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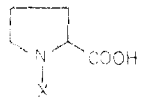
Nitramino Acids. Synthesis and Biological Evaluation of 1-Nitroproline, 1-Nitropipecolic Acid, and *N*-Nitrosarcosine

Herbert T. Nagasawa,* William P. Muldoon, and Frances N. Shirota

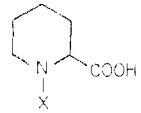
Medical Research Laboratories, Veterans Administration Hospital, and the Department of Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55417. *Received July 11, 1977*

The *N*-nitro derivatives of secondary α-amino acids, viz., 1-nitroproline (**1a**) (L and D), 1-nitro-DL-pipecolic acid (**2a**), and *N*-nitrosarcosine (**3a**), were prepared by the oxidation of the corresponding nitrosamino acids with peroxytrifluoroacetic acid. These nitramino acids (**1a–3a**) were not active against *Escherichia coli*, *Candida albicans*, *Pseudomonas aeruginosa*, or *Mycobacterium smegmatis*, and **1a** and **2a** did not show mutagenic activity in a *Salmonella typhimurium* TA-100 system, with or without added rat liver 9000g supernatant fraction. The marginal mutagenicity of **3a** in this system suggests that additional work should be done to assess its carcinogenic–mutagenic potential.

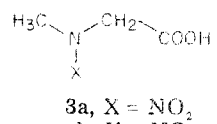
Although the *N*-nitroso derivatives of the naturally occurring cyclic or secondary α-amino acids such as proline and sarcosine, e.g., 1-nitrosoproline (**1b**) and *N*-nitrososarcosine (**3b**), have been known for some time,[1] the corresponding *N*-nitro-*sec*-α-amino acids, e.g., **1a** and **3a**, have not been described. The nitramino acids (**1a–3a**) are chemically related to the nitrosamino acids (**1b–3b**) and differ only in the oxidation state of the exocyclic nitrogen.



1a, X = NO_2
b, X = NO

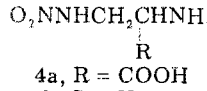


2a, X = NO_2
b, X = NO

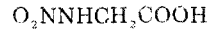


3a, X = NO_2
b, X = NO

Contemporary interest in the nitrosamino acids derives from the possibility of their formation in processed meat and other foodstuff that have been preserved with nitrite,[2] their decarboxylation to *N*-nitroso-*sec*-amines under normal cooking conditions,[3] and the implications of carcinogenic hazards thereby.[4] Nitrosamines such as dimethylnitrosamine and 1-nitrosopyrrolidine are highly carcinogenic[5] and have also been shown to be mutagenic.[6] Biochemical rationale for the formation in food of nitramino acids or their decarboxylation products, the nitramines themselves, has not been advanced. However, the natural occurrence of β-nitraminoalanine (**4a**), the *N*-nitro derivative of β-aminoalanine, and its decarboxylated product, *N*-nitroethylenediamine (**4b**), in mushrooms (*Agaricus silvaticus*) appears to be well documented[7] and the production of *N*-nitroglycine (**5**) by *Streptomyces norsei* has also been described.[8]



4a, R = COOH
b, R = H



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Whether these nitramino acids can be reduced in vivo to the corresponding nitrosamino acids, e.g., **1a** to **1b**, by mammalian enzymes is not known. It appears possible that bacterial systems might effect such reductions on account of the ubiquitous presence of nitro,[9] nitrate, and nitrite reductases[10] in bacteria. In vivo decarboxylation of the nitramino acids to secondary nitramines should also be considered. The recent report that dimethylnitramine is carcinogenic to rodents[11] suggests that such decarboxylations may yield highly toxic products. Moreover, many C-nitro heterocyclic compounds, while possessing potent antibacterial, antiprotozoal, and anthelminthic properties,[12] also elicit disturbing degrees of mutagenic activities which are correlative with their carcinogenicities.[13] These considerations prompted us to examine the titled nitramino acids for (a) growth inhibition against a cross section of clinically endemic bacteria and fungi in culture and (b) mutagenicity in the *Salmonella typhimurium* system of Ames et al.[14] The preparation of the nitramino acids, 1-nitro-L- and -D-proline (L- and D-**1a**), 1-nitro-DL-pipecolic acid (**2a**), and *N*-nitrosarcosine (**3a**), is presented herewith, together with an evaluation of their biological properties.

