Bioorganic & Medicinal Chemistry 23 (2015) 3297–3302

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Transnitrosation of non-mutagenic *N*-nitrosoproline forms mutagenic *N*-nitroso-*N*-methylurea

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ARTICLE INFO

Article history:
Received 9 February 2015
Revised 17 April 2015
Accepted 18 April 2015
Available online 25 April 2015

Keywords:
N-Nitrosoproline
N-Nitroso-*N*-methylurea
Transnitrosation
Thioureas

ABSTRACT

N-Nitroso-*N*-methylurea (NMU) is a potent carcinogen and suspected as a cause of human cancer. In this study, mutagenic NMU was detected by HPLC after the transnitrosation of non-mutagenic *N*-nitrosoproline (NP) to *N*-methylurea in the presence of thiourea (TU) under acidic conditions. The structure of NMU was confirmed by comparing ^{1}H NMR and IR spectra with that of authentic NMU after fractionation by column chromatography. Furthermore, a fraction containing NMU formed by transnitrosation was mutagenic in *Salmonella typhimurium* TA1535.

NMU was formed in the reaction of NP and *N*-methylurea in the presence of 1,1,3,3-tetramethylthiourea (TTU) or 1,3-dimethylthiourea in place of TU as an accelerator. The reaction rate constants (k) for NMU formation were correlated with their nucleophilicity of sulfur atom in thioureas. The *N*-methylurea concentration did not affect the NMU formation, whereas the rate of NMU formation correlated linearly with concentrations of NP, TTU and oxonium ion. The observed kinetics suggests a mechanism by which the nitroso group was transferred directly from the protonated NP to the thiourea then to *N*-methylurea to form NMU. The rate-determining step was the formation of the complex with the protonated NP and thiourea.

1. Introduction

N-Nitroso compounds represent a major class of important chemical carcinogens and mutagens that have been implicated as a hazard to human health.[1] These compounds and their precursors are present in the human environment and diet. They are also found in tobacco products, cosmetics, pharmaceutical products, and they form endogenously in the human body from dietary components.[1]

N-Nitroso compounds are divided into two categories: nitrosamines, which require activation to exert their genotoxicity, and nitrosamides including *N*-nitroso-*N*-methylurea (NMU), which spontaneously decompose to form alkylating agents.[1]

NMU is a potent direct-acting carcinogen that has been shown to induce cancers in a wide variety of animal species and in various organs, mainly the forestomach, brain, and the nervous system.[2] Humans are exposed to NMU exogenously through food.[3,4] In addition, NMU can be formed endogenously in stomach[5–8] and intestine.[9] In fact, NMU has been formed under acidic conditions from creatinine, which is present at fairly high concentrations in meat and fish.[8] The microbiota of the intestine can reduce nitrate to nitrite, which can then react to form NMU.[9]

There are non-mutagenic and non-carcinogenic *N*-nitrosamines, for example, *N*-nitrosoproline (NP) and *N*-nitrosothioproline.[10,11] NP was formed in stomach[12–14] and detected in foods.[15,16] The presence of these compounds in urine is considered as an indicator of nitrosation exposure in vivo.[17]

Transnitrosation, in which a nitroso group is transferred from nitrosamine to another amine, is a reaction characteristic of *N*-aryl- and *N*-alkyl-*N*-nitrosoureas,[18–21] and *N*-aryl-*N*-nitrosamines.[22–27] Furthermore, transnitrosation by aliphatic nitrosamines under appropriate conditions have also been reported.[23,28–31] Thus a non-carcinogenic *N*-nitrosamine can give carcinogenic compound by transnitrosation under the acidic conditions of the mammalian stomach. In this study, we report that the non-mutagenic NP transnitrosated to an alkylurea and formed the mutagenic NMU in the presence of accelerator under acidic conditions. We also report on our investigation of the mechanism of this transnitrosation.

Abbreviations: DTU, 1,3-dimethylthiourea; h, hour; HPLC, high performance liquid chromatography; IR, infrared; NMR, nuclear magnetic resonance; NMU, *N*-nitroso-*N*-methylurea; NP, *N*-nitrosoproline; R_f, retention factor; TLC, thin layer chromatography; TTU, 1,1,3,3-tetramethylthiourea; TU, thiourea.

