactone.

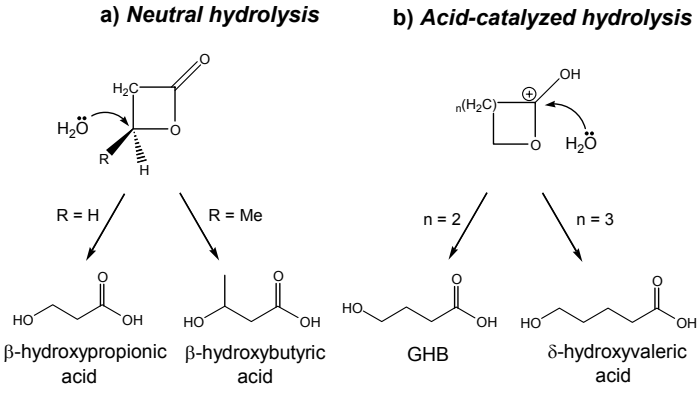
γ-Butyrolactone is used widely as an industrial solvent and is marketed as a dietary supplement. Since GHB is a drug of abuse and is also used as a dietary supplement claimed to improve physical performance and to reduce stress [43], investigation of its formation is of biological/biochemical interest.

The behavior of lactones in their hydrolysis reactions is a good indicator of their reactivity as electrophilic molecules.

Our investigation was carried out in two stages: i) A kinetic study of the hydrolysis reactions of β-propiolactone (BPL), β-butyrolactone (BBL), γ-butyro lactone (GBL), and δ-valerolactone (DVL); ii) a kinetic and thermodynamic study of the formation of GHB [44].

6. *Reactivity of lactones*

6.1. ***Neutral hydrolysis: Water reactions.*** We investigated the hydrolysis of β-propiolactone and β-butyrolactone (Scheme 9).



Scheme 9 Hydrolysis of lactones.

Experiments designed to determine the influence of the lactone concentration revealed the reactions to be first-order with respect to this reagent. Thus:

$$r = k\,[H_2O]\,[L] = k_1\,[L] \qquad (4.8)$$

where [L] represents the concentration of lactone and $k_1 = k\,[H_2O]$ is the pseudo-first-order rate constant.

Figure 10 represents the integrated form of eq 4.8 in terms of $[L]_t$, the concentration of lactone at time ***t***, $[L]_o$ being the initial concentration.

Table 12 gives the k_{BPL} and k_{BBL} rate constants (as k in eq 4.8) for the hydrolysis reactions of BPL and BBL, respectively, as a function of temperature.

The results given in Table 12 show that the rate constants are more than four times shorter for β-butyrolactone than for β-propiolactone.

Table 13 shows the values of the activation parameters $\Delta H^{\#}$ and $\Delta S^{\#}$ for both hydrolysis reactions.

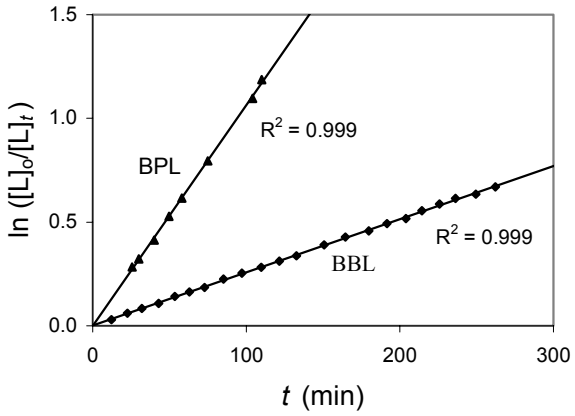


Fig. 10 Integrated form of the pseudo-first-order rate equation (eq 4.8) for the hydrolysis of β-propiolactone and β-butyrolactone. $[L]_o$ = 0.08 M, T = 35 ºC.

With the aim of gaining deeper insight into the transition state, the hydrolysis of BPL and BBL in water/dioxane was investigated. Tables 13 and 14 show the results obtained.

The very similar $\Delta S^{\#}$ values found for the hydrolysis of BPL and BBL (Table 14) are consistent with the analogous geometry of these lactones. They also show that the reactivity of these β-lactones is mainly enthalpy-controlled.

The greater $\Delta H^{\#}$ value obtained for the hydrolysis of β-butyrolactone must be caused by the methyl group as a donor of charge on the β-carbon, with a decrease in its electrophilic character (Scheme 9a). Since in general terms substitution on carbon atoms -2 or -3 in lactones results in a decrease or loss in carcinogenic activity [45], the steric hindrance of the BBL methyl group must also contribute to the higher value of $\Delta H.^{\#}$

It can be observed that when the percentage of dioxane increased: i) The rate constant decreased appreciably; ii) the enthalpy of activation became smaller, and iii) the absolute value of the entropy of activation increased.

Table 12 Rate constants[a] as a function of temperature for the neutral hydrolysis of β-propiolactone and β-butyrolactone.

T (ºC)	$10^7 \cdot k_{BPL}$	$10^7 \cdot k_{BBL}$
	$M^{-1} s^{-1}$ [b]	
15.0	3.54 ± 0.05	-
17.5	4.30 ± 0.04	0.94 ± 0.01
22.5	7.6 ± 0.4	1.82 ± 0.01
25.0	10.6 ± 0.1	2.22 ± 0.03
27.5	14.9 ± 0.3	3.63 ± 0.02
30.0	20.2 ± 0.7	4.38 ± 0.05
32.5	27.4 ± 0.9	5.89 ± 0.08
35.0	31.9 ± 0.9	7.69 ± 0.07

$[BPL]_o$ = 0.08 M; $[BBL]_o$ = 0.08 M [a]As k in eq 4.8. [b]Values of rate constants are given within the 95% confidence interval.