Chemistry. All attempts to N-nitrate proline directly with nitronium tetrafluoroborate[15] in the presence of pyridine in the manner used to N-nitrosate this cyclic amino acid with nitrosyl tetrafluoroborate[16] were unsuccessful, possibly due to competitive ring-opening reactions undergone by pyridine with this reagent.[15] However, use of other amines such as tri-*n*-butylamine or dimethylaniline in highly polar solvents such as nitromethane or sulfolane did not give **1** nor did the use of the more soluble nitronium hexafluorophosphate (NO_2PF_6).[17] Direct nitration of L-proline with HNO_3–H_2SO_4 or HNO_3–Ac_2O was also unsuccessful.

The nitramino acids (**1a–3a**) were successfully prepared by oxidation of the corresponding nitrosamino acids (**1b–3b**, respectively)[16] with peroxytrifluoroacetic acid. We were unable to prepare 1-nitro-L-azetidine-2-carboxylic acid by this method due to the instability of the 1-nitroso-L-azetidine-2-carboxylic acid under these strongly acid conditions. The Emmons procedure[18] for the oxidation of nitrosamines to nitramines required some modification (procedure B) due to the water solubility of these nitramino acids. For the more lipophilic 1-nitropipecolic acid (**3a**), a standard workup procedure (procedure A) gave satisfactory yields (Table I). Use of less potent oxidizing agents such as *m*-chloroperoxybenzoic acid or peroxyacetic acid gave only low yields of the desired products.

The electron-ionization mass spectra (EI–MS) of these nitramino acids gave molecular ions ($M^{+\cdot}$) of only feeble and sometimes undiscernible intensities, but all gave characteristic (and therefore diagnostic) fragment ions at M – 45 $[M - CO_2H]^+$, M – 75 $[M - CO_2H - NO]^{+\cdot}$, and M – 91 $[M - CO_2H - NO_2]^{+\cdot}$ (Table I). These fragmentation paths were verified by the presence of appropriate metastable peaks. In addition, trace amounts of fragment ions

Table I. Nitramino Acids

Compd	Formula	Procedure	Yield, %	Recrystn solvent	Mp, °C	Analyses	IR (NO_2), cm^{-1}	UV_{max}, MeOH nm	UV_{max}, MeOH Log ε	$[\alpha]^{25}D$, deg	EI-MS, m/e (rel intensity) $[M-45]^+$	EI-MS, m/e (rel intensity) $[M-75]^+\cdot$	EI-MS, m/e (rel intensity) $[M-91]^+\cdot$	CI-MS, m/e (rel intensity) MH^+	CI-MS, m/e (rel intensity) $[MH-16]^+$	LD_{50} (mice), mmol/kg
L-1a	$C_5H_8N_2O_4$	A	70	Et_2O–hexane	98–99	C, H, N	1490, 1300	238	3.87	−126.1	115 (100)	85 (91.6)	69 (46.3)	161 (100)	145 (15)	1.17 (0.77–1.63)[a]
		B	80													
D-1a	$C_5H_8N_2O_4$	A	40	Et_2O–hexane	99–100	C, H, N	1490, 1300	238	3.87	+126.0						1.39[b]
		B	49													
2a	$C_6H_{10}N_2O_4$	A	72	Et_2O–hexane	125–126	C, H, N	1525, 1300	246	3.78		129 (53.6)	99 (7.3)	83 (100)	175 (100)	159 (86)	2.10 (1.53–4.47)[a]
		B	73													
3a	$C_3H_6N_2O_4$	A	67.4	$CHCl_3$	98–99	C, H, N	1525, 1285	240	3.79		89 (91.2)	59 (5.9)	43 (75.0)	135 (100)	119 (22)	1.84 (1.04–3.38)[a]
		B	78													

[a] 95% confidence limits. [b] Fiducial limits could not be calculated here because of the variable responses observed at the higher dose levels. Represents range of data from six groups of mice, five mice per group.

represented by $[M-16]^+\cdot$ were present. Since this [M − 16] ion was also present in the mass spectrum of gas chromatographically purified 1-nitropyrrolidine (i.e., by GC–MS), the [M − 16] ions in the EI–MS of the nitramino acids did not represent the molecular ions of the corresponding nitrosamino acids which might conceivably have been present as trace components. The chemical ionization mass spectra (CI–MS) of the nitramino acids were equally decisive in structural elucidations. Quasimolecular ions (MH^+) were present in abundance and the most prominent fragment ions were those corresponding to the loss of elemental oxygen from these quasimolecular ions, viz., $[MH-16]^+$. These nitramino acids absorbed maximally in the UV between 240 and 245 nm and had infrared absorptions near 1500 cm^{-1} (sym) and near 1300 cm^{-1} (unsym) (Table II) characteristic for nitramines.[19]

