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NITROSATION OF PEPTIDE BONDS. CLEAVAGE OF NITROSATED PEPTIDES BY PYRROLIDINE AND α-AMINO ESTERS

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Abstract—The reaction of several α-amino acids and peptides (containing Gly, L-Ala, L-Leu, L- or DL-Phe, and/or L- or D-Val) with air-diluted nitrogen oxides has been studied to roughly mimic the N-nitrosation of peptide bonds that the contaminated urban air might produce in pulmonary tissues. Most N-protected α-amino acids give practically quantitative yields of N-nitroso derivatives. N-Protected dipeptides afford either dinitrosated peptides, mixtures of di- and mononitrosated compounds, selectively mononitrosated products, or no reaction at all, depending mainly on steric effects. The same trends are observed for some higher peptides. The (poly)nitrosated peptides, which retain the chirality of the starting materials, have been characterized by ^1H and ^{13}C NMR spectroscopy and are cleaved by pyrrolidine and amino esters under mild conditions to give (new) amides or peptides plus diazo derivatives.

The reaction of nitrous acid with proteins is the basis of an analytical procedure aimed at estimating the number of free amino groups that they contain.[1] Apart from this deamination reaction, attention has been focused in recent years onto the transformations of side chains of peptides by the action of (ingested) $NaNO_2$ in acid media.[2] By contrast, the reaction of peptides and proteins with nitrogen oxides has been hardly studied despite the fact that NO_2 is a ubiquitous atmospheric pollutant, which suggest that such reactions might take place in living tissue. Since N_2O_3 and N_2O_4 under neutral or only slightly acidic conditions are much more reactive than $NaNO_2$ in mineral acid toward amide bonds,[3] it could be envisioned that proteins may be damaged by nitrogen oxides under certain conditions, since not only side chains may react with them[2] but also their amide bonds may be N-nitrosated.[4]

Before studying the reaction of proteins with nitrogen oxides in natural environments, there seemed reasonable to investigate that of more simple models under conditions that permitted the isolation and/or characterization of the (poly)nitrosated products. By similar reasons, peptides constituted from α-amino acids containing free amino groups and other functions able to react with nitrosating agents (tyrosine, tryptophane, etc.)[2] were ruled out in this preliminary work. Thus, we report here that several amine bonds of protected α-amino acids and short peptides containing glycine, L-alanine, L-leucine, L- or DL-phenylalanine, and/or L- or D-valine react very rapidly, in cold organic solvents, with a stream of air-diluted nitrogen oxides (mainly NO_2) arising from copper wire and conc. HNO_3 to give either completely or partially amide-nitrosated products. These compounds can be readly cleaved by nucleophiles, e.g. amines, to afford the expected fragments.[5]

Results and discussion—The compounds shown in Table I—14 protected α-amino acids, 10 protected dipeptides, 2 protected tripeptides, and 1 protected octapeptide—, most prepared by standard procedures[6] or obtained from commercial sources, were submitted to the usual nitrosation conditions (CH_2Cl_2 at -20 °C, NaAcO).[3,5] In the case of PhCO-GlyOMe (2) the reaction was also carried out in cold DMF, in cold acetonitrile (with NaAcO added in both cases), and in cold CH_2Cl_2/pyridine; since good, similar results were obtained in all the experiments, the ease of isolation of the nitrosated products from the CH_2Cl_2 solutions decided us to reserve DMF, pyridine, etc. for future work (e.g., with peptides insoluble in CH_2Cl_2).

N-Nitrosation can be monitored by TLC (appearance of yellow spots of large Rf as compared with those of starting compounds) and ^1H NMR spectroscopy (disappearance of CONH signals, simplification of NH-bonded CH_2 and/or CH multiplets, and downfield shifts of several sets of protons—see below). Starting from ca. 200 mg of the peptide the complete nitrosation was usually accomplished in 1/2 - 1 h; even though some reluctant amide bonds required more time, TLC of the mixture did not change after 2 h.

The influence of steric effects is worth noting (see Table I). Most simple amino acids and unbranched peptides (such as PhCO-Gly-GlyOMe, 15) were nitrosated in less than 1 h, whereas Z-Val-LeuOMe (24) gave no reaction at all. The behavior shown by the remaining compounds may be summarized as follows: (i) the approach of the nitrosating species to each CONH bond is hindered by the presence of branched chains on both sides, so that, in the case of PhCO-PheOMe (11), the conjunction of a phenyl group linked to the carbonyl and a benzyl group replacing a methylene hydrogen of a glycine produces a remarkable decrease in the nitrosation yield; in fact, the phenyl group alone (see PhCO-GlyOMe, 2), as well as a branched chain alone in the α-amino acid (see, e.g., EtOCO-PheOMe, 9), does not block the nitrosation; (ii) for the same apparent reason the Phe-