Table 13 Rate constants[a] as a function of the composition of the media and the temperature for the neutral hydrolysis of β-propiolactone and β-butyrolactone.

	Water/Dioxane (vol. ratio)							
T	8/2		6/4		5/5		4/6	
(ºC)	$10^7 \cdot k_{BPL}$	$10^7 \cdot k_{BBL}$	$10^7 \cdot k_{BPL}$	$10^7 \cdot k_{BBL}$	$10^7 \cdot k_{BPL}$	$10^7 \cdot k_{BBL}$	$10^7 \cdot k_{BPL}$	$10^7 \cdot k_{BBL}$
	$M^{-1}s^{-1}$[b]							
25.0	10.3 ± 0.3	1.75 ± 0.01	6.84 ± 0.07	0.87 ± 0.01	5.1 ± 0.1	0.61 ± 0.01	3.43 ± 0.04	0.387± 0.008
27.5	13.40 ± 0.06	2.34 ± 0.01	8.46 ± 0.07	1.20 ± 0.01	7.02 ± 0.03	0.81 ± 0.02	4.50 ± 0.01	0.500± 0.007
30.0	17.5 ± 0.4	3.10 ± 0.03	11.8 ± 0.2	1.55 ± 0.02	8.6 ± 0.3	1.09 ± 0.01	5.66 ± 0.08	0.67 ± 0.02
32.5	23.2 ± 0.6	4.10 ± 0.04	14.7 ± 0.4	2.09 ± 0.03	11.0 ± 0.5	1.39 ± 0.01	7.31 ± 0.03	0.87 ± 0.01
35.0	30.4 ± 0.9	5.41 ± 0.05	18.9 ± 0.5	2.63 ± 0.06	14.6 ± 0.3	1.85 ± 0.01	9.3 ± 0.1	1.11 ± 0.03

$[BPL]_o$ = 0.08 M; $[BBL]_o$ = 0.08 M; [a]As k in eq 4.8. [b]Values of rate constants are given within the 95% confidence interval.

Mixed solvents behave regularly only insofar as the components are similar; thus, they can be characterized by the bulk relative permittivity, which is a monotonic function of component permittivities. If one solvent is protic and highly structured, its structure is broken by a foreign solvent and the solvation power diminishes rapidly, increasing the disorder of the protic solvent. This latter being the current case: i) The increase in the dioxane percentage relaxing the intermolecular *O···H* hydrogen bonds

in the ordered structure of the water increases its hydrolytic activity by reducing $\Delta H^{\#}$ (see Table 14); ii) as a consequence of the considerable disorder induced by the dioxane in the reagent water, $-\Delta S^{\#}$ values should decrease when increasing those of the water/dioxane ratio, as was also observed (Table 14).

Table 14 Activation parameters as a function of the composition of the media for the neutral hydrolysis of β-propiolactone and β-butyrolactone.

Water/Dioxane	$\Delta H^{\#a}$, kJ mol^{-1}		$-\Delta S^{\#a}$, J K^{-1} mol^{-1}	
(vol. ratio)	BPL	BBL	BPL	BBL
10/0	83 ± 2	88 ± 3	80 ± 8	78 ± 10
8/2	80 ± 1	84 ± 1	90 ± 3	94 ± 1
6/4	76 ± 3	82 ± 2	106 ± 10	105 ± 7
5/5	75 ± 3	82 ± 1	112 ± 9	109 ± 4
4/6	73 ± 1	79 ± 2	123 ± 3	122 ± 6

[a]Values are given with their standard deviations.

Since the strong effect of the dioxane on the kinetic parameters is a consequence of a strong lactone-water interaction, the enthalpy and entropy will both tend to be linear functions of the volume fraction of that solvent, and an isokinetic relationship should result [46]. Figure 11 shows that this effect was indeed observed.

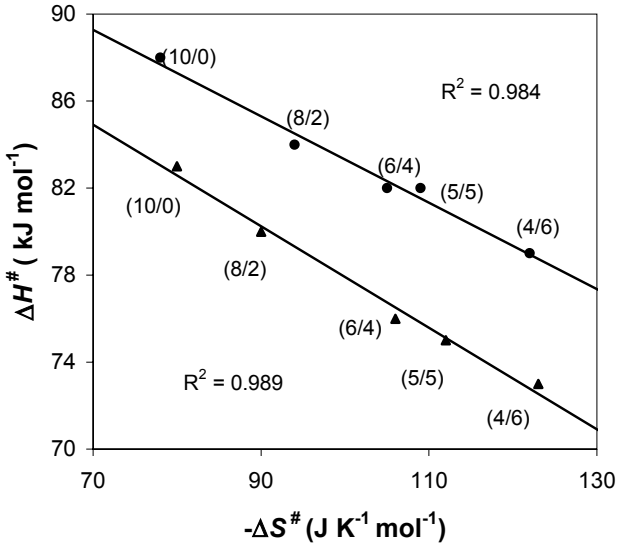


Fig. 11 Isokinetic relationship in the hydrolysis of β-propiolactone (▲) and β-butyrolactone (●) in water/dioxane media.

Because the tumorigenicity of lactones would be correlated with their alkylating capacity on nucleophilic substrates [37, 45], the greater reactivity of β-propiolactone would imply a greater carcinogenic potential. The results are consistent with such a conclusion.

6.2. ***Acid-catalyzed hydrolysis.*** Under the same conditions as for BPL and BBL, no neutral hydrolysis reactions of GBL and DVL were observed.

The loss of reactivity of GBL and DVL at neutral *pH* is in agreement with their inactivity as carcinogens [37] as well as with our previous results concerning the reactivity of lactones resulting in the nitrosation of amino acids [25, 26]. The alkylation time of NBP by β-lactones generated in the nitrosation of β-amino acids was $\boldsymbol{t}_{\text{alkylation}}$ = 2 - > 70 h, with $\boldsymbol{t}_{\text{alkylation}}$ > 150 h for the γ-butyrolactone derived from the nitrosation of γ-amino butyric acid.

