Amino Acid Nitrosation Products as Alkylating Agents

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Abstract: Nitrosation reactions of α-, β-, and γ-amino acids whose reaction products can act as alkylating agents of DNA were investigated. To approach in vivo conditions for the two-step mechanism (nitrosation and alkylation), 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. These conclusions were drawn: (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 power is: α-lactones > β-lactones > γ-lactones, coming respectively from the nitrosation of α-, β-, and γ-amino acids; and (iii) the results obtained may be useful in predicting the mutagenic effectiveness of the nitrosation products of amino acids.

Introduction

Since the report by Magee and Barnes[1] that dimethylnitrosamine induces liver cancer when fed to rats this finding has prompted and expanded study of the series of nitroso compounds. Biologists were mainly interested in the use of these compounds as models for producing a broad range of cancers,[2−4] whereas chemists were more interested first in the mechanisms of formation of nitroso compounds[5−8] and then in blocking or inhibiting the mechanisms of formation of these species.[9−13]

Studies showing that dimethylnitrosamine is converted enzymically into a methylating agent[14,15] sparked interest in the mechanism of carcinogenesis through the alkylation of proteins and nucleic acids by *N*-nitroso compounds.

Some nitroso compounds decompose or can be metabolized in vivo to form strongly alkylating electrophiles that may damage DNA, in which process the cytotoxic and mutagenic effects of carcinogenic compounds are believed to reside.[16,17]

The nitrosation of amino acids is particularly interesting. In the case of those with a secondary amino group, besides direct nitrosation by NO$^+$ and N$_2$O$_3$, a nitrosation mechanism through the initial formation of a nitrosyl carboxylate followed by a slow intramolecular rearrangement has been reported.[18,19]

Regarding the nitrosation of amino acids with a −NH$_2$ group, some results suggested that mutagenic products arose primarily from nitrosation of the primary amine rather than the amide or indole group.[20,21] It thus became of interest to identify the alkylating agent as well as its alkylating potential.

This research was carried out in two stages: (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[22]). NBP has nucleophilic characteristics similar to those of DNA.

Experimental Section

Amino acid solutions were made up by weight from Merck 99% glycine and valine; Aldrich 99% alanine, α-aminobutyric acid, α-aminoisobutyric acid, norvaline, and β-alanine, 97% β-aminobutyric acid and γ-aminobutyric acid, and 98% aspartic acid. NBP was from Sigma. Triethylamine (99%) was obtained from Aldrich. Perchloric acid, sodium hydroxide used for preparing buffer solutions, and the ionic strength controller sodium perchlorate monohydrate were obtained from Merck. Sodium dihydrogen phosphate 2-hydrate was from Panreac as well as the NBP solvents, 99% acetone and 99% ethylene glycol.

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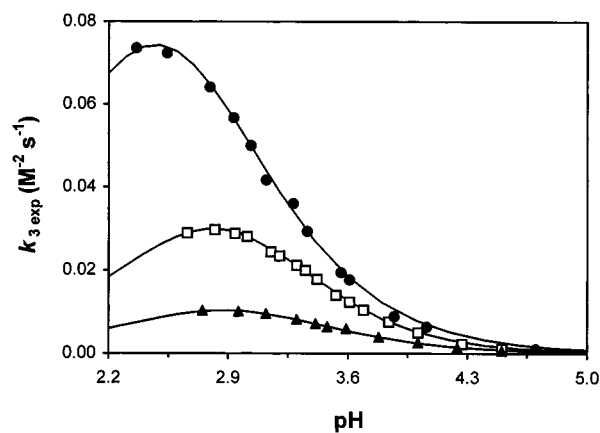(15) Magee, P. N.; Farber, E. *Biochem. J.* **1962**, *83*, 114.

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Figure 1. Nitrosation rate as a function of pH. (●) [nor-Val]$_o$ = 0.300 M, (□) [β-Ala]$_o$ = 0.300 M, (▲) [γ-Amb]$_o$ = 0.300 M; [Nit]$_o$ = 0.010 M, [NaH$_2$PO$_4$] = 0.50 M, I = 1.00 M, T = 298 K.

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.