Table I. Reaction of Peptides[a] with Nitrogen Oxides

starting compound	nitrosated product	yield(%)[b]
1, MeOCO–GlyOEt	1a, MeOCO–(NO)GlyOEt[c]	100
2, PhCO–GlyOMe	2a, PhCO–(NO)GlyOMe	100
3, PhCO–Gly–N⬡	3a, PhCO–(NO)Gly–N⬡	100
4, MeOCO–AlaOEt	4a, MeOCO–(NO)AlaOEt	100
5, Ac–LeuOMe	5a, Ac–(NO)LeuOMe	98
6, EtOCO–LeuOMe	6a, EtOCO–(NO)LeuOMe	100
7, Boc–LeuOMe[d]	7a, Boc–(NO)LeuOMe	80
8, Z–Leu–N⬡	8a, Z–(NO)Leu–N⬡	15[e]
9, EtOCO–PheOMe	9a, EtOCO–(NO)PheOMe	100
10, EtOCO–Phe–N⬡	no reaction	-
11, PhCO–PheOMe	11a, PhCO–(NO)PheOMe	20[e]
12, Ac–ValOMe	no reaction	-
13, EtOCO–ValOMe	13a, EtOCO–(NO)ValOMe	75
14, EtOCO–Val–N⬡	14a, EtOCO–(NO)Val–N⬡	20
15, PhCO–Gly–GlyOMe	15ab, PhCO–(NO)Gly–(NO)GlyOMe	100
16, Z–Ala–GlyOEt	16ab, Z–(NO)Ala–(NO)GlyOEt 16b, Z–Ala–(NO)GlyOEt	40 54
17, Boc–Ala–GlyOEt	17ab, Boc–(NO)Ala–(NO)GlyOEt 17b, Boc–Ala–(NO)GlyOEt	62 30
18, Z–Ala–AlaOMe	18ab, Z–(NO)Ala–(NO)AlaOMe 18b, Z–Ala–(NO)AlaOMe	10 85
19, PhCO–Gly–PheOMe	19ab, PhCO–(NO)Gly–(NO)PheOMe	100
20, PhCO–DL-Phe–GlyOEt	20b, PhCO–DL-Phe–(NO)GlyOEt	96
21, Z–Phe–GlyOMe	21ab, Z–(NO)Phe–(NO)GlyOMe 21b, Z–Phe–(NO)GlyOMe	70 26
22, PhCO–Gly–ValOMe	22a, PhCO–(NO)Gly–ValOMe	100
23, Boc–Ala–LeuOMe	23a, Boc–(NO)Ala–LeuOMe	95
24, Z–Val–LeuOMe	no reaction	-
25, PhCO–DL-Phe–Gly–GlyOEt	25bc, PhCO–DL-Phe–(NO)Gly–(NO)GlyOEt	100
26, Z–Phe–Gly–LeuOMe	26abc, Z–(NO)Phe–(NO)Gly–(NO)LeuOMe 26bc, Z–Phe–(NO)Gly–(NO)LeuOMe	60 30
27, Boc(–Val–D-Val)$_4$OMe	no reaction	-

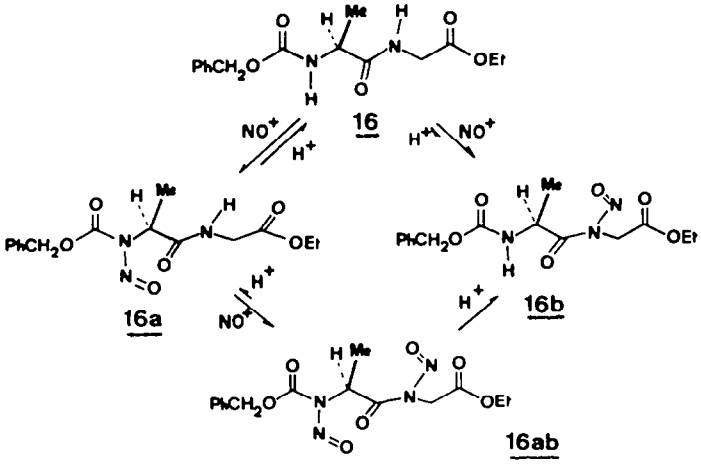
[a] Along this work dashes indicate the CO–NH bonds; all chiral amino acids as natural enantiomers unless noted; letters a, b, and c point out that the first, the second, and the third CO–NH bonds, respectively, are nitrosated. [b] Yields after 1-2 h of reaction. [c] MeOCO–N(NO)–CH_2–COOEt. [d] Bu^{t}OCO–LeuOMe. [e] Unreacted material (ca. 80%) recovered after 2 h of reaction. [f] $PhCH_2OCO$–Ala–GlyOEt.

Table II. Specific Rotations in CH_2Cl_2, before and after the N-Nitrosation

compd	$[\alpha]_D^{20}$, degrees	compd	$[\alpha]_D^{20}$, degrees
5	-4.8 (c 2.3)	5a	-43.0 (c 0.9)
6	-7.7 (c 1.0)	6a	-36.8 (c 1.0)
7	-7.1 (c 2.0)	7a	-44.5 (c 2.0)
9	+51.2 (c 1.7)	9a	-72.0 (c 1.3)
11	+76.2 (c 2.0)	11a	-156.0 (c 0.9)
13	+5.6 (c 1.0)	13a	-105.4 (c 1.0)
16	-11.7 (c 1.6)	16ab	-18.2 (c 0.9)
19	+39.0 (c 1.0)	19ab	-93.5 (c 1.0)
23	-32.7 (c 1.2)	23a	-38.5 (c 1.5)

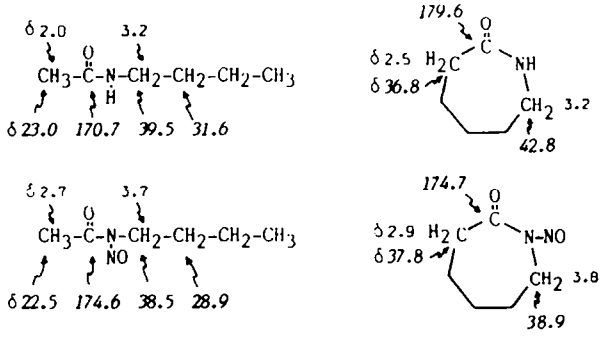
Gly and Gly-Gly bonds of 20 and 25 are completely nitrosated while the first amide bonds (PhCO-Phe) remain practically unchanged; (iii) whereas 20 gives mononitrosated product 20b, peptide 19 yields dinitrosated compound 19ab, which indicated that branching at position α with respect to the CO group makes the nitrosation more difficult than the same side chains α to the NH;[7] (iv) branching appears to be important even if it occurs far away from the amide or carbamate functions, as noted by comparing methyl esters with pyrrolidine-derived amides (compare, especially, the pairs 9-10 and 13-14).