Upon carrying out the reactions in weak hydrochloric acid solutions, the following results were obtained.

Since the hydrolysis reaction of GBL is reversible, the first-order rate constant for the forward reaction ($\vec{k}_1 = \vec{k}_{\text{GBL}}\left[\text{H}_3\text{O}^+\right]$, $\vec{k}_{\text{GBL}}$ being the catalytic constant) is given by

$$\vec{k}_1 = \frac{1}{t}\frac{\left[\text{GHB}\right]_{\text{eq}}}{\left[\text{GHB}\right]_{\text{o}}}\ln\frac{\left[\text{GHB}\right]_{\text{eq}}}{\left[\text{GHB}\right]_{\text{eq}} - \left[\text{GHB}\right]} \qquad (4.9)$$

where $[\text{GBL}]_{\text{o}}$ represents the initial lactone concentration, and $[\text{GHB}]_{\text{eq}}$ and [GHB] are the concentrations of the hydroxy acid at equilibrium and at time ***t***, respectively.

Figure 12a shows a typical kinetic run, allowing one to know $[\text{GHB}]_{\text{eq}}$ as the plateau value of [GHB]. Figure 12b shows the good fit of the results to eq 4.9.

In order to check the hydrolysis of GBL occurring through an acid-catalyzed mechanism, experiments at different *pH* values were carried out. The slope value of α = 1.00 in Figure 13 is evidence of this.

The values of $\vec{k}_{\text{GBL}}$ at different temperatures are reported in Table 15.

With the experimental value of $[\text{GHB}]_{\text{eq}}$, knowledge of K_{eq}, the concentration equilibrium constant of the hydrolysis of GBL,

$$K_{\text{eq}} = \vec{k}_{\text{GBL}} / \overleftarrow{k}_{\text{GHB}} = [\text{GHB}]_{\text{eq}}/[\text{GBL}]_{\text{eq}} = [\text{GHB}]_{\text{eq}} / ([\text{GBL}]_{\text{o}} - [\text{GHB}]_{\text{eq}}) \qquad (4.10)$$

is straightforward ($\vec{k}_{\text{GBL}}$ and $\overleftarrow{k}_{\text{GHB}}$ represent the second-order forward and backward rate constants, respectively).

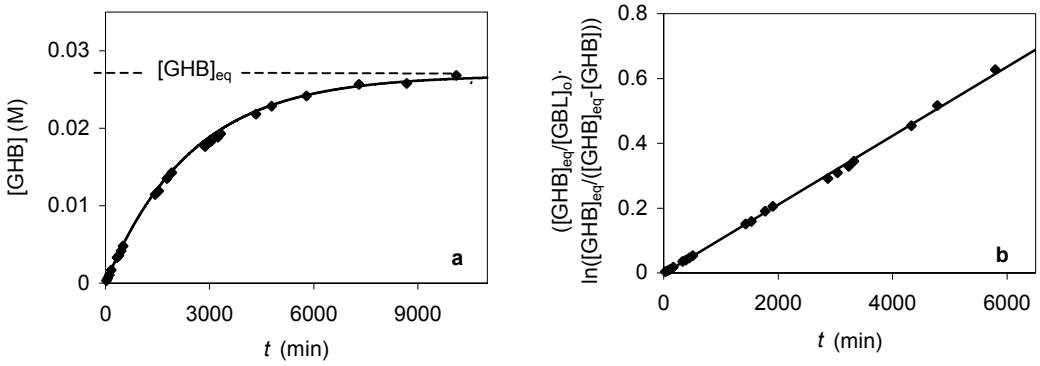


Fig. 12 Formation of GHB in the acid hydrolysis of γ-butyrolactone. (a) Variation in [GHB] with the reaction time; (b) Integrated form of the rate equation (eq 4.8). $[GBL]_o$ = 0.1 M, [HCl] = $1.18 \cdot 10^{-2}$ M, T = 20 ºC.

Table 15 Rate constants as a function of temperature for the acid-catalyzed hydrolysis of γ-butyrolactone and δ-valerolactone.

T (ºC)	$10^4 \cdot \vec{k}_{GBL}$	$10^2 \cdot \vec{k}_{DVL}$
	$M^{-1} s^{-1}$ [a]	
15.0	0.99 ± 0.01	1.97 ± 0.03
17.5	1.27 ± 0.01	2.35 ± 0.06
20.0	1.55 ± 0.03	2.76 ± 0.05
22.5	1.98 ± 0.03	3.24 ± 0.05
25.0	2.40 ± 0.02	4.06 ± 0.07
27.5	3.11 ± 0.05	4.7 ± 0.2
30.0	3.82 ± 0.04	5.4 ± 0.1
32.5	4.95 ± 0.07	6.7 ± 0.2
35.0	6.22 ± 0.06	7.3 ± 0.2

$[GBL]_o$ = 0.1 M; $[DVL]_o$ = 0.1 M $[HCl]_{GBL}$ = $1.18 \cdot 10^{-2}$ M; $[HCl]_{DVL}$ = $4.72 \cdot 10^{-3}$ M. [a]Values of rate constants are given within the 95% confidence interval.

Regarding DVL, the experiments revealed that the reactions were first-order with respect to this reagent: rate = k_{DVL} $[H_3O^+]$ [DVL]. The results obtained with this lactone are summarized in Tables 15 and 16, and show that: i) The rate constant of the acid-catalyzed hydrolysis of DVL (k_{DVL}) is two-orders of magnitude higher than that (k_{GBL}) of GBL hydrolysis; ii) the Gibbs energy of activation for DVL hydrolysis (81 kJ mol^{-1}; 25 ºC) is lower than that seen for GBL (94 kJ mol^{-1}; 25 ºC). The data reveal

that the activity of DVL as an electrophilic reagent is greater than that of GBL; so much so that the hydrolysis of the latter is a clearly reversible reaction.

