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Formation and Chemical Characterization of Some Nitroso Dipeptides N Terminal in Proline

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The reaction products from sodium nitrite in dilute acid and six dipeptides that were N terminal in proline were investigated. Chemical identity of the reaction products was based on spectral characteristics using ultraviolet, infrared, and mass spectrometry correlated with spot tests obtained from thin-layer chromatographic plates. The nitrosation products from the dipeptides were *N*-nitroso dipeptides with the nitroso group on the imino nitrogen.

Dipeptides are biologically important compounds that occur in foods and can be formed upon digestion of proteins in the upper gastrointestinal tract. The potential for formation of *N*-nitroso derivatives of peptides in foods would be of considerable concern since *N*-nitrosamines and *N*-nitrosamides represent two classes of potent carcinogens (Magee et al., 1976).

Several investigators have considered the possibility of nitrosation of amide bonds. White (1955) and Bonnett and Nicolaidou (1977) demonstrated that the amide linkage is relatively unreactive toward nitrosation by nitrite in aqueous solution. Kakuda and Gray (1980) reported that N-nitrosation of amides decreased rapidly as the pH increased, and little reaction occurred above pH 3. Pollock (1982) reported nitrosation of the peptide bond in a series of dipeptides. These same investigators, however, have more recently claimed that the reaction products were (*N*-nitrosoimino)alkanoic acids rather than *N*-nitroso peptides (Outram and Pollock, 1983).

Bonnett and Nicolaidou (1977) suggested that peptides with the amino acids proline, hydroxyproline, or sarcosine in the N-terminal position could form stable *N*-nitroso derivatives. For this investigation we chose to study the formation of *N*-nitroso derivatives of a number of peptides that are N terminal in proline. Such dipeptides would be expected to undergo nitrosation at the imino nitrogen to produce a stable *N*-nitrosamine. These compounds have neither been synthesized nor been characterized. The existence in nature of *N*-nitroso derivatives of dipeptides N terminal in proline has not been reported, and the carcinogenic properties of these compounds are not known. The purpose of this study was to synthesize and characterize products from the nitrosation of dipeptides N terminal in proline.

EXPERIMENTAL SECTION

Chemicals. L-Proline (Pro), L-prolylglycine (Pro-Gly), L-prolylhydroxyproline (Pro-Hyp), L-prolylisoleucine (Pro-Ile), L-prolylphenylalanine (Pro-Phe), L-glycylglycine (Gly-Gly), and L-glycylproline (Gly-Pro) were obtained from Aldrich Chemical Co. L-Prolylglutamic acid (Pro-Glu) and L-prolylserine (Pro-Ser) were purchased from Bachem, Inc.

Purity of the above dipeptides was checked by thin-layer chromatography on silica gel 60, F-254, using propanol-water (7:3) as a developing system. Griess and ninhydrin reagents were used separately to visualize the plates. Commercial dipeptides were free of *N*-nitroso compounds and free amino acids. The lower limit for detection of *N*-nitroso compounds and free amino acids in the dipeptides was 1%. All solvents were analytical grade.

Synthesis of *N*-Nitroso Derivatives of Dipeptides. The method of Hansen et al. (1974) previously developed for the synthesis of *N*-nitrosoamino acids was modified for the synthesis of *N*-nitroso derivatives of dipeptides. One gram of dipeptide was dissolved in 50 mL of water. Ten grams of sodium nitrite was added, and the solution was acidified to pH 3 with hydrochloric acid. Since Pro-Phe and Pro-Ile were not soluble in water, they were added directly to the acidified sodium nitrite solution. The reaction was allowed to proceed in the dark while being stirred at room temperature for 3 h. The mixture was then acidified to pH 1, water was evaporated under reduced