The peptides which give mixtures of nitrosated compounds, i.e. 16-18, 21, and 26, may be commented together. The ratios of pernitrosated to partially nitrosated products reflect their relative stabilities under the reaction conditions, since they could not been enhanced by means of longer reaction times or by nitrosating again the isolated mixture. There seems that the nitrosation of the second CO-NH bond increases the steric hindrance, preventing the complete nitrosation of the first one (carbamate group); this effect may be related to that observed for terminal-pyrrolidine peptides 8, 10, and 14, after considering that N-alkyl-N-nitroso and N,N-dialkyl substituents should show similar hinderings. Compound 16 was choiced as the representative of such peptides and was dealt with in more depth. Thus, its nitrosation was repeated under the usual conditions, but samples were removed and analyzed by ^{1}H NMR after 10 min, 20 min, 1 h, and 2 h. The first sample contained only starting material and Z-(NO)Ala-GlyOEt (16a, 15%). The second one contained 16 (19%), 16a (38%), dinitrosated product (16ab, 32%), and 16b (11%). After 1 h, the ratio was 12% of 16, ca. 0% of 16a, 38% of 16ab, and 50% of 16b. After 2



h, no 16 or 16a remained, the reaction mixture being 16ab and 16b in a 42:58 ratio. Therefore, (i) the Z-Ala bond is more rapidly nitrosated than the Ala-Gly bond, which agrees with the relative nitrosation rates observed for EtCONHMe, PhCONHMe, and ZNHMe under our conditions (1: 1.2 : 4.7, respectively), and (ii) the nitrosation of Z-Ala bond, in contrast with the above-mentioned reverse case, does not hinder that of the Ala-Gly bond. It can be said, in short, that the carbamate nitrosation is kinetically favored whereas that of the amide bond (at least in 16 and similar peptides) is favored when the equilibrium conditions are reached.[8]

Several features of the ^{1}H and ^{13}C NMR spectra are worthy of mention. The protons on both sides of CONH groups undergo downfield shifts as a consequence of the N-nitrosation, the observed δ values lying between 0.4-0.6 for NCH_2, 0.6-1.0 for NCHR, 0.8-1.0 for CH_2CO, and 1.3-1.4 for CHRCO; these values (larger for the protons α to CO than for the protons α to N) agree with those observed in simple nitrosoamides,[5] for which a transoid arrangement involving the CO-N bond is assumed.

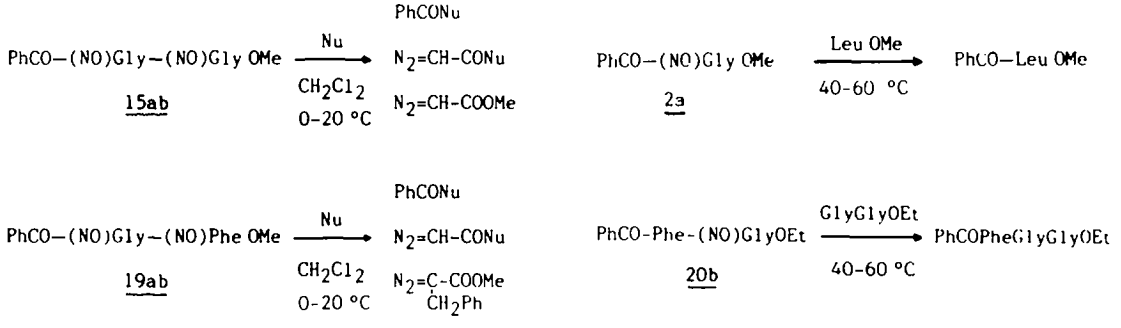


and differ from those measured from N-nitrosobutirolactame, N-nitrosovalerolactame, and N-nitrosocaprolactame[9] (see figures above) in which the carbonyl oxygen atom and the NO group are obviously forced to be cisoid. When methylene or methine protons are flanked by two amide groups some compensation occurs since $\Delta\delta$= 1.2-1.4 for $CONCH_2CON$ and $\Delta\delta$= 1.7-1.9 for CONCHRCON. Shifts to lower field ($\Delta\delta$= 0.4-0.5) of CH_2OCON are also structurally important as they confirm that the nitrosation of the carbamate function has been carried out.

With regard to ^{13}C spectra, it should be noted that only the CO carbon atoms of the amide groups are significantly shifted to lower field ($\Delta\delta$ = 2.3-4.0). Indeed, the CO carbon atoms of the carbamate groups are shifted to higher field ($\Delta\delta$= -2.4 to -3.0), which suggests that the transoid-transoid arrangement may not be predominant in these cases. Also, the other carbon atoms are either slightly shifted to higher field ($\Delta\delta$ between -0.5 and -2 for CH_2CO, CHRCO, $CONCH_2$, and CONCHR) or undergo even larger upfield shifts ($\Delta\delta$= -5 for NCHRCOOR and $NCHCH_2$).