For studying alkylation reactions, equimolar concentrations of amino acid and sodium nitrite were incubated in NaH$_2$PO$_4$/HClO$_4$ buffer solution ("nitrosation mixture"). At different times ("nitrosation time"), aliquots were removed and added to a NBP solution (NBP was dissolved in acetone, ethylene glycol, and sodium phosphate buffer, pH 8, at a ratio of 1:4:1).
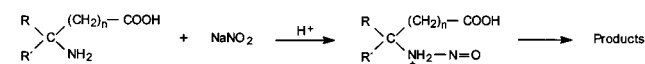
The "alkylation mixture" (neutral pH) was prepared with 1 mL of nitrosation mixture and 3 mL of NBP solution. The mixture was incubated at 298 K.

Aliquots of 2.4 mL of the alkylation mixture were removed at different times ("alkylation time") and added to the cuvette containing 0.6 mL of the 99% triethylamine reagent (to stop the alkylation reaction and render the adduct stable), measuring absorbance at the wavelength of maximum absorption.

Spectrophotometric measurements were carried out on a Shimadzu UV-2101 PC spectrophotometer equipped with a thermoelectric six-cell holder temperature-control system (±0.1 °C). IR spectra were obtained with a Bonem FT MB-100 spectrometer. NMR proton spectra were obtained with a 200 MHz Bruker, model WP 200 SY spectrometer.

Results and Discussion

Nitrosation Reactions. We investigated the nitrosation of six α-amino acids: 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).

Since the HNO$_2$/NO$_2^-$ system (henceforth nitrite, Nit) shows a maximum in the absorption spectrum at λ = 371 nm, nitrite was used as the control species for the continuous measurement of the amino acid nitrosation.

All reactions studied exhibited experimental rate equations of the form:

$$\text{rate} = k_{3\text{exp}} [\text{amino acid}] [\text{Nit}]^2 \qquad (1)$$

$k_{3\text{exp}}$ showing a strong dependence on pH (Figure 1).

Table 1. Nitrosation Rate of Amino Acids

amino acid	pH	$k_{3\,\text{exp}} \cdot 10^2$ (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

Table 2. Alkylating Potential of the Amino Acid Nitrosation Products

amino acid	λ_{max} (nm)	$t_{\text{nitrosation}}$ (min)[b]	$t_{\text{alkylation}}$ (min)[b]
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

[a] Nitrosation reactions (pH ≈ 3.5), [amino acid]$_o$ = 0.060 M, [NaNO$_2$]$_o$ = 0.060 M, [NaH$_2$PO$_4$] = 0.50 M, I = 1.00 M (NaClO$_4$), T = 298 K. Alkylation reactions (pH ≈ 7), [NBP]$_o$ = 0.116 M, T = 298 K. [b] Times for maximum adduct concentration to be reached.

Second-order with respect to the nitrite concentration shows that the effective nitrosating agent should be dinitrogen trioxide.[23]

Table 1 shows the values of $k_{3\text{exp}}$ obtained in the nitrosation of α-, β-, and γ-amino acids. The observed sequence of $k_{3\text{exp}}$ values is: α-amino acids > β-amino acids > γ-amino acids.

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 (see Experimental Section).

Table 2 shows the wavelength of maximum absorption for each adduct. Figure 2, a−c, shows the evolution of the NBP alkylation process as a function of the nitrosation and alkylation times.

Table 2 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 $k_{3\text{exp}}$ values found in the nitrosation reaction (Table 1).

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.

Figure 2d shows the results obtained on studying the alkylation potential of the nitrosation products of aspartic acid.

As may be seen from Table 2, 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