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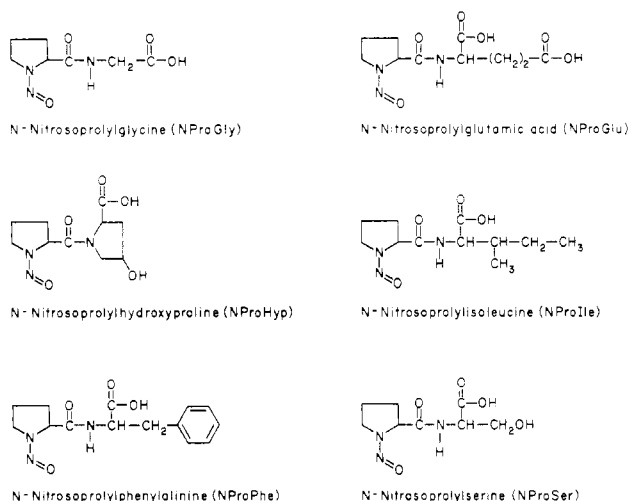
Figure 1. Structure of N-nitroso derivatives of dipeptides N terminal in proline.

pressure, and the residual solids were extracted with four 25-mL portions of acetone. The extract was dried over sodium sulfate and filtered, and the acetone was removed by rotary evaporation at reduced pressure. Each compound was placed in a vacuum desiccator over potassium hydroxide and stored overnight.

Chemical Characterization of Reactants and Products. Dipeptides and reaction products were chromatographed on 0.25 mm thick silica gel 60F-254 plates (20 × 20 cm or 20 × 10 cm) by using propanol–water (7:3 v/v) as a mobile phase. The plates were subjected to UV light and then sprayed with Griess reagent to give a pink spot for the N-nitroso derivatives of the dipeptides. Ninhydrin in acetone (0.4%) was used for color development of dipeptides and free amino acids.

Thin-layer chromatography (TLC), ultraviolet spectroscopy (UV), infrared spectroscopy (IR), and mass spectrometry (MS) were used to characterize the dipeptides and the N-nitroso derivatives. The structures are shown in Figure 1. UV absorption spectra of the N-nitroso dipeptides were taken on a Perkin-Elmer 550 spectrophotometer in the range 400–190 nm. Dipeptides and their N-nitroso derivatives were analyzed for IR absorption neat between NaCl disks or as a Nujol mull using a Beckman Model IR-18A spectrophotometer. Most MS spectra were obtained by using electron impact (EI) on a Finnigan Model 1015C quadrupole. Samples were introduced with the solid probe inlet. Data were acquired and processed by using a Riber 400 data system. Those N-nitroso dipeptides that failed to yield a discernible molecular ion under EI were run on a Finnigan Model 4023 quadrupole by using positive ion–negative ion chemical ionization (PINICI) with methane as the reactant gas. Data were acquired and processed by using the INCOS data system.

Safety Precautions. Precautions were taken in the handling of N-nitroso derivatives of dipeptides to prevent inhalation and skin contact. Gloves were used and work was done in efficient fume hoods whenever possible. All glassware exposed to N-nitroso compounds was decontaminated with 5% HBr in acetic acid.

RESULTS

All six N-nitroso dipeptides were yellow oils that were highly soluble in water and a variety of polar organic solvents. Methods used for identification indicated that the products possessed properties of N-nitrosamines.

Thin-Layer Chromatography. Table I summarized the TLC qualitative tests used to characterize the N-

Table I. Analysis of N-Nitroso Derivatives of Dipeptides by TLC

compound	UV light[a]	Griess test[b]	ninhydrin
NPro-Gly	+	+	<1% Pro-Gly
NPro-Glu	+	+	
NPro-Hyp	+	+	
NPro-Ile	+	+	
NPro-Phe	+	+	
NPro-Ser	+	+	<1% Pro-Ser
Gly-Gly (after nitrosation)	–	–	
Gly-Pro (after nitrosation)	–	–	

[a] (+) means fluorescent reaction. [b] (+) means development of a pink color.

Table II. Absorption Maxima of N-Nitroso Derivatives of Dipeptides

compound	nm
NPro-Gly	344; 238
NPro-Glu	344; 226
NPro-Hyp	344; 234
NPro-Ile	343; 234
NPro-Phe	342; 228
NPro-Ser	344; 229
Gly-Gly (after nitrosation)	225
Gly-Pro (after nitrosation)	225

nitroso derivatives of the dipeptides. The results indicate that the nitrosated derivatives contain a N-nitroso group since they reacted positively to Griess reagent and they fluoresced under UV light. In contrast nitrosated dipeptides without Pro in the N-terminal position reacted negatively to these tests. This indicates that the imino group in the Pro but not the peptide bond was derivatized under these conditions. The N-nitroso derivatives of the peptides had less than 1% contamination with unreacted dipeptide as demonstrated by the ninhydrin tests. In addition, the inability to detect the presence of the N-nitrosoproline indicates that no hydrolysis of peptide bonds occurred under the reaction and workup conditions.