Racemization apparently did not occur during the nitrosation since very net changes to negative $[\alpha]_D^{20}$ values are observed for chiral molecules studied (see Table II). Nevertheless, to prove that some racemization did not take place, a cold CH_2Cl_2 of pure 5a was treated with a stream of dry HCl for a few min; evaporation in vacuo gave chromatographically pure 5 with the expected specific rotation. In other words, neither N-nitrosation nor N-denitrosation under these conditions seem to affect the chiral center (linked to the nitrogen atom).[3] Furthermore, when the mixture of 16ab and 16b arising from the nitrosation of 16, $[\alpha]_D$ -11.7° (c 1.56, CH_2Cl_2), was treated with HCl(g) as above, the denitrosated peptide, $[\alpha]_D$ -10.8° (c 1.21, CH_2Cl_2), was recovered in 93% yield. Therefore, it can be stated that racemization is not significant.

The nitrosopeptides obtained in this work react with amines and α-amino esters under mild conditions.[10] Thus, addition of a slight excess of pyrrolidine to a CH_2Cl_2 solution of 2 at room temperature caused a fast colour change, affording quantitatively N-benzoylpyrrolidine and methyl diazoacetate, as shown by TLC and NMR comparison with authentic samples. Similarly, 4 was converted to N-(methoxycarbonyl)pyrrolidine and ethyl diazoalaninate. Etc. Most of these reactions were also followed by HPLC (see Experimental Section). Other representative examples of the preference of amines to attack the carbonyl carbon atom rather than to effect the denitrosation are outlined below. It should be noted that amino esters react far more slowly than pyrrolidine (Nu), so that the percentages of conversion are only moderate unless long refluxing times are used.



EXPERIMENTAL SECTION

General——The NMR spectra were obtained in $CDCl_3$ on Varian XL-200 (200 MHz for ^1H, 50.3 MHz for ^{13}C) or Perkin-Elmer R-24B (60 MHz, ^1H) spectrometers; chemical shifts are reported in parts per million with respect to internal Me_4Si in all the cases, and J values are given in hertz. The IR spectra were taken as films or $CHCl_3$ solutions with a Perkin-Elmer 681 instrument; only the most significant absorptions (in cm^{-1}) are listed. Mass spectra were recorded on a Hewlett-Packard 5930 spectrometer. Melting points were determined on a Büchi apparatus and are uncorrected. Silica gel sheets (Kieselgel 60, F_{254}, Merck) were used for analytical TLC. Chromatographic separations were carried out on 70-230 mesh Merck silica gel (column at atmospheric pressure) or on 230-400 mesh Merck silica gel by "flash" chromatography. HPLC analyses were performed on a Waters instrument using a Radial-PAK C-18 column (2 ml/min, from 60% MeOH aq to 80% MeOH aq in 10 min; UV detector at 262 nm). Optical rotation measurements were obtained on a Perkin-Elmer 141 polarimeter.

Starting Compounds——Protected α-amino acids and peptides used in this work were known (with the exception, to our knowledge, of 3, 4, 8, 10, 14, 20, and 23) and have been prepared from natural L-amino acids according to standard procedures.[6] Esterifications have been performed with MeOH-HCl or EtOH-HCl; amino esters have been treated with PhCOCl, MeOCOCl, EtOCOCl, $PhCH_2OCOCl$ (ZCl), AcCl, or

$(Bu^tOCO)_2O$ in the presence of NEt_3 to afford the amino-protected derivatives. Dipeptides were synthesized by coupling the corresponding protected fragments in CH_2Cl_2 or THF with the aid of DCCI. The reaction of 4-benzyl-2-phenyl-5-oxazolone with HGlyOMe and with HGly-GlyOEt furnished 20 and 25, respectively. Tripeptide 19 was obtained from Z-Phe-GlyN$_3$ (prepared from 21) and HLeuOMe. Dipeptide 24 was bought to Sigma, whereas 27 was prepared by G. P. Lorenzi et al.[11] Compounds 3, 8, 10, and 14 were obtained by heating the corresponding esters with an excess of pyrrolidine in a sealed tube at 100°C for 1 day, and were then purified by dilution with CH_2Cl_2, rinsing with diluted HCl aq. and column chromatography (CH_2Cl_2-MeOH). N-Methylpropanamide, N-methylbenzamide, and O-benzyl-N-methylcarbamate have been prepared from the corresponding acid chlorides and aqueous methylamine.

Nitrosation Products——The nitrosation procedure has been described in a precedent paper.[5] Usually, 2-5 mmols of peptide in 80 mL of CH_2Cl_2 containing 2-3 g of NaAcO were treated with a smooth stream of nitrogen dioxide for 1-2 h at -20 °C. Mixtures of "all-nitrosated" and partially nitrosated compounds were separated by "flash" chromatography with CH_2Cl_2 or CH_2Cl_2-MeOH 99:1 as the eluents to give yellow oils (or, in few cases, yellow solids of low melting point), unstable to the heat and light. Compound 2 (150 mg in each case) was also nitrosated in 50 mL of DMF (containing ca. 1 g of NaAcO), in 50 mL of acetonitrile (plus 1 g of NaAcO), and in a mixture of 30 mL of CH_2Cl_2 and 10 mL of pyridine; before washing with water the final solutions, 100 mL of CH_2Cl_2 were added in all cases; after the usual workup quantitative yields of 2a were always obtained.