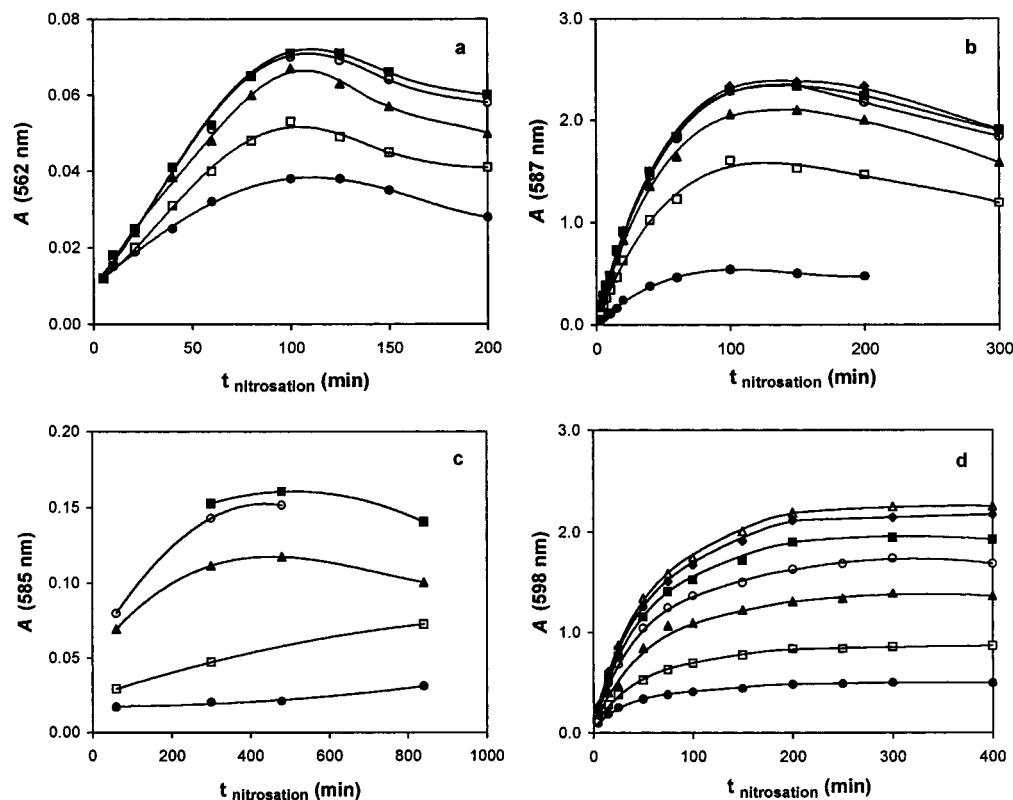(23) Cachaza, J. M.; Casado, J.; Castro, A.; López-Quintela, M. A. *Z. Krebsforsch.* **1978**, *91*, 279.

Figure 2. Formation of NBP adduct with the nitrosation time, at different alkylation times. (a) **Glycine**, $[Gly]_o = 0.060$ M, $[Nit]_o = 0.060$ M, pH (nitrosation) = 3.55; $[NBP]_o = 0.116$ M, pH (alkylation) = 7.0. Alkylation time: (●) 3 min, (□) 5 min, (▲) 10 min, (○) 15 min, (■) 20 min. (b) β-**Alanine**, $[\beta\text{-Ala}]_o = 0.060$ M, $[Nit]_o = 0.060$ M, pH (nitrosation) = 3.53; $[NBP]_o = 0.116$ M, pH (alkylation) = 7,0. Alkylation time: (●) 5 min, (□) 25 min, (▲) 50 min, (○) 75 min, (■) 100 min, (◆) 125 min. (c) γ-**Aminobutyric acid**, $[\gamma\text{-Amb}]_o = 0.116$ M, $[Nit]_o = 0.116$ M, pH (nitrosation) = 2.78; $[NBP]_o = 0.116$ M, pH (alkylation) = 7.0. Alkylation time: (●) 60 min, (□) 300 min, (▲) 1440 min, (○) 2880 min, (■) 8640 min. (d) **Aspartic acid**, $[Asp]_o = 0.060$ M, $[Nit]_o = 0.060$ M, pH (nitrosation) = 3.53; $[NBP]_o = 0.116$ M, pH (alkylation) = 7.0. Alkylation time: (●) 25 min, (□) 50 min, (▲) 100 min, (○) 150 min, (■) 200 min, (◆) 300 min, (△) 400 min.

located at the β position with respect to the amino group exerts a greater influence than that situated at the α position.

Scheme 1 shows the proposed mechanism. Following nitrosation, the corresponding diazo compound is formed, which rapidly loses N_2.

The time required for the maximum concentration of adduct to be reached (Table 2) 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 2), 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, and their alkylation time would increase, as was, in fact, observed (Table 2).