Ultraviolet Spectroscopy. N-Nitroso derivatives of dipeptides show two absorption bands in water, one with a maximum near 345 nm and the other near 230 nm (Table II).

The results show that the compounds are N-nitroso derivatives as they have an absorption band near 345 nm, indicating the existence of the N=O group (n → π^*), and near 230 nm, demonstrating the existence of the N=N group (π → π^*). The absorption maximum near 345 nm is the most important since other compounds such as nitrite, peptides, and amino acids absorb in the region of 230 nm. Lack of an absorption maximum for nitrosated Gly-Gly and Gly-Pro near 345 nm confirmed the results obtained by TLC, namely, that these peptides do not form N-nitroso compounds.

Infrared Spectroscopy. The identity of N-nitroso derivatives of dipeptides was confirmed by obtaining IR spectra of the dipeptides and the N-nitroso dipeptides. The most important bands are presented in Table III.

Nitrosation of dipeptides to their N-nitroso derivatives caused the disappearance of the N–H stretch (near 3080 cm⁻¹) from the pyrrolidine ring and the appearance of the N=O stretch (near 1430 cm⁻¹). In addition, the fact that the peptide bond NH stretch remained after nitrosation indicates that the peptide bond does not undergo nitrosation.

Mass Spectrometry. Mass spectral data for the six N-nitroso dipeptides are presented in Table IV. The EI

Table III. Major Characteristic IR Absorption Bands of Dipeptides and the Corresponding *N*-Nitroso Dipeptides (cm⁻¹)

compound	NH stretch from five-membered ring	NH stretch from peptide bond	N=O stretch
Pro-Gly	3080	3280	
NPro-Gly		3320	1430
Pro-Glu	3080	3260	
NPro-Glu		3310	1430
Pro-Hyp	*a*		
NPro-Hyp			1430
Pro-Ile	3080	3260	
NPro-Ile		3330	1430
Pro-Phe	3090	3230	
NPro-Phe		3310	1430
Pro-Ser	3080	3260	
NPro-Ser		3320	1430

a Obscured by OH stretch.

spectra were condensed by the method of Hertz et al. (1971). Since it was considered crucial to observe the molecular ion, the four samples that gave very weak or missing molecular ions with EI were also examined by CI. For the CI data only four ions for each sample are reported. From the positive ion spectra M + 1 and M − 30 are given, and for the negative ion spectra M − 1 and M − 30 are given. The CI tended to compliment the EI data, particularly at higher masses. The negative ion CI peaks at high mass were diagnostically useful and of moderate intensity. The EI spectra generally exhibited a weak or missing M⁺; the M − 30 ion was usually present in EI spectra and was more diagnostically useful.

DISCUSSION

The results of this investigation show that peptides that are N terminal in Pro can be easily nitrosated on the imino nitrogen to produce the corresponding stable *N*-nitroso derivatives. The peptide bonds in the dipeptides did not form stable *N*-nitrosamides under our conditions of nitrosation and workup. The results of our work, however, do not preclude the possibility that *N*-nitroso peptides could be formed under different conditions than those used in our experiments. In fact Challis et al. (1983) very recently claimed to have synthesized and characterized the first authentic *N*-nitroso peptide. *N*-(*N*-Acetylprolyl)-*N*-nitrosoglycine was formed by nitrosation and hydrogenolysis of *N*-(*N*-acetylprolyl)benzylglycine, a compound on which nitrosation of the imino nitrogen had been blocked. The authors first synthesized the *N*-nitroso peptide in methylene chloride, but these investigators also described experiments using aqueous media in which they claimed to have studied the rate of formation and decomposition of the *N*-nitroso peptide.

It is apparent that additional research will be required before we will have a satisfactory understanding of the conditions effecting *N*-nitroso peptide formation and stability. Additional work will be required, for instance, in order to allow an accurate prediction of the possible hazard of *N*-nitroso peptide formation in the gastrointestinal tract.