Ethyl N-methoxycarbonyl-N-nitrosoglycinate (1a): ^{1}H NMR δ 1.28(t, J=7, 3H), 4.07 (s, 3H), 4.10 (q, J=7, 2H), 4.39 (s, 2H); ^{13}C NMR δ 14.0 ($CH_2\underline{C}H_3$), 41.8 (N(NO)$\underline{C}H_2$), 55.2 ($\underline{C}H_3OCO$), 62.0 (COO$\underline{C}H_2$), 153.8 (CON(NO)), 165.4 (COO). Methyl N-nitrosohippurate (2a): ^{1}H NMR δ 3.75 (s, 3H), 4.60 (s, 2H), 7.3-7.9 (m, 5H); ^{13}C NMR δ 40.5 (N(NO)$\underline{C}H_2$), 52.7 ($\underline{C}H_3$), 132.2 (C ipso), 128.3 (C meta), 130.9 (C ortho), 132.9 (C para), 165.9 (COO), 172.0 (CON(NO)); IR 1735, 1700; MS, m/z 222 (M^+). N,N-Tetramethylene-2-(N-nitrosobenzamido)acetamide (3a): ^{1}H NMR δ 1.87 (m, 4H), 3.40 (m, 4H), 4.60 (s, 2H), 7.2-7.8 (m, 5H); ^{13}C NMR δ 24.1, 26.2 ($CH_2\underline{C}H_2\underline{C}H_2CH_2$), 41.2 (N(NO)$\underline{C}H_2$), 45.7, 46.2 ($\underline{C}H_2CH_2CH_2\underline{C}H_2$), 132.9 (C ipso), 128.2 (C meta), 130.8 (C ortho), 132.6 (C para), 166.7 (CON(NO)), 167.6 (CON). Ethyl N-methoxycarbonyl-N-nitrosoalaninate (4a) ^{1}H NMR δ 1.22 (t, J=7, 3H), 1.40 (d, J=6.5, 3H), 4.05 (s, 3H), 4.14 (q, J=7, 2H), 5.20 (q, J=6.5, 1H). Methyl N-acetyl-N-nitrosoleucinate (5a): ^{1}H NMR δ 0.85 (d, J=6.5, 3H), 0.96 (d, J=6.5, 3H), 1.6-2.2 (m, 3H), 2.81 (s, 3H), 3.65 (s, 3H), 5.22 (m, 1H); ^{13}C NMR δ 21.7, 21.9 (($\underline{C}H_3)_2$CH), 22.7 (CH_3CO), 25.2 (($CH_3)_2\underline{C}$H), 36.7 ($C\underline{H}CH_2$CH), 50.2 (N(NO)$\underline{C}$HCO), 52.6 (CH_3O), 168.5 (COO), 174.0 ($CH_3\underline{C}$O); MS, m/z 217 (M+1$^+$). Methyl N-ethoxycarbonyl-N-nitrosoleucinate (6a): ^{1}H NMR δ 0.80 (d, J=5.8, 6H), 1.4 (t, J=7.0, 3H), 1.6-2.0 (m, 3H), 3.58 (s, 3H), 4.45 (q, J=7.0, 2H), 5.20 (t, J=6.0, 1H); ^{13}C NMR 14.2 ($\underline{C}H_3CH_2$), 21.6, 22.9 (($CH_3)_2$CH), 25.0 (($CH_3)_2\underline{C}$H), 37.1 ($C\underline{H}CH_2$CH), 51.5 (N(NO)$\underline{C}$HCO), 52.6 (OCH_3), 64.8 (CH_2O), 153.3 (OCON(NO)), 168.7 (COO); MS, m/z 247 (M+1$^+$). Methyl N-(tert-butoxycarbonyl)-N-nitrosoleucinate (7a): ^{1}H NMR δ 0.85 (d, J=6.5, 3H), 0.87 (d, J=6.5, 3H), 1.3-1.9 (m, 3H), 1.64 (s, 9H), 3.68 (s, 3H), 5.30 (t, J=7, 1H); ^{13}C NMR δ 21.8, 22.8 (($\underline{C}H_3)_2$CH), 25.1 (($CH_3)_2\underline{C}$H), 28.0 (($\underline{C}H_3)_3$C), 37.4 ($C\underline{H}CH_2$CH), 50.7 (N(NO)$\underline{C}$HCO), 52.5 (OCH_3), 86.0 (($CH_3)_3\underline{C}$), 151.2 OCON), 169.0 (COO); MS, m/z 275 (M+1$^+$). Methyl N-ethoxycarbonyl-N-nitrosophenylalaninate (9a): ^{1}H NMR δ 1.32 (t, J=7, 3H), 2.95, 3.19, 5.43 (ABX system, JAB=14.0, JAX=12.5, JBX=3.5), 3.65 (s, 3H), 4.40 (q, J=7, 2H), 6.9-7.3 (m, 5H); ^{13}C NMR δ 14.1 ($\underline{C}H_3CH_2$), 33.9 ($\underline{C}H_2Ph$), 52.7 (OCH_3), 54.0 (N(NO)$\underline{C}$HCO), 64.7 (CH_2O), 127.0 (C para), 128.5 (C meta), 128.9 (C ortho), 135.9 (C ipso), 153.1 (OCON(NO)), 167.8 (COO). Methyl N-benzoyl-N-nitrosophenylalaninate (11a): ^{1}H NMR 3.0-3.4 (m, 2H), 3.68 (s, 3H), 5.75 (m, 1H), 6.9-7.9 (m, 10H). Methyl N-ethoxycarbonyl-N-nitrosovalinate (13a): ^{1}H NMR δ 0.65 (d, J=6.5, 3H), 1.15 (d, J=6.5, 3H),1.50 (t, J=7.0, 3H), 2.1-2.7 (m, 1H), 3.60 (s, 3H), 4.50 (q, J=7.0, 2H), 4.80 (d, J=9, 1H); ^{13}C NMR δ 14.2 ($\underline{C}H_3CH_2$), 18.7, 21.5 (($CH_3)_2\underline{C}$H), 27.6 (($CH_3)_2\underline{C}$H), 52.4 (OCH_3), 58.1 (N(NO)$\underline{C}$H), 64.9 ($CH_3\underline{C}H_2O$), 153.6 (OCON), 168.0 (COO); IR 1755; MS, m/z 233 (M+1$^+$). Dinitrosated benzoylglycylglycine methyl ester (15ab): mp 88 °C; ^{1}H NMR δ 3.75 (s, 3H), 4.45 (s, 2H, N(NO)$\underline{C}H_2$COO), 5.50 (s, 2H, N(NO)$\underline{C}H_2$CON), 7.4-8.0 (m, 5H); ^{13}C NMR δ 39.9 (N(NO)$\underline{C}H_2$COO), 41.8 (N(NO)$\underline{C}H_2$CON), 52.9 (OCH_3), 128.4 (C meta), 131.0 (C ortho), 132.0 (C ipso), 133.1 (C para), 165.0 (COO), 167.7 ($CH_2\underline{C}$ON), 172.0 (PhCON); IR 1735, 1705. Dinitrosated benzoxycarbonylalanylglycine ethyl ester (16ab): ^{1}H NMR δ 1.25 (t, J=7.2, 3H), 1.52 (d, J=6.5, 3H), 4.05 (q, J=7.2, 2H), 4.30 (s, 2H), 5.40 (s, 2H), 5.98 (q, J=6.5, 1H), 7.29 (s, 5H). Mononitrosated benzoxycarbonylalanylglycine ethyl ester (16b): ^{1}H NMR δ 1.25 (t, J=7.2, 3H), 1.47 (d, J=6.5, 3H), 4.05 (q, J=7.2, 2H), 4.40 (s, 2H), 5.05 (s, 2H), 5.55 (q, J=6.5, 1H), 5.60 (br d, 1H), 7.25 (s, 5H). Dinitrosated tert-butoxycarbonylalanylglycine ethyl ester (17ab): ^{1}H NMR 1.25 (t, J=7.2, 3H), 1.53 (d, J=6.8, 3H), 1.56 (s, 9H), 4.05 (q, J=7.2, 2H), 4.50 (s, 2H), 5.98 (q, J=6.8, 1H). Mononitrosated tert-butoxycarbonylalanylglycine ethyl ester(17b): ^{1}H NMR δ 1.25 (t, J=7.2, 3H), 1.46 (s, 9H), 1.47 (d, J=6.5, 3H), 4.05 (q, J=7.2, 2H), 4.50 (s, 2H), 5.55 (q, J=6.5, 1H), 5.60 (br d, 1H). Dinitrosated benzoxycarbonylalanylalanine methyl ester (18ab): ^{1}H NMR δ 1.18 (d, J=6.5, 3H), 1.50 (d, J=6.5, 3H), 3.58 (s, 3H), 5.10(q, J=6.5, 1H, N(NO)C$\underline{H}$COO), 5.40 (s, 2H), 5.87 (q, J=6.5, 1H, N(NO)C$\underline{H}$CON), 7.28 (s, 5H). Mononitrosated benzoxycarbonylalanylalanine methyl ester (18b): ^{1}H NMR δ 1.28 (d, J=7.0, 3H), 1.48 (d, J=6.8 3H), 3.55 (s, 3H), 5.05 (s, 2H), 5.20 (q, J=6.8, 1H), 5.55 (m, 1H), 5.90 (br d, 1H), 7.22 (s, 5H). Dinitrosated benzoylglycylphenylalanine methyl ester (19ab): ^{1}H NMR δ 3.07, 3.43, 5.51 (ABX system, JAB=14.3, JAX=12.0, JBX=4.0), 3.72 (s, 3H), 5.33 (center of an AB system, N(NO)$\underline{C}H_2$CON), 6.9-8.0 (m, 10H); ^{13}C NMR δ 33.6 (Ph$\underline{C}H_2$), 41.8 (N(NO)$\underline{C}H_2$), 52.9 (OCH_3 and C$\underline{H}$COOMe; separated by means of a DEPT experiment), 127.3, 128.3, 128.8, 128.9, 130.9, 132.1, 133.0, 135.4 (aromatic) 167.1 (COO), 168.0 (CON(NO)), 171.9 (PhCO); IR 1750, 1710. Mononitrosated DL-benzoylphenylalanylglycine ethyl ester (20b): ^{1}H NMR 1.25 (t, J=7.2, 3H), 3.21, 3.42 (AB part of an ABX system, JAB=14.2, JAX=5.0, JBX=3.2), 4.05 (q, J=7.2, 2H), 4.50 (center of an AB system, $\underline{C}H_2$COO), 6.35 (m, CONHCH), 6.90 (d, J=7.8, CONHCH); ^{13}C NMR δ 14.0 ($CH_2\underline{C}H_3$), 39.9 (N(NO)$\underline{C}H_2$CO), 38.2 (Ph$\underline{C}H_2$), 53.0 (NH$\underline{C}$HCO), 62.1 ($\underline{O}CH_2$), 127.1, 127.5, 128.6, 128.8, 129.5, 132.0, 133.5, 135.0 (aromatic), 165.0 (COO), 167.1 (Ph$\underline{C}$O), 174.9 (CON(NO)). Dinitrosated benzoxycarbonylphenylalanylglycine methyl ester (21ab): ^{1}H NMR δ 3.1-3.4 (m, 2H),