1-Nitro-L-proline (L-**1a**), a representative example of this series, behaved like a typical organic acid and unlike an amino acid. It was stable to acid, even when heated on the steam bath in 4 N HCl for 12 h. However, under strongly alkaline conditions ninhydrin chromogenic decomposition products were formed from L-**1a**, presumably by the loss of the elements of HNO_2, a normal elimination reaction for secondary nitramines.[20]

Catalytic hydrogenolysis of the *N*-nitro group of **1a** gave L-proline with 97% retention of optical purity, indicating that the integrity of the single chiral center α to the carboxyl group was preserved during the oxidation of L-**1b** to L-**1a**, as well as during the preceding nitrosation reaction. It has been shown that nitrosation of L-proline with nitrosyl tetrafluoroborate and pyridine[16] or with nitrous acid[21] gives optically active **1b**, but of unspecified chiral purity.

Biological Properties of the Nitramino Acids. The single-dose LD_{50} for male Swiss–Webster mice observed at 72 h ranged from 1.17 mmol/kg for 1-nitro-L-proline (**1a**) to 2.10 mmol/kg for 1-nitro-DL-pipecolic acid (**2a**) (Table I) and, hence, were of comparable magnitude to the LD_{50} values reported[16] for 1-nitroso-L-proline (**1b**, 1.41 mmol/kg) and 1-nitroso-DL-pipecolic acid (**2b**, 1.29 mmol/kg). These moderate in vivo toxicities of the nitramino acids were not manifested against certain selected bacteria and fungi in culture as none of the nitramino acids listed in Table I were effective in inhibiting the growth in vitro of bacterial strains represented by *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium smegmatis* H607 or of two fungal strains of *Candida albicans*—up to concentrations of 100 μg/mL.

The histidine-dependent tester mutant strain, *S. typhimurium* TA-100, was selected for assessing the mutagenicity of the nitramino acids. This strain detects point mutations in DNA due to base-pair substitution and is sensitive toward nitrosamines.[6a] The viability of the test method in our hands was attested by the potent mutagenic effect observed for metronidazole, nitrofurantoin, and *N*-2-fluorenylacetamide (FAA), all known mutagens which served as internal controls (Table II). As shown by other work, FAA[14b] required oxidative metabolism for mutagenicity, while the intervention of nitroreductases was required for activation of metronidazole and nitrofurantoin.[13b,22] These latter test compounds were all mutagenic in the 10–300 nmol per plate range. The nitramino acids, **1a** and **2a**, were essentially devoid of mutagenic activity in this system at concentrations 1000-fold higher than the test mutagens, viz., 300 μmol per plate (Table II). The mutagenicity of **3a** can be considered marginal at these concentrations (number of revertants less than twice background), but solubility considerations precluded testing at much higher concentrations.

Table II. Mutagenicity of Nitramino Acids toward *S. typhimurium* TA-100

Compd	Concn/plate	No. of revertants per plate[a]			
		Aerobic		Anaerobic	
		Without S-9	With S-9	Without S-9	With S-9
1a (L)	300 μmol	9 ± 6	11 ± 3	36 ± 6	⩽0
2a	300 μmol	⩽0	⩽0	7 ± 11	⩽0
3a	300 μmol	49 ± 18	72 ± 19	9 ± 2	18 ± 4
Metronidazole	290 nmol	130 ± 7		185 ± 12	1821[b]
Nitrofurantoin	8.4 nmol	1014 ± 38		123 ± 10	1206[b]
N-2-Fluorenylacetamide (FAA)	57 nmol		351 ± 112	⩽0	⩽0

[a] Mean ± SD; $n = 3$ except where indicated. Where backgrounds equaled or exceeded the values obtained with the test substances, they are indicated as ⩽0. [b] Single determination.

Discussion and Conclusions

The complete absence of antibacterial and antifungal activity of the nitramino acids **1a–3a** in the test systems to which they were subjected was somewhat surprising. In contradistinction, *N*-nitroglycine (**5**), the simplest of the α-nitramino acids and which is also a primary nitramine, has been reported to inhibit the growth of *E. coli*, *Pseudomonas tabaci*, and *Mycobacterium phlei*, although it was ineffective against *C. albicans*.[8]

The absence of mutagenic activity of the nitramino acids **1a** and **2a** can be compared to the lack of carcinogenic activity of the nitrosamino acids **1b** and **2b**.[16,23] Likewise, the marginal mutagenicity of *N*-nitrosarcosine (**3a**) may be related to the weak carcinogenic activity of *N*-nitrososarcosine (**3b**). The possibility that these latter compounds may be decarboxylated by enzymic or other mechanisms to dimethylnitramine and dimethylnitrosamine, respectively, both proven carcinogens, merits further investigation.