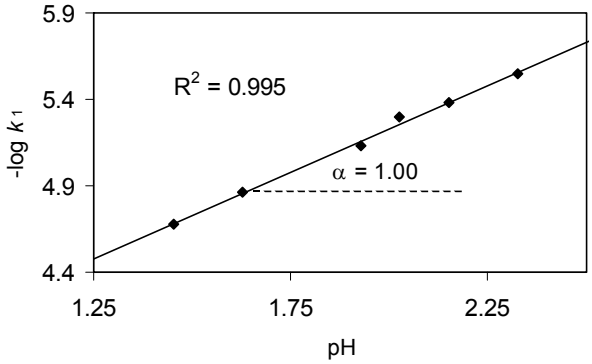


Fig. 13 Variation in the GBL hydrolysis rate constant with the acidity of the medium. $[GBL]_o = 0.1$ M, $T = 35$ ºC.

Another piece of information consistent with the notion of acylic cleavage is the solvent kinetic isotope effect, SKIE. Table 17 shows this effect to be inverse. This is indicative of pre-equilibrium protonation of the lactone molecule prior to the reaction, as would be anticipated for an acid-catalyzed mechanism [47]. Brown *et al.* [48] reported a number of examples of transition states for acid acyl-cleavage mechanisms with inverse solvent effects (it should be noted that SKIE was not observed in the non-catalyzed hydrolysis of BPL and BBL).

Table 16 Activation parameters for the acid-catalyzed hydrolysis of γ-butyrolactone and δ-valerolactone.

Lactone	$\Delta\vec{H}^{\#a}\left(\Delta\bar{H}^{\#}\right)^b$ **kJ mol^{-1}**	$\Delta\vec{S}^{\#a}\left(\Delta\bar{S}^{\#}\right)^b$ **J K^{-1} mol^{-1}**
γ-Butyrolactone	65 ± 1 (61 ± 1)	96 ± 3 (100 ± 4)
δ-Valerolactone	47 ± 1	113 ± 5

[a]Values are given with their standard deviations.
[b]Magnitudes within parentheses correspond to the back reaction.

Table 17 Solvent kinetic isotope effect in the acid-catalyzed hydrolysis of lactones.

Lactone	k_{H_2O}/k_{D_2O}
γ-Butyrolactone	0.67
δ-Valerolactone	0.41

Comparison of the results from the study of neutral and acid-catalyzed reactions provided the following conclusions:

i) Lower $\Delta H^{\#}$ values for the acid-catalyzed reactions. The acyl-oxygen fission route being the normal path, the presence of H^{+} ions enhancing the electrophilic character of the carbonyl carbon should imply lower $\Delta H^{\#}$ values, as was observed.

ii) More negative $\Delta S^{\#}$ values for the acid-catalyzed reactions. In the acyl-fission mechanism, cleavage of the *C-O* bond is not required in the transition state. In addition, nucleophilic attack by water on the lactone molecule occurs on the previously protonated carbonyl *C*-atom, i.e., a C^{+}-atom. This involves a compact tetrahedral intermediate in the case of the acid-catalyzed hydrolysis, *i.e.*, a more negative $\Delta S^{\#}$ value (Table 16).

At molecular level, there are arguments that rationalize the kinetic and thermodynamic parameters found in the acid-catalyzed hydrolysis of GBL and DVL. Some of the major effects of protonation are a change in the direction of the dipole component at the carbonyl oxygen (Fig.14a) and a contraction of the lone pair orbitals (Fig. 14b) [49]. Accordingly, the hydrolytic encounter between the protonated lactones and water molecules will be favored by diminishing intermolecular lone pair-lone pair repulsion. This should cause a lower enthalpy of activation and a more negative entropy of activation in the reactions occurring with protonation of the carboxyl carbon, as was observed (Table 16).

Study of the reactions of triethyloxonium ion with GBL and DVL to form the ethylated lactone has shown [49] that the basicity of DVL is 13 kJ mol^{-1} greater than that of GBL. Ab initio calculations [49] also reveal that the proton affinity of DVL is 8 kcal mol^{-1} greater than that of GBL. This allows one to assume a lower Gibbs energy of activation for DVL catalyzed-hydrolysis reaction than that for GBL, as was in fact observed.

It may be concluded that the kinetic and thermodynamic results can be used as a criterion to discern between mechanisms occurring through alkyl-oxygen or acyl-oxygen cleavage.

When we studied the hydrolysis of BPL and BBL, the possibility of acid-catalyzed hydrolysis was also investigated. Results showed that, in the working conditions, that catalyzed hydrolysis can be discarded.