3.60 (s, 3H), 4.35 (AB system), 5,35 (s, 2H), 6.35 (m, 1H), 7.1-7.5 (m, 10H). Mononitrosated benzoxycarbonylphenylalanylglycine methyl ester (21b): ^{1}H NMR δ 3.1-3.4 (m, 2H), 3.60 (s, 3H), 4.35 (AB system), 5.05 (s, 2H), 6.10 (m, 1H), 6.70 (d, J=6.0, NHCH), 7.1-7.5 (m, 10H). Mononitrosated benzoylglycylvaline methyl ester (22a): ^{1}H NMR δ 0.88 (d, J=7.0, 3H), 0.91 (d, J=7.0, 3H), 1.9-2.3 (m, 1H), 3.60 (s, 3H), 4.3-4.6 (m, 1H), 4.60 (s, 2H), 6.60 (d, J=8.0, NHCH), 7.2-7.8 (m, 5H); ^{13}C NMR δ 17.8, 18.9 ($(CH_3)_2CH$), 31.5 ($(CH_3)_2CH$), 42.2 ($N(NO)CH_2$), 52.4 (OCH_3), 57.4 (NHCH), 128.2, 130.9, 132.4, 132.8 (aromatic), 164.3 (CONH), 172.3 (COO), 172.5 (CON(NO)). Mononitrosated tert-butoxyalanylleucine methyl ester (23a): ^{1}H NMR δ 0.90 (d, J=5, 6H), 1.35 (d, J=7, 3H), 1.40 (s, 9H), 1.4-1.8 (m, 3H), 3.68 (s, 3H), 4.3-4.7 (m, 1H), 5.20 (br q, 1H), 6.90 (d, J=8, 1H). Dinitrosated DL-benzoylphenylalanylglycylglycine ethyl ester (25bc): ^{1}H NMR 1.21 (t, J=7.0, 3H), 3.2-3.6 (m, 2H), 4.10 (q, J=7, 2H), 4.40 (s, 2H), 5.34 (s, 2H), 6.2-6.6 (m, 1H), 6.90 (d, J=8.5, 1H), 7.1-7.8 (m, 10H). Trinitrosated (benzoxycarbonil)phenylalanylglycylleucine methyl ester (26abc): ^{1}H NMR 0.85 (d, J=6.5, 3H), 0.87 (d, J=6.5, 3H), 1.2-1.9 (m, 3H), 3.2-3.5 (m, AB part of an ABX system), 3.64 (s, 3H), 5.10-5.25 (AB system), 5.20 (m, 1H), 5.40 (s, 2H), 6.37 (m, X part of the ABX system), 7.1-7.5 (m, 10 H). Dinitrosated (benzoxycarbonyl)-phenylalanylglycylleucine methyl ester (26bc): ^{1}H NMR δ 0.85 (d, J=6.5, 3H), 0.87 (d, J=6.5, 3H), 1.2-1.9 (m, 3H), 3.2-3.5 (m, AB part of an ABX system), 3.65 (s, 3H), 5.05 (s, 2H), 5.20 (m, 1H), 5.30 (s, 2H), 5.50 (br d, 1H), 6.10 (m, X part of the ABX system), 7.1-7.5 (m, 10H).