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 been found to be a potent, although slow-acting, carcinogen.[24] 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.[24]

The times of alkylation by α-lactones are shorter than those corresponding to β- and γ-lactones (Scheme 2) owing to ring

stability. All of 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

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Scheme 1. Alkylation Mechanism
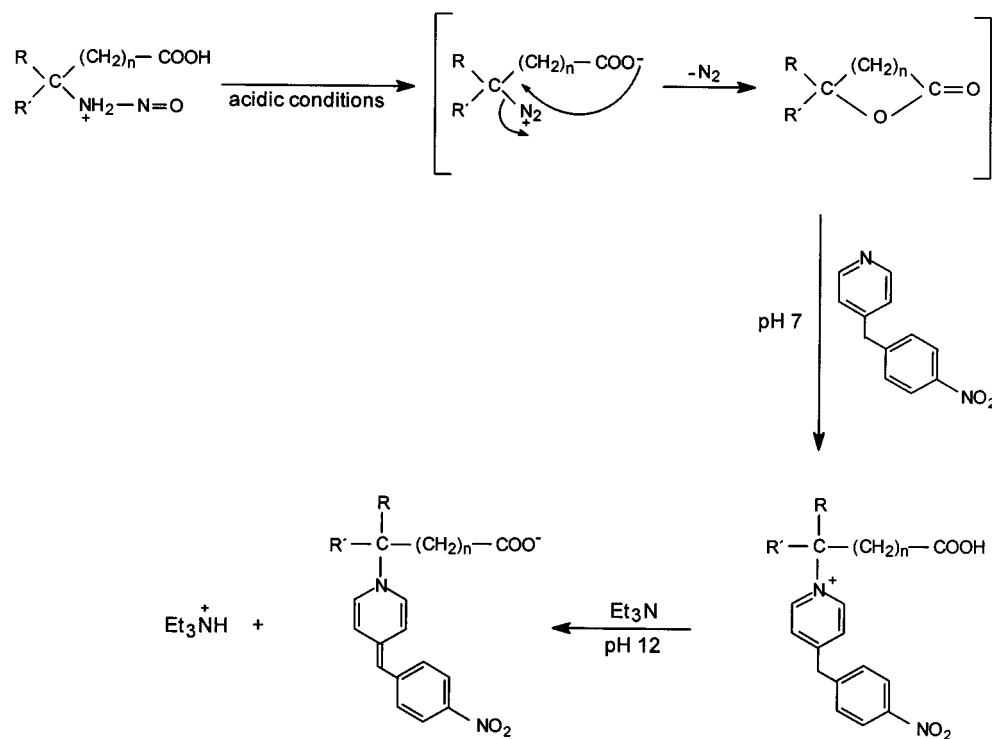
Scheme 2. Lactones Resulting from Nitrosation of α-, β-, and γ-Amino Acids; Arrow ↓ Shows Electrophilic Center; $t_{alkylation}$ Corresponds to the Maximum Adduct Concentration
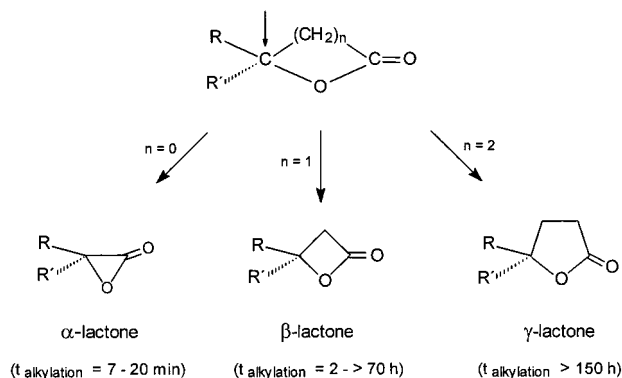
with CHCl$_3$ (β-lactones) or CH$_2$Cl$_2$ (γ-butyrolactone), separating the organic phase, which was then washed with a saturated solution of NaCl and dried with anhydrous Na$_2$SO$_4$. This was then filtered and the solvent evaporated off under reduced pressure.

Identification of β-Propiolactone. The ^{1}H 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.[25]

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.[26,27]

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}[25,28] 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.[25,28]

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 a spectrum ($\lambda_{max} = 587$ nm) analogous 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[29]).

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, that is the most common natural amino acids are the precursors of the most powerful alkylating agents.

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(iii) In view of the existence of a correlation between the mutagenic index of nitrosation products and the alkylating potential index,[30] 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.

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