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http://dx.doi.org/10.1016/j.bmc.2015.04.058
0968-0896/

2. Results

2.1. Identification of NMU from NP and methylurea in the presence of thiourea (TU)

To a solution of NP (28 mmol) in acetonitrile was added a solution of *N*-methylurea (28 mmol) in 0.1 M HCl aqueous solution (pH 1.5) and a solution of TU (11 mmol) in 0.1 M HCl aqueous solution (pH 1.5), and then the mixture was stirred at 37 °C for 4 h. The reaction mixture was extracted with chloroform, and the organic phase was dried over anhydrous sodium sulfate, and the solvent subsequently evaporated. The residue was fractionated by silica gel column chromatography [*n*-hexane/diethyl ether/dichloromethane (4:3:2), UV 254 nm]. A fraction which included a substance with the same R_f value on TLC as authentic NMU was collected, the organic solvent evaporated and the residue analyzed. The retention time by HPLC, R_f value on TLC, ^{1}H NMR and IR spectra were identical with those of authentic NMU.

The fraction containing the NMU was mutagenic in *Salmonella typhimurium* TA1535 (Fig. 1). The products formed in the absence of either NP, *N*-methylurea or TU were not mutagenic (Table S1 in Supplementary data).

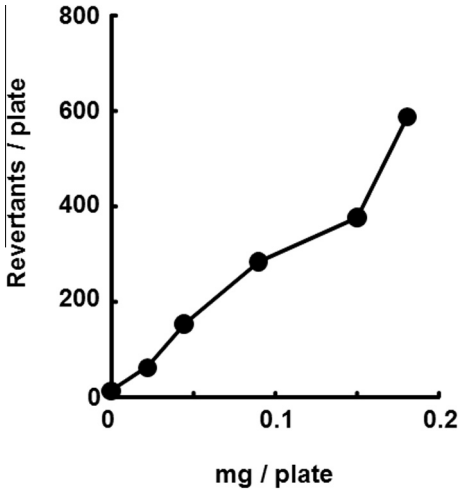


Figure 1. Mutagenicity of reaction extract from NP and *N*-methylurea in the presence of TU in *Salmonella typhimurium* TA1535.

2.2. Effect of accelerators on NMU formation from NP and methylurea under acidic conditions

An accelerator was necessary for the formation of NMU from NP and *N*-methylurea as described above. Thiocyanate is one of the accelerators for transnitrosation,[21,28,29,31] however, it has been reported to have lower catalytic activity than thioureas.[23,25,27,32] In addition to 1,1,3,3-tetramethylthiourea (TTU) and 1,3-dimethylthiourea (DTU), other sulfur compounds; thioacetamide, cysteine, methionine and glutathione, were tested as possible accelerators for transnitrosation from NP to *N*-methylurea in this study. NMU was not detected following the reaction with cysteine, methionine and glutathione, and only a trace amount of NMU was detected after 22 h reaction with thioacetamide (data not shown).

The effect of three thioureas on NMU formation was compared, and the reaction was followed by initial rate methods.[33,34] NMU was formed in a dose- and time-dependent manner in the presence of three thioureas (Fig. 2). A plot of $\ln[k_{in}]$ versus ln[a thiourea] was linearly correlated with a slope (approximately 1.0), indicating that the order of the reaction was first-order with respect to thioureas (Fig. 3).

The reaction rate constant (k_{in}) was obtained from a *y*-intercept in plot of $\ln[k_{in}]$ versus ln[a thiourea] (Fig. 3). The accelerating effect increased in the following order: TU (1.51×10^{-4} M s^{-1}) < DTU (2.94×10^{-4} M s^{-1}) < TTU (5.23×10^{-4} M s^{-1}). The