Nitrosation of N-Methylpropanamide / N-Methylbenzamide / O-Benzyl-N-methylcarbamate Mixtures-(i) A mixture of 226 mg (2.59 mmols) of N-methylpropanamide and 350 mg (2.59 mmols) of N-methylbenzamide in 50 mL of CH_2Cl_2 was submitted to the usual nitrosation conditions for a few min. The ^{1}H NMR spectrum of the crude product showed 75% of nitrosation for the alifatic amide and 90-95% for the aromatic amide. (ii) In a similar way, an equimolar mixture of N-methylbenzamide and O-benzyl-N-methylcarbamate yielded 12% of nitrosation for the benzamide and 56% for the carbamate. (iii) Finally, an equimolar mixture of N-methylpropanamide, N-methylbenzamide, and O-benzyl-N-methylcarbamate gave their corresponding N-nitroso compounds in 53%, 73%, and 90% yields, respectively.

Denitrosation——A solution of 227 mg of 5a in 80 mL of CH_2Cl_2 was treated with a stream of HCl(g) for 20 min at 0 °C, the light yellow solution becoming quickly dark yellow; evaporation in vacuo yielded 5 (189 mg, 96%) as a colourless oil. In a similar way, 223 mg of a mixture of 16ab and 16b in a 42:58 ratio gave 183 mg (93%) of 16.

Reaction of Nitroso Compounds with Pyrrolidine——All the nitrosated products (1a-26bc) were treated with an excess of pyrrolidine in CH_2Cl_2, a colour change being almost instantaneously observed. The reactions were checked by TLC (with CH_2Cl_2 or CH_2Cl_2-MeOH 9:1 as the eluents), by comparing the products with authentic samples. In some cases, the resulting α-diazo compounds were isolated by "flash" chromatography (see Preparation of α-Diazo Compounds). In most cases, the reaction was also followed by HPLC.

In a typical procedure, 10 mg of the nitrosated peptide in 1 mL of CH_2Cl_2 were treated with an excess (one drop) of pyrrolidine either at 0 °C or at room temperature. After ca. 5 min, a TLC anc-dicated the complete conversion of nitroso compounds 4a, 5a, 13a, 15ab, 19ab, 20b, and 25bc to the following derivatives: 4a ⟶ ethyl diazoalaninate (volume retention: 11.4 mL); 5a ⟶ methyl diazo-leucinate (20.6 mL); 13a ⟶ methyl diazovalinate (17.0 mL); 15ab ⟶ N-benzoylpyrrolidine (7.2 mL), α-diazo-N,N-tetramethyleneacetamide (6.2 mL), and methyl diazoglycinate (5.7 mL); 19ab ⟶ N-benzoylpyrrolidine, α-diazo-N,N-tetrametileneacetamide, and methyl diazophenylalaninate (19.8 mL); 20b ⟶ (N,N-tetramethylene)benzoylphenylalaninamide (19.4 mL) and ethyl diazoglycinate (7.1 mL); 25bc ⟶ (N,N-tetramethylene)benzoylphenylalaninamide, α-diazo-N,N-tetramethyleneacetamide, and ethyl diazoglycinate.