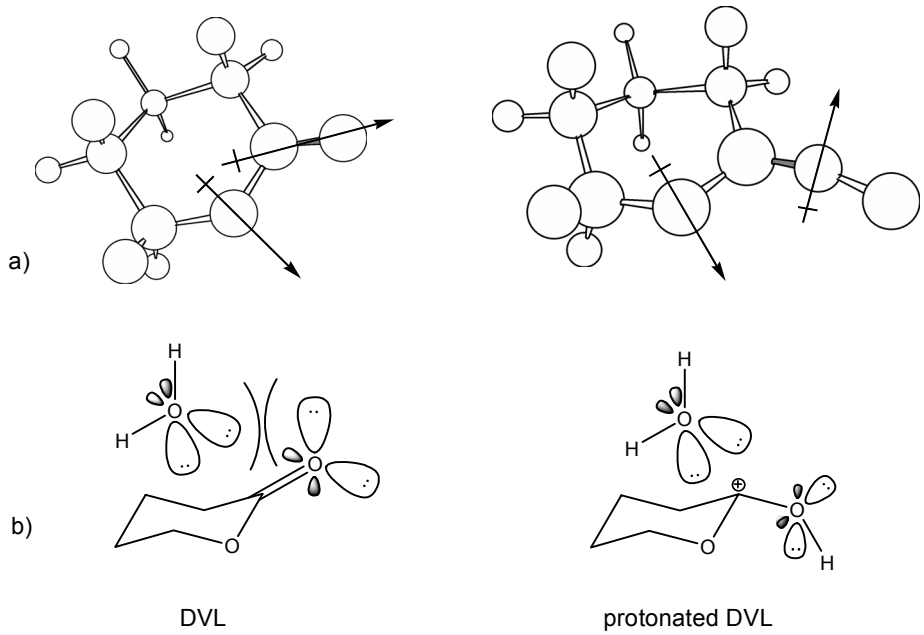


Fig. 14 (a) Orientation of the dipoles at oxygen for unprotonated and protonated δ-valerolactone. In the protonated molecule, the dipoles' orientation is more favorable; (b) contraction of the lone pair orbitals leading to decreased lone pair-lone pair repulsion.

6.3. ***Formation of GHB: Heat of reaction.*** Since the fraction of lactone and hydroxy acid existing at equilibrium is dependent on the relative thermodynamic stability of the acid and its precursor lactone, the concentration equilibrium constant K_{eq} was measured.

With eq 4.10, K_{eq} values were determined at different temperatures. Table 18 shows the results obtained by using the van't Hoff equation.

From the equilibrium constants of Table 8, $\Delta_r H$ for the hydrolysis reaction is calculated to be $\Delta_r H$ = 3.6 ± 0.3 kJ mol.$^{-1}$ This result shows that the acid-catalyzed hydrolysis of GBL to form GHB is an endothermic reaction (Scheme 10).

Such a conclusion is contrary to that of Coffin and Long [50], who reported a value of $\Delta_r H$ = -710 cal (≅ -3 kJ mol^{-1}), implying that the reaction would be exothermic.

Because the kinetic experiments were carried out under *pH* conditions comparable to those existing in the stomach, from the data in Tables 15 and 16 it can be deduced that at human body temperature the amount of GBL converted into GHB is less than 3% after two hours (as shown in Fig. 12a, one week is necessary for the equilibrium situation to be reached). This means that when ingested GBL flows into the blood in the non-hydrolyzed, non-opened form.

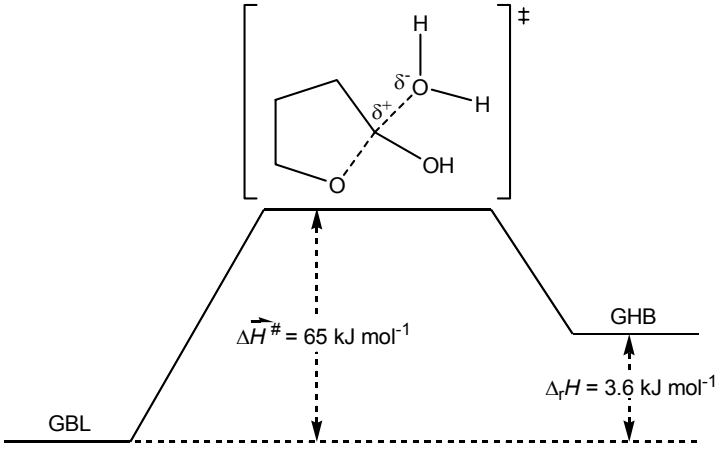
Although GBL is rapidly hydrolyzed in the blood by a γ-lactonase (with a half-life of less than 1 min [51-53]) it is more liposoluble than GHB, which helps it to pass through the lipid layers of tissues before it can be hydrolyzed. Such tissues may act as a GBL reservoir and extend the duration of its action as compared to GHB [54-56].

These results would also help to explain the fact that the oral bioavailability of GBL (the degree to which it becomes available to target tissues) in rats is 85%, higher than that of GHB [57], and also explains why GBL has a longer duration of action than GHB, possibly due to differences in drug distribution [58-60].

Table 18 Variation in the equilibrium constant with temperature in the GHB formation.

T (ºC)	$10 \cdot K_{eq}$
15.0	3.66 ± 0.04
20.0	3.73 ± 0.04
25.0	3.79 ± 0.04
30.0	3.93 ± 0.04
35.0	4.03 ± 0.04

$[GBL]_o = 0.1$ M; $[HCl] = 1.18 \cdot 10^{-2}$ M.



Scheme 10 Reaction path of the GHB formation in the acid-catalyzed hydrolysis of γ-butyrolactone [44].

6.4. ***Hydrolysis and alkylation as competitive mechanisms.*** While carcinogenesis elicited by lactones has long been known, the alkylating potential of these species has not been investigated in quantitative chemical terms and even less so when concurrent

with other reaction pathways, such as hydrolysis, which should diminish the efficiency of lactones as alkylating agents.

We performed a kinetic study of the alkylating potential of β-propiolactone (BPL), β-butyrolactone (BBL), γ-butyrolactone (GBL), and δ-valerolactone (DVL) (Fig. 15).

The nucleophile NBP was used as the alkylation substrate.

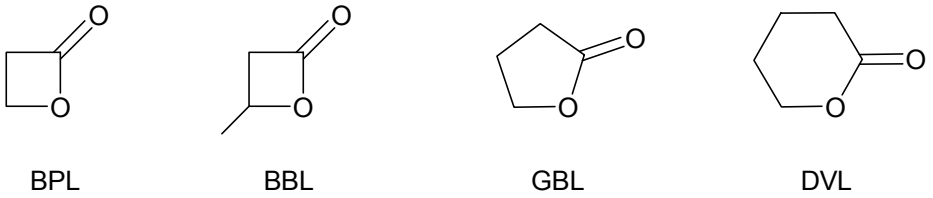


Fig. 15 Chemical structures of the studied lactones.