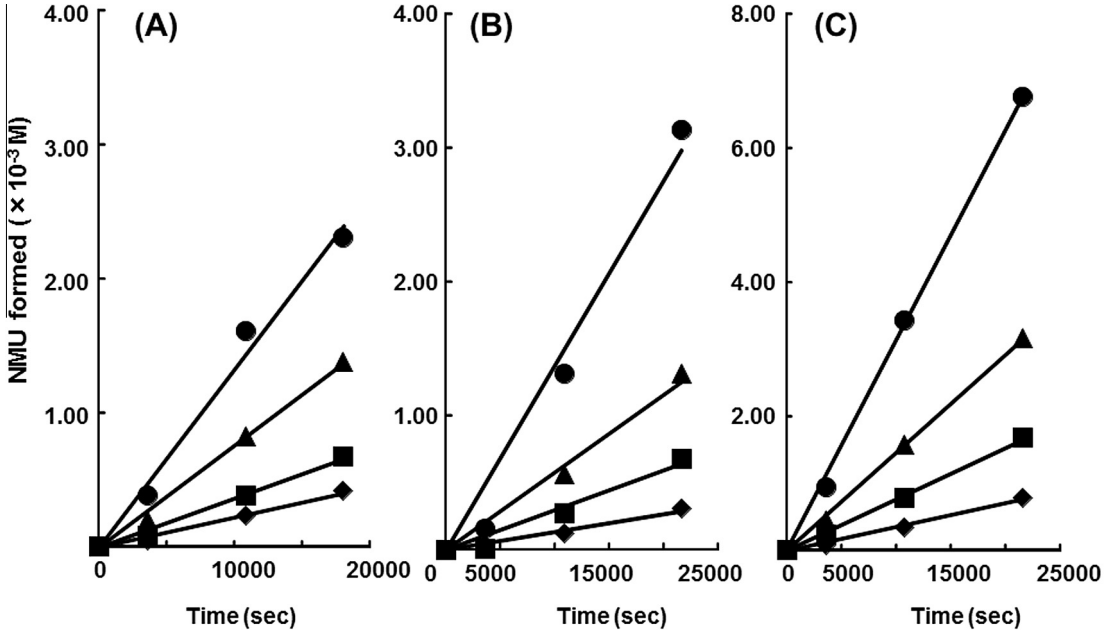


Figure 2. Effect of accelerator on NMU formation by transnitrosation of NP. (A) NMU formation from NP and *N*-methylurea in the presence of TU at several concentrations [0.005 M (♦), $y = 2.24 \times 10^{-7}$ ($R^2 = 0.99$); 0.01 M (■), $y = 3.66 \times 10^{-7}$ ($R^2 = 0.99$); 0.02 M (▲), $y = 7.60 \times 10^{-7}$ ($R^2 = 0.99$); 0.05 M (●), $y = 13.3 \times 10^{-7}$ ($R^2 = 0.99$)]. (B) NMU formation from NP and *N*-methylurea in the presence of DTU at several concentrations [0.005 M (♦), $y = 1.33 \times 10^{-7}$ ($R^2 = 0.96$); 0.01 M (■), $y = 2.94 \times 10^{-7}$ ($R^2 = 0.95$); 0.02 M (▲), $y = 5.79 \times 10^{-7}$ ($R^2 = 0.97$); 0.05 M (●), $y = 13.8 \times 10^{-7}$ ($R^2 = 0.97$)]. (C) NMU formation from NP and *N*-methylurea in the presence of TTU at several concentrations [0.005 M (♦), $y = 3.53 \times 10^{-7}$ ($R^2 = 0.99$); 0.01 M (■), $y = 7.66 \times 10^{-7}$ ($R^2 = 1.00$); 0.02 M (▲), $y = 14.6 \times 10^{-7}$ ($R^2 = 1.00$); 0.05 M (●), $y = 31.3 \times 10^{-7}$ ($R^2 = 1.00$)].

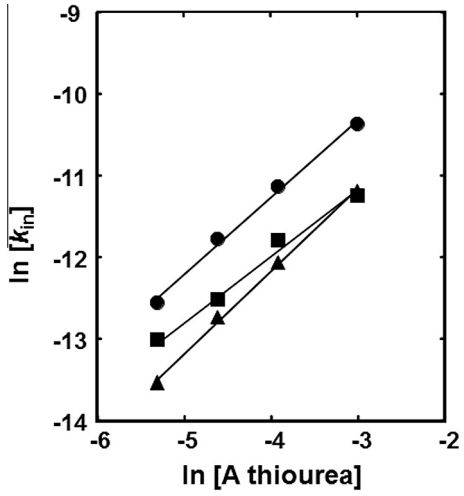


Figure 3. Plot of ln[k_{in}] versus ln[a thiourea]. Reaction conditions were as follows: NP, 0.05 M; *N*-methylurea, 0.05 M; thioureas [TU (■), $y = 0.88\ x - 8.38$ ($R^2 = 0.99$); DTU (▲), $y = 1.01\ x - 8.13$ ($R^2 = 1.00$); TTU (●), $y = 0.94\ x - 7.50$ ($R^2 = 1.00$)], 0.005–0.05 M; pH 2.0; 37 °C.

data was in good agreement with the order of nucleophilicity of sulfur atom in thioureas.

2.3. Mechanism of the transnitrosation for NP and *N*-methylurea in the presence of TTU

To elucidate the mechanism of transnitrosation by NP and *N*-methylurea in the presence of TTU, the effect of concentration of *N*-methylurea, NP and oxonium ion on NMU formation was investigated.

In the reaction with NP and *N*-methylurea in the presence of TTU, the concentration of *N*-methylurea as NO acceptor did not affect NMU formation under those conditions (Fig. 4).

The effect of the concentrations of NP on NMU formation was investigated. NMU was formed in a dose- and time-dependent manner in the reaction (Fig. 5A). A slope of plot of ln[k_{in}] versus ln[NP] showed the order of reaction with respect to [NP] (Fig. 5B). The results of kinetics experiments were linearly correlated with the slope of approximately 1.08 ($r^2 = 1.00$), indicating that good first-order plots on NP concentration.