Experimental Section

Melting points were taken on a Fisher-Johns melting point apparatus and are corrected. Spectrophotometers used were IR, Beckman IR-10; UV, Beckman Acta VI; NMR, Varian T-60D; electron-ionization mass spectra (EI-MS), Hitachi Perkin-Elmer RMU-6 (ionization energy, 70 eV, ion source temperatures as indicated). Chemical ionization mass spectra (CI-MS) were provided by Dr. Roger Foltz, Battelle Columbus Laboratories, Columbus, Ohio, using an AEI-MS-902 mass spectrometer equipped with an SRIC Model CIS-2 combined CI–EI ion source. For the GC–MS of 1-nitropyrrolidine, a 10% Carbowax 20M on Chromosorb W column at 180 °C was used with an LKB-9000 GC–MS computor system. *Warning: N-nitroso and N-nitro compounds are potentially carcinogenic and should be handled with care.*

Procedure A. 1-Nitro-DL-pipecolic Acid (2a). (*Caution: although we have not encountered any problems, potential explosion hazards exist and these reactions should be conducted in a hood behind a safety shield.*) To a cooled (ice bath), stirred mixture of 100 mL of CH_2Cl_2 and 2.0 mL (0.080 mol) of 90% H_2O_2 was added 12.0 mL (0.090 mol) of trifluoroacetic anhydride. After stirring in the cold for 5 min the ice bath was removed and a solution of 7.91 g (0.050 mol) of 1-nitroso-DL-pipecolic acid (**4b**)[16] in 180 mL of CH_2Cl_2 was treated with decolorizing charcoal and then added dropwise to the peracid mixture over 45 min. After the addition was complete the reaction mixture was heated under reflux for 1 h, then diluted to 500 mL with CH_2Cl_2, and washed with 50 mL of H_2O. After drying (Na_2SO_4) the solvent was evaporated to dryness on a rotating evaporator using a water aspirator and the solid residue remaining was recrystallized (Table I).

Procedure B. 1-Nitro-L-proline (L-1a). The procedure was identical with that of A above except that the quantities were 1.58 mL (0.055 mol) of 90% H_2O_2, 5.18 mL (0.060 mol) of trifluoroacetic anhydride, and 7.20 g (0.050 mol) of 1-nitroso-L-proline (L-**1b**).[16] After heating under reflux for 1 h the solvent was removed in a rotating evaporator using water aspiration. The colorless liquid residue was dissolved in 100 mL of 1 N HCl and heated on the steam bath for 5 min to hydrolyze traces of unreacted **1b** to proline. The aqueous solvent was then removed by lyophylization and the resulting solid recrystallized (Table I) to give crystalline L-**1a**.

Catalytic Hydrogenolysis of 1-Nitro-L-proline (L-1a) to L-Proline. L-**1a** (140 mg, 0.875 mmol) in 50 mL of absolute EtOH in a glass hydrogenation apparatus was reduced with H_2 in the presence of 0.29 g of 10% Pd/C at room temperature and atmospheric pressure until the theoretical quantity of H_2 was absorbed (11 h). The catalyst was removed by filtration and the filtrate was evaporated to dryness under reduced pressure. The solid product was redissolved in absolute EtOH, and the solution was decolorized with charcoal, filtered, and evaporated once again. Recrystallization of the residue from absolute EtOH–ether gave 65 mg (65% yield) of L-proline, $[\alpha]^{26}_D$ −79.6° (*c* 2.0, H_2O). The optical rotation of the starting L-proline used in this three-step sequence of reactions was $[\alpha]^{24}_D$ −84.7° (*c* 2.0, H_2O). The hydrogenolysis product was identical with authentic L-proline on TLC (BuOH–HOAc–H_2O, 4:1:1) and gave a single, yellow chromogenic spot with ninhydrin.