To gain quantitative knowledge about the effect of the hydrolysis of lactones on their alkylating capacity, both competing reactions -hydrolysis and alkylation- were studied in parallel [61]. Previous work on the hydrolysis of lactones had been carried out in neutral and acid media ([44]).

The reactions were performed at various temperatures between 25 and 35 ºC.

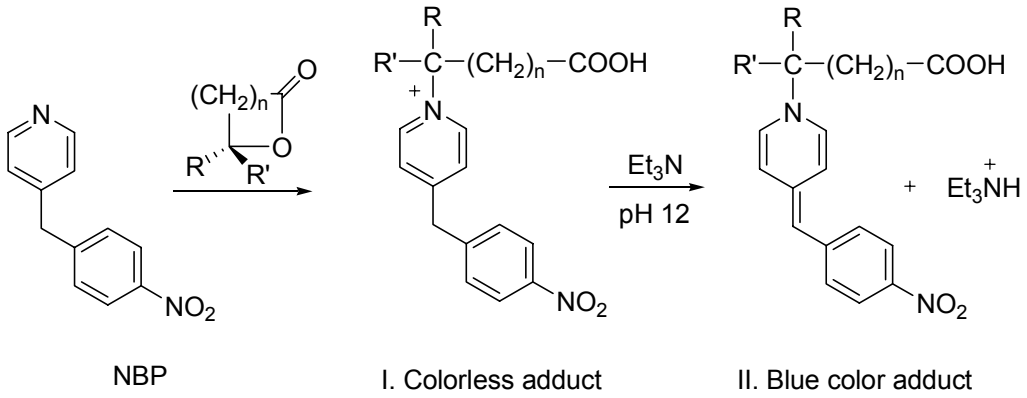
The hydrolysis of lactones (performed in 7:3 water/dioxane media) was monitored by titration of the resulting hydroxy acids.

To monitor the alkylation reactions, 2.4-mL aliquots of the alkylation mixture (lactone+NBP) were removed at different times and added to a cuvette containing 0.6 mL of 99% triethylamine reagent (Et_3N) to stop the alkylation process (Scheme 11), measuring absorbance at the wavelength of maximum absorption. In order to render NBP soluble, the lactone+NBP alkylation mixtures were prepared in 7:3 water/ dioxane medium.

6.4.1. ***Hydrolysis reactions.*** No hydrolysis of γ-butyrolactone or δ-valerolactone was observed. This result is consistent with our previous results reporting that these two lactones require acid media for their hydrolysis to occur (*vide supra*).

Regarding β-propiolactone and β-butyrolactone, experiments performed to determine the influence of the lactone concentration revealed the reactions to be first-order with respect to this reagent (eq 4.8).

Table 19 gives the values of *k* (BPL) and *k* (BBL) (as *k* in eq 4.8) for the hydrolysis reactions as a function of temperature. As can be observed, the hydrolysis rate constant of β-propiolactone is about 6-fold greater than that of β-butyrolactone.



Scheme 11 Method of monitoring the alkylation reactions [61].

Table 19 Hydrolysis rate constants as a function of temperature for β-propiolactone and β-butyrolactone in 7:3 water/dioxane medium.

T **(°C)**	$10^5 \cdot k$ **(BPL)**[a] $M^{-1}\ min^{-1}$ [b]	$10^5 \cdot k$ **(BBL)**[a]
17.5	2.17 ± 0.06	-
20.0	2.97 ± 0.05	-
22.5	3.85 ± 0.06	0.594 ± 0.002
25.0	4.88 ± 0.05	0.806 ± 0.007
27.5	6.64 ± 0.04	1.08 ± 0.01
30.0	8.7 ± 0.1	1.385 ± 0.007
32.5	11.4 ± 0.4	1.86 ± 0.02
35.0	15.2 ± 0.4	2.47 ± 0.02

$[BPL]_o = [BBL]_o = 0.08$ M; [a]As *k* in eq 4.8.
[b]Values of rate constants are given within the 95% confidence interval.

Table 20 shows the values obtained for the activation parameters.

Table 20 Activation parameters for the hydrolysis of β-propiolactone and β-butyrolactone in 7:3 water/dioxane medium.

Lactone	$\Delta H^{\#a}$ kJ mol^{-1}	$-\Delta S^{\#a}$ J K^{-1} mol^{-1}	$\Delta G^{\#a}$ (35°C) kJ mol^{-1}
β-Propiolactone	79 ± 1	95 ± 3	108 ± 1
β-Butyrolactone	83 ± 1	98 ± 3	113 ± 1

[a]Values are given with their standard deviations.

6.4.2. ***Alkylation reactions.*** No alkylation by either γ-butyrolactone or by δ-valerolactone was observed after two weeks. This lack of reactivity is in agreement with their loss of ring strain (Fig. 15).

The blue-colored adducts NBP-BPL and NBP-BBL showed maximum absorption at λ = 584 nm and λ = 586 nm, respectively.