The research described in this paper also raises the question as to whether peptides N terminal in Pro can be nitrosated on the imino nitrogen in food systems or in the acidic environment of the human stomach. Future work in our laboratory will be directed toward studying the rate of nitrosation of dipeptides N terminal in Pro.

Table IV. Mass Spectra of *N*-Nitroso Dipeptides[a–d]

NPro-Gly

EI: 28 (93), 30 (77), 41 (97), 42 (94), 55 (35), 56 (93), 69 (99), 70 (99), 83 (12), 84 (11), 99 (100), 100 (18), 110 (4), 111 (6), 125 (3), 127 (4), 153 (1), 154 (1), 171 (30), 172 (3), *201* (1)[c]

CI: not needed

NPro-Glu

EI: 28 (51), 29 (17), 41 (52), 42 (21), 55 (12), 56 (9), 70 (100), 71 (20), 84 (18), 85 (15), 99 (22), 100 (5), 110 (5), 114 (1), 128 (1), 137 (0.6), 146 (0.7), 151 (0.4), 156 (0.9), 165 (0.2), 174 (0.2), 179 (0.2), 197 (0.8), 198 (0.2), 206 (0.2), 208 (0.2), 225 (1), 226 (0.3), 243 (1), 244 (0.2), *273* (0.1)[c]

CI
positive ions: 274, M + 1 (8)
243, M − 30 (59)
negative ions: 272, M − 1 (5)
243, M − 30 (98)

NPro-Hyp

EI: 28 (50), 30 (51), 41 (73), 43 (42), 55 (24), 56 (20), 68 (62), 70 (100), 84 (5), 86 (17), 96 (6), 99 (17), 112 (2), 124 (5), 126 (1), 130 (12), 140 (1), 144 (0.9), 158 (0.6), 165 (1), 181 (2), 182 (1), 188 (0.3), 192 (0.4), 209 (1), 210 (3), 227 (3), 228 (0.4), *257* (0.1)[c]

CI
positive ions: 258, M + 1 (0.2)
227, M − 30 (30)
negative ions: 256, M − 1 (19)
227, M − 30 (100)

NPro-Ile

EI: 28 (47), 29 (28), 30 (28), 41 (82), 43 (46), 55 (18), 57 (10), 69 (68), 70 (100), 83 (3), 86 (11), 99 (50), 100 (17), 112 (10), 113 (2), 125 (1), 130 (16), 140 (0.6), 153 (0.6), 158 (0.7), 169 (0.2), 171 (0.3), 181 (1), 212 (0.1), 227 (5), 228 (1), *257* (0.2)[c]

CI: not needed

NPro-Phe

EI: 28 (94), 30 (42), 41 (71), 43 (69), 51 (16), 55 (14), 70 (100), 71 (84), 91 (72), 92 (19), 99 (42), 103 (15), 118 (4), 120 (13), 128 (6), 131 (5), 146 (16), 147 (10), 164 (1), 166 (0.8), 174 (2), 182 (0.7), 187 (0.4), 192 (3), 206 (0.2), 214 (0.2), 215 (0.7), 217 (0.4), 261 (8), 262 (1), 275 (0.1), *291* (0)[c]

CI
positive ions: 292, M + 1 (10)
261, M − 30 (12)
negative ions: 290, M − 1 (38)
261, M − 30 (100)

NPro-Ser

EI: 28 (35), 30 (54), 43 (96), 44 (100), 55 (10), 60 (8), 69 (52), 70 (90), 83 (5), 86 (8), 99 (70), 100 (9), 113 (2), 114 (2), 127 (2), 132 (5), 141 (0.9), 144 (2), 155 (0.7), 156 (0.3), 171 (0.7), 183 (0.8), 185 (0.5), 186 (0.2), 201 (2), 202 (0.4), *231* (0.1)[c]

CI: not needed

a Mass spectra were condensed by the method of Hertz et al. (1971). *b* *m/z* (rel intensity). *c* Molecular ion italicized. *d* Ions <0.1% not reported.