Preparation of α-Diazo Compounds——Ethyl diazoacetate, ethyl 2-diazopropanoate, methyl 2-diazo-4-methylpentanoate, methyl 2-diazo-3-phenylpropanoate, and methyl 2-diazo-3-methylbutanoate were prepared from 1a, 4a, 5a, 9a, and 13a, respectively, according to the following procedure: 200-400 mg of the nitroso compound were treated with 1.1 equiv. of pyrrolidine in 20-30 mL of CH_2Cl_2 at room temperature. After 5 min, the reaction mixture was separated by "flash" chromatography (CH_2Cl_2), the yellow fraction being collected. Elimination of the solvent under vacuum afforded the diazo esters in 57-74% yields. Ethyl diazoacetate: ^{1}H NMR δ 1.28 (t, J=7.0, 3H), 4.15 (q, J=7.0, 2H), 4.65 (s, 1H); IR 2110, 1705. Ethyl 2-diazopropanoate: ^{1}H NMR δ 1.30 (t, J=7.0, 3H), 1.92 (s, 3H), 4.20 (q, J=7.0, 2H); IR 2100, 1700. Methyl 2-diazo-4-methylpentanoate: ^{1}H NMR δ 0.95 (d, J=6.5, 6H), 1.4-1.8 (m, 1H), 2.30 (d, J=6.0, 2H), 3.65 (s, 3H); IR 2100, 1695. Methyl 2-diazo-3-phenylpropanoate: ^{1}H NMR δ 3.57 (s, 2H), 3.72 (s, 3H), 7.18 (s, 5H); IR 2100, 1695. Methyl 2-diazo-3-methylbutanoate: ^{1}H NMR δ 1.12 (d, J=6.5, 6H), 2.65 (m, J=6.5, 1H), 3.65 (s, 3H); IR 2100, 1690. 2-Diazo-N,N-tetramethyleneacetamide was prepared from 3a (116 mg, 0.47 mmols), solved in 30 mL of CH_2Cl_2, and NaMeO-MeOH (0.1 M, 2 mL); after 5 min, the mixture was separated by "flash" chromatography with CH_2Cl_2 to afford 36 mg (62%) of a yellow oil: ^{1}H NMR δ 1.85 (m, 4H), 3.35 (m, 4H), 4.70 (s, 1H); IR 2100, 1605.

Reaction of 2a with Methyl Leucinate——Compound 2a (177 mg, 0.80 mmols) and methyl leucinate (145 mg, 1.0 mmol) were refluxed in 50 mL of CH_2Cl_2 for 3 days or in 50 mL of $CHCl_3$ overnight. (In both cases, TLC indicated the disappearance of 2a and the formation of methyl diazoacetate and methyl benzoylleucinate, as compared with authentic samples.) After washing the final solutions with cold diluted HCl aq and water, drying them over Na_2SO_4, and removing the solvents and volatile products in vacuo, 219 mg (95%) of chromatographically pure methyl benzoylleucinate were obtained.

Reaction of 20b with Ethyl Glycylglycinate—Compound 20b (50 mg, 0.14 mmols) and ethyl glycylglycinate (25 mg, 0.16 mmols) in 20 mL of solvent, working as above, afforded quantitatively ethyl benzoylphenylalanylglycylglycinate (25).

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(7) It is generally accepted[3] that electrophilic attacks to CONH bonds take first place on the oxygen and are followed by a rapid O- to N- rearrangement of the electrophile. This may explain the facts commented in (iii).

(8) Another set of experiments corroborated that the conversions occurred as outlined in the Figure: (i) when a drop of AcOH was added to a pure sample of 16ab in $CDCl_3$, denitrosation of the carbamate function to give 16b took place in a few min at the NMR-probe temperature (ca. 30 °C); AcOH was not able, however, to denitrosate significantly the amide group (16b ⟶ 16) after 5 days; (ii) an equimolar mixture of 16ab and 16 in $CDCl_3$ was not transformed into 16a and 16b in the absence of N_2O_4/AcOH/NaAcO; an equivalent amount of AcOH denitrosated 16ab to 16b, but 16 was not nitrosated. No efforts have been done, at present, to investigate whether some intramolecular transnitrosation (16a ⟶ 16b) takes also place or not under the reaction conditions.

(9) F. Urpí, J. Vilarrasa, unpublished results.

(10) The reaction of tetraethylenepentamine with ethyl N-acetyl-N-nitrosoglycinate aimed at preparing ethyl diazoglycinate was reported by White and Baumgarten.[4] For studies on the catalysis of bases such as imidazole, lysine, or arginine regarding the hydrolysis of N-alkyl-N-nitrosocarboxamides, see: C. N. Berry, B. C. Challis, A. D. Gribble, S. P. Jones, in "N-Nitroso Compounds", ACS Symposium Series-174, R. A. Scalan, S. R. Tannenbaum, Eds., American Chemical Society, Washington, 1981.

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(12) Diazo esters are reasonably stable in the presence of the excess of pyrrolidine: the HPLC peaks of the diazo compounds did not decrease after maintaining the reaction mixture in cold for a day.