By designating the fraction of lactone converted into adduct (AD) in the alkylation reaction as *x*, and that disappeared in the hydrolysis reaction as *y* (Scheme 12), one has eqs 4.11 and 4.12.

$$\mathrm{L}\quad (\,[\mathrm{L}]_\mathrm{o} - x - y) \left\{ \begin{array}{l} +\,\mathrm{H_2O} \\ \left[\mathrm{H_2O}\right] - y \approx \left[\mathrm{H_2O}\right] \xrightarrow{k_1} y \\ \\ +\,\mathrm{NBP} \\ \left[\mathrm{NBP}\right]_\mathrm{o} - x \xrightarrow{k_\mathrm{alk}} \mathrm{AD} \equiv x \end{array} \right.$$

Scheme 12 Concurrent hydrolysis and alkylation reactions [61].

$$\frac{\mathrm{d}x}{\mathrm{d}t} = \frac{\mathrm{d}[\mathrm{AD}]}{\mathrm{d}t} = k_\mathrm{alk}\left(\left[\mathrm{NBP}\right]_\mathrm{o} - x\right)\left(\left[\mathrm{L}\right]_\mathrm{o} - x - y\right) \tag{4.11}$$

$$\frac{\mathrm{d}y}{\mathrm{d}t} = k_1\left(\left[\mathrm{L}\right]_\mathrm{o} - x - y\right) \tag{4.12}$$

The combination of eqs 4.11 and 4.12 yields

$$\frac{\mathrm{d}[\mathrm{AD}]}{\mathrm{d}t} = k_\mathrm{alk}\left(\left[\mathrm{NBP}\right]_\mathrm{o} - [\mathrm{AD}]\right)\left(\left[\mathrm{L}\right]_\mathrm{o} - [\mathrm{AD}]\right) - k_1\left(\left[\mathrm{NBP}\right]_\mathrm{o} - [\mathrm{AD}]\right)\ln\frac{\left[\mathrm{NBP}\right]_\mathrm{o}}{\left[\mathrm{NBP}\right]_\mathrm{o} - [\mathrm{AD}]} \tag{4.13}$$

Integration of eq 4.13 and substitution of [AD] by the absorbance, $A = [\mathrm{AD}]/\varepsilon$, ε being the molar absorption coefficient of the adducts, yields eq 4.14,

$$\ln \frac{A_\infty}{A_\infty - A_t} = \frac{k_{alk}[L]_o}{k_1}\left(1 - e^{-k_1 t}\right) \quad (4.14)$$

where A_∞ is the absorbance of the adducts when the plateau is reached.

The results in Table 21 show that the alkylating potential (expressed as the alkylating rate constant k_{alk}, eq 4.11) of BPL is about 10-fold higher than that of BBL.

Table 22 shows the values of the activation parameters.

The values of the activation parameters, as well as those reported in Table 20 referring to the hydrolysis reactions, clearly demonstrate that the reactivity of these lactones is essentially enthalpy-controlled. The higher $\Delta H^{\#}$ value obtained for alkylation by β-butyrolactone must be caused, as in the case of its hydrolysis, by the methyl group as a donor of charge on the β-carbon, with a decrease in its electrophilic character.

Table 21 Alkylation rate constants as a function of temperature for β-propiolactone and β-butyrolactone in 7:3 water/dioxane medium.

T (°C)	$10 \cdot k_{BPL}$	$10 \cdot k_{BBL}$
	M^{-1} min^{-1} [a]	
25.0	4.7 ± 0.4	0.40 ± 0.02
27.5	5.2 ± 0.2	0.48 ± 0.02
30.0	6.2 ± 0.4	0.55 ± 0.02
32.5	7.1 ± 0.4	0.64 ± 0.03
35.0	8.2 ± 0.3	0.78 ± 0.04

[a]Values of rate constants (as k_{alk} in eq 4.11) are given within the 95% confidence interval.

Table 22 Activation parameters for NBP alkylation by β-propiolactone and β-butyrolactone in 7:3 water/dioxane medium.

Lactone	$\Delta H^{\#a}$	$-\Delta S^{\#a}$	$\Delta G^{\#a}$ (35°C)
	kJ mol^{-1}	J K^{-1} mol^{-1}	kJ mol^{-1}
β-Propiolactone	41 ± 2	148 ± 6	87 ± 2
β-Butyrolactone	47 ± 2	148 ± 6	93 ± 2

[a]Values are given with their standard deviations.

Since β-propiolactone and β-butyrolactone are possibly carcinogenic for humans, we wondered about the effect of their transformation into the respective hy-

droxy acids by hydrolysis on their effectiveness as alkylating agents. Figure 16 shows the comparative yields of hydrolysis and alkylation reactions over time.

These results allow one to assume that in the formation of lactones by nitrosation of primary amino acids [25, 26], the transformation of lactones into the corresponding hydroxy acid is not sufficiently effective to prevent alkylation (it should be noted that since hydrolysis of BPL and BBL occurs through alkyl cleavage, the hydrolysis rate constants are practically invariable in the $0 < pH < 9$ range [62-64].

The kinetic results obtained are consistent with the biological activity of these lactones (Table 23): *Sufficient evidence* in experimental animals for the carcinogenicity of β-propiolactone and β-butyrolactone, and *evidence suggesting a lack of carcinogenicity* for γ-butyrolactone [65].

These results also suggest that the NBP test is a simple and reliable primary assay for the evaluation of carcinogenic potential.