1-Nitropyrrolidine. To 400 mL of cold CH_2Cl_2 (ice bath) were added, with rapid stirring, 15.8 mL (0.55 mol) of 90% H_2O_2 and 91 mL (0.60 mol) of trifluoroacetic anhydride. The ice bath was removed, the peracid solution was allowed to reach room temperature, and 50.0 g (0.50 mol) of 1-nitrosopyrrolidine [bp 49 °C (0.35 mmHg); reported[5b] 98 °C (12 mm)] in 150 mL of CH_2Cl_2 was added dropwise over 1.5 h. The reaction mixture was heated under reflux for 1 h, then diluted with H_2O, and extracted several times with Et_2O. The combined Et_2O extracts were dried (Na_2SO_4) and evaporated to a syrupy residue, which when added to water crystallized to give a product with mp 56–57 °C. Recrystallization from hexane gave 29.4 g (67.8% yield) of 1-nitropyrrolidine: mp 57–58 °C (lit.[19b] 58–59 °C); GC–MS *m/e* >50 amu (rel intensity) 116 (39.1, $M^{+\cdot}$), 100 (1.9), 99 (1.8), 86 (2.5). 85 (5.4), 69 (35), 68 (20), 55 (7.5).

Biological Evaluation. For the LD_{50} determinations, the nitramino acids were administered ip to male Swiss-Webster mice during their waking period (5:00–7:00 p.m.). For **1a** and **3a** the vehicle was water; the less soluble **2a** required 4% (w/v) aqueous ethanol. The corresponding controls received the highest volume of vehicle administered. The LD_{50} at 72 h, calculated by probit analysis,[24] are given in Table I.

1a (L and D), **2a**, and **3a** were also assayed for growth-inhibitory activity against *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853), *M. smegmatis* H607, and *C. albicans* (ATCC11651 and a clinical isolate) at 10, 25, 50, and 100 μg/mL. *E. coli* and *P. aeruginosa* were assayed in Mueller-Hinton broth, *C. albicans* in yeast nitrogen base (pH 7.0), and *M. smegmatis* in Sauton synthetic medium. Incubation times (37 °C with rotary shaking at 150 rpm) varied from 7 h for the fast-growing *P. aeruginosa* to 16 h for *E. coli* and *C. albicans* and 22 h for *M. smegmatis*. Cell growth was measured turbidimetrically at 600 nm in a Coleman Junior spectrophotometer.

Mutagenic activity was determined according to the assay procedure of Ames et al.[25] using a histidine-requiring mutant of *S. typhimurium* TA-100 as the indicator organism, either coupled to or in the absence of a mammalian activating system. This tester strain was cultivated overnight in sterile test tubes containing

5 mL of nutrient broth and 100 μL (about 10^7 cells) of this bacterial culture was added per plate. Bacterial cell growth was measured by serial dilution of the original culture, and 1 mL of the dilutions corresponding to 10^6, 10^7, and 10^8 cells was plated in triplicate in 10 mL of nutrient agar broth. The bacterial colonies were counted after incubation for 2 days at 37 °C.

The S-9 fraction was prepared from the livers of male Sprague–Dawley rats which had been administered a 1.0% aqueous solution of phenobarbital in place of drinking water for 1 week. Sterile 25% liver homogenates were prepared in 0.15 M KCl and 2-mL aliquots of the 9000g supernatant fraction (S-9 fraction) were frozen over dry ice and kept at −100 °C until needed. The activating system or "S-9 mix" referred to in Table II contained 4 μmol of $MgCl_2$, 16.5 μmol of KCl, 2.5 μmol of glucose 6-phosphate, 2.0 μmol of NADP (yeast β-NADP, Sigma), 50.0 μmol of sodium phosphate buffer (pH 7.4), and 150 μL of S-9 fraction per 500 μL.

The known mutagens used as standards were dissolved in $(CH_3)_2SO$ and the test compounds in H_2O. Aqueous solutions which were adjusted to pH 7 were sterilized by passage through a 0.45-μm sterile Millex filter. The test compound, indicator organism, and the S-9 mix were incorporated into an agar overlay containing a minimal amount of L-histidine–biotin and plated over a layer of minimal glucose–agar medium. The plates were incubated aerobically at 37 °C in the dark for 2 days or anaerobically[23] in a Gas-Pak system (BBL Division of Becton, Dickenson and Company, Cockeysville, Md.) for 16–17 h, followed by 48 h under aerobic conditions. After this time the number of revertants to histidine independence was counted (in triplicate at each concentration) and these numbers were averaged. Specific controls (test compounds omitted) were matched according to the individual systems (e.g., combinations of Me_2SO–H_2O, aerobic–anaerobic, with S-9–without S-9) and the corresponding backgrounds were substracted from each experimental set. Although the actual background levels varied from system to system, they were consistent within an experimental set and overall averaged 74 ± 5 revertants/plate (mean ± SE, n = 52).

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