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Kinetics of Nitrosation of Four Dipeptides N Terminal in Proline

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The kinetics of nitrosation for the imino nitrogen of dipeptides N terminal in proline were studied. The pH optima for nitrosation of four dipeptides were, for Pro-Gly, pH 2.7, for Pro-Hyp, pH 2.7, for Pro-Ser, pH 2.9, and, for Pro-Glu, pH 3.0. On the basis of a determination of the initial rates of nitrosation, the pH-dependent rate constants at optimal pH and 25 °C were 0.26 M^{-2} s^{-1} for Pro-Gly, 0.19 M^{-2} s^{-1} for Pro-Hyp, 0.29 M^{-2} s^{-1} for Pro-Glu, and 0.18 M^{-2} s^{-1} for Pro-Ser.

Recent studies from our laboratory have shown that the nitrosation products from dipeptides N terminal in proline were N-nitroso dipeptides with the nitroso group on the imino nitrogen, (Kubacka et al., 1984). Mirvish et al. (1973) examined the nitrosation of prolyglycine and found that the nitrosation kinetics obeyed the rate expression

$$\text{rate} = k_1[\text{amine}][\text{nitrite}]^2 \qquad (1)$$

According to eq 1, the pH-dependent rate constant k_1 is a function of the total concentrations of amine and nitrite. Mirvish et al. (1973) did not synthesize the nitroso derivative of Pro-Gly; rather, they used the molar absorptivity for N-nitrosoproline in their investigation. On this basis they reported a pH-dependent rate constant for N-nitrosoprolylglycine of 0.25 M^{-2} s^{-1} at pH 3.0, which is approximately 7 times larger than the pH-dependent rate constant for proline. This suggests that dipeptides N terminal in proline might undergo nitrosation more rapidly than free proline.

The purpose of this study was to determine the nitrosation kinetics of four dipeptides that are N terminal in proline: prolylglycine, prolylglutamic acid, prolylhydroxyproline, and prolylserine.

EXPERIMENTAL SECTION

Chemicals. L-Proline (Pro), L-prolylglycine (Pro-Gly), and L-prolylhydroxyproline (Pro-Hyp) were purchased from Aldrich Chemical Co. L-Prolylglutamic acid (Pro-Glu) and L-Prolylserine (Pro-Ser) were obtained from Bachem, Inc. Ammonium sulfamate was obtained from

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J. T. Baker Chemical Co. Seventy percent acid and sodium nitrite were from Mallinckrodt Chemical Works. Nitrosoproline (NPro) was synthesized according to Hansen et al. (1974). All reagents were analytical grade.

Decomposition Rate of HNO₂. An aqueous solution 0.4 M in sodium nitrite was adjusted to pH 3.0 with 70% perchloric acid and placed in a 25 °C water bath. After 0, 5, 10, 15, 30, 45, 60, 90, 120, and 180 min, 2-mL aliquots were transferred to 1 cm path length quartz cells for absorption readings at 359 nm. The experiment was performed twice, and the rate of nitrous acid decomposition was calculated by using a first-order kinetic equation.

Standard Curve for NPro. The following aqueous solutions of NPro were prepared: 6.0, 4.0, 3.0, 2.0, 1.5, 0.5, and 0.25 (all 10^{-3} M). Absorbance readings of the solutions were obtained at 340 nm by using a 1 cm path length quartz cell. The experiment was repeated twice, and a linear regression analysis produced eq 2 with a coefficient

$$y = 110.8x + 15.4 \qquad (2)$$

of determination of 0.99. Our experimental design assumes that the molar absorptivities of nitrosated dipeptides that are N terminal in Pro are essentially the same as the molar absorptivity for NPro. Accordingly, the relationship between absorbance and NPro concentration as expressed by eq 2 was used to determine concentrations of the nitrosated dipeptides.

Assay Procedure. Solutions of sodium nitrite were prepared immediately before use to minimize decomposition. Solutions of dipeptides and sodium nitrite were adjusted to the desired pH with 70% perchloric acid or 1 M sodium hydroxide. The reaction was initiated by pipetting sodium nitrite solution into a reaction vial containing the dipeptide solution. The initial dipeptide concentration at the initiation of the reaction was 0.02 M while the concentration of sodium nitrite was 0.01 M. The reaction was carried out in a constant-temperature bath