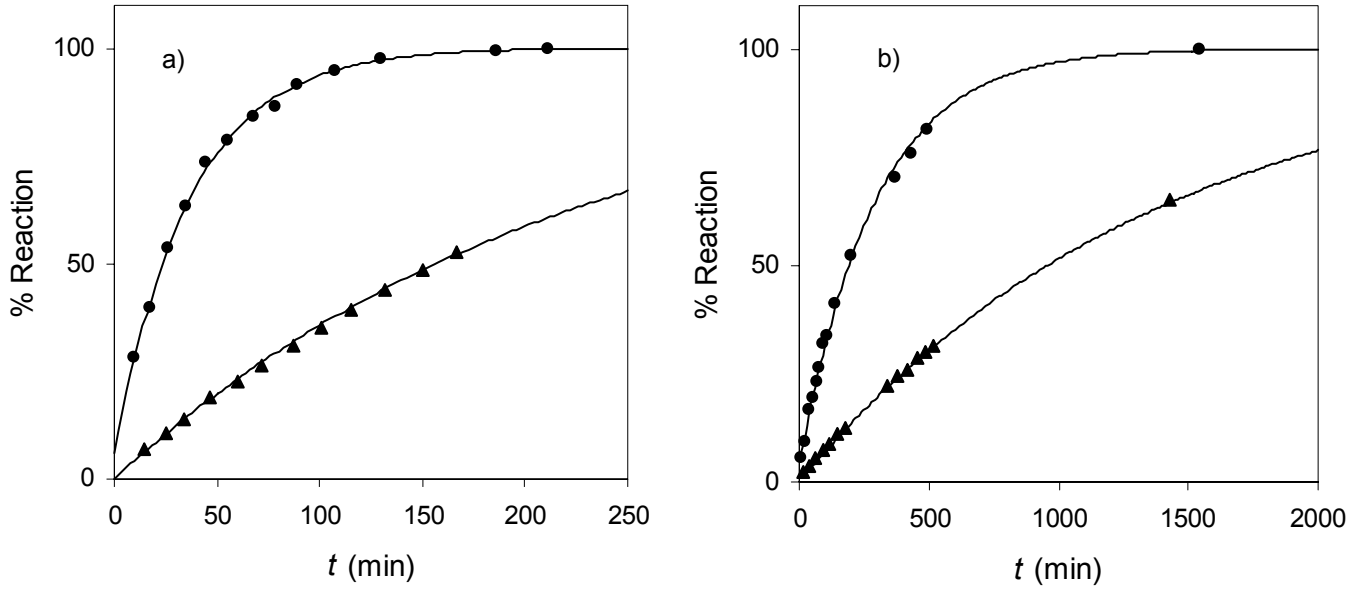


Fig. 16 Hydrolysis of lactones (▲) and NBP alkylation reactions (●) for a) β-propiolactone and b) β-butyrolactone, in 7:3 water/dioxane medium. Hydrolysis: $[BPL]_o = [BBL]_o = 0.08$ M; $T = 32.5$ ºC; %Reaction = $100 \cdot [L]_t/[L]_o$. Alkylation: $[BPL]_o = 4.3 \cdot 10^{-2}$ M; $[BBL]_o = 6.2 \cdot 10^{-2}$ M; $[NBP]_o = 2 \cdot 10^{-4}$ M; $T = 32.5$ ºC; %Reaction = $100 \cdot [NBP]_t/[NBP]_o$.

A kinetic study of alkylation and competing hydrolysis by BPL and BBL was also performed at several water/dioxane ratios. Table 24 shows the results.

As may be seen, the efficiency of alkylation expressed as the alkylation rate/hydrolysis rate ratio (k_{alk}/k) clearly decreases when the amount of dioxane in the

reaction medium increases. A possible explanation for this is that the organic solvent molecules would stabilize the lactones' ground state more than water, resulting in lower activities of BPL and BBL as alkylating agents.

Table 23 Alkylating potential of β-propiolactone and β-butyrolactone and their tumorigenicity/carcinogenicity [61].

Lactone	*Alkylating potential* (this work; Table 21) $10 \cdot k_{alk}^{a}$ $M^{-1}\ min^{-1}$	*Tumorigenicity* [45]			
		Subcutaneous Injection in Mice[b]		Subcutaneous Injection in Rats[c]	
		Dose (mg)	Number of Malignant Tumors at Injection Site / Number of Animals	Dose (mg)	Number of Malignant Tumors at Injection Site / Number of Animals
β-Propiolactone	8.2 ± 0.3	0.73	18/30	4	13/20
β-Butyrolactone	0.78 ± 0.04	10	18/30	100	9/20
γ-Butyrolactone	No reaction	Not classifiable as to its carcinogenicity to humans [65]			
δ-Valerolactone	No reaction	No data on carcinogenicity available			

[a] T = 35 ºC. [b] 0.05 mL Tricaprylin as vehicle. [c] 0.1 mL Tricaprylin as vehicle.

This result may be useful when working with hydrophilic/lipophilic media, such as in the food science. For instance, the results may be significant in cases of the presence in the human stomach of mixtures of alcoholic spirits and food containing vegetal oils, such as salads, or food coming from the preserves industry. When the water/organic component ratio decreases, a slowing down of the efficiency of the alkylation reactions would be expected.

Table 24 Relative efficiency of alkylation by lactones when compared to their hydrolysis at different water/dioxane ratios.

Water/Dioxane	β-*Propiolactone*		β-*Butyrolactone*	
	$10 \cdot k_{alk}^{a}$ $M^{-1}\ min^{-1}$ [c]	k_{alk}^{a}/k^{b}	$10 \cdot k_{alk}^{a}$ $M^{-1}\ min^{-1}$ [c]	k_{alk}^{a}/k^{b}
7/3	5.2 ± 0.2	7843	0.48 ± 0.02	4444
6/4	3.9 ± 0.2	7677[d]	0.17 ± 0.01	2396[d]
5/5	3.0 ± 0.1	7126[d]	0.08 ± 0.01	1765[d]
4/6	1.6 ± 0.1	5926[d]	0.03 ± 0.01	967[d]
2/8	0.150 ± 0.003	1500	0.0060 ± 0.0004	735

$[BPL]_o = 4.3 \cdot 10^{-2}$ M; $[BBL]_o = 6.2 \cdot 10^{-2}$ M; T = 27.5 ºC.
[a] As k_{alk} in eq 4.11. [b] As k in eq 4.8. [c] Values of rate constants are given within the 95% confidence interval.
[d] values from [44].

Finally, it should be pointed out that the above NBP method used to investigate the alkylating potential of lactones has also seen to be applicable to determine the reactivity of much weaker alkylating molecules such as sorbates [66]. Based on these results we are currently attempting to inhibit some alkylation reactions in a way parallel to that previously performed to block/inhibit nitrosation reactions [34, 67-70]. Some of these reactions converge in the area of preservatives in Food Science.

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