The rate of NMU formation in the reaction with NP and *N*-methylurea in the presence of TTU was linearly correlated with oxonium ion concentration (slope = 1.19, $r^2 = 1.00$), indicating that the transnitrosation proceeds by specific acid catalysis (Fig. 6).

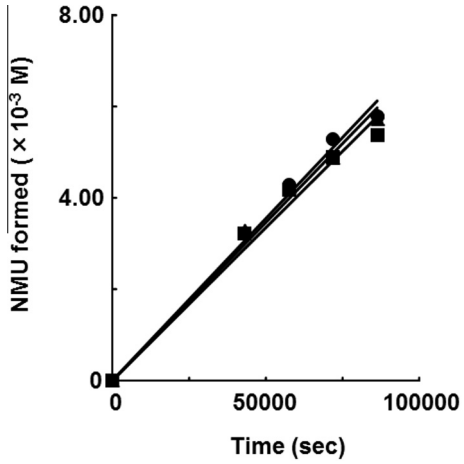


Figure 4. Effect of *N*-methylurea concentration on NMU formation by transnitrosation of NP. Reaction conditions were as follows: NP, 0.05 M; *N*-methylurea [0.05 M (■), $y = 69.0\ x \times 10^{-6}$ ($R^2 = 0.99$); 0.07 M (▲), $y = 66.9\ x \times 10^{-6}$ ($R^2 = 0.98$); 0.1 M (●), $y = 70.9\ x \times 10^{-6}$ ($R^2 = 0.99$)]; TTU, 0.01 M; pH 2.0; 37 °C.

3. Discussion

Transnitrosation of alicyclic *N*-nitrosamines has been reported to occur in the presence of accelerator under acidic conditions.[23,28–31] Many reports have shown kinetic data obtained by decreasing the initial concentration of starting *N*-nitrosamines, whereas only a few reports have shown kinetic data obtained by formation of *N*-nitrosamines[28,29] or *S*-nitroso compounds[35,36] produced by transnitrosation of *N*-nitrosamines. Although NMU has been reported to form by nitrosation in vivo,[5–8] there have been few reports of NMU formation by transnitrosation of non-mutagenic *N*-nitrosamine. Herein we report that the nitroso group of the non-carcinogenic NP is transnitrosated to *N*-methylurea in the presence of a thiourea, forming carcinogenic NMU under acidic conditions. We also report on our investigation of the transnitrosation mechanism.

NMU produced in the reaction of NP with *N*-methylurea in the presence of TU was identified by HPLC retention time and by R_f value on TLC. The reaction mixture of NP and *N*-methylurea with TU was fractionated by silica gel chromatography, and the fraction containing NMU was obtained. The ^{1}H NMR and IR spectra for this fraction were also identical to those of authentic NMU. Furthermore, this fraction was mutagenic in *Salmonella typhimurium* TA1535 (Fig. 1).

The NMU formation rate from NP and *N*-methylurea were compared among accelerators. Methionine, glutathione and cysteine were not effective in forming NMU in the reaction of NP and methylurea (data not shown). Three thioureas accelerated the reaction to form NMU following first order with respect to a thiourea (Fig. 2). The accelerating effect of thioureas is in the order TTU > DTU > TU, which was in good agreement with nucleophilicity of sulfur atom in thioureas (Fig. 3). The data indicates that nucleophilicity of sulfur atom in thioureas plays a key role for the transnitrosation. When thioacetamide was used instead of a thiourea, NMU formed in a trace amount after 22 h under acidic conditions. The results showed that thioamide structure was necessary for the formation of NMU by transnitrosation.

The mechanism for the transnitrosation of NP in the presence of TTU was investigated. *N*-Methylurea concentration did not affect the rate of NMU formation (Fig. 4), whereas oxonium ion and TTU were both necessary for the transnitrosation to proceed. The NMU formed in a time- and dose-dependent manner with NP (Fig. 5A). A plot of ln[k_{in}] versus ln[NP] showed a linear correlation with a slope of approximately 1.0, which indicated that the order of reaction was 1 with respect to NP (Fig. 5B). The transnitrosation reaction preceded by specific acid catalysis, indicating that the protonated NP was involved in the rate-determining step (Fig. 6). The experimentally observed rate equation for the transnitrosation is shown in Eq. 1.

$$\text{Rate} = k\,[\text{TTU}][\text{NP}][\text{H}_3\text{O}^+] \quad (1)$$

NMU stability in 0.1 M HCl solution (pH 1.5) was measured by the decrease in UV absorption at 254 nm. NMU decomposed by approximately 25% over 18 h. Since our kinetics experiments were typically followed for 5 or 6 h, the decomposition rate of NMU was negligible.

A presumptive mechanism is shown in Scheme 1. The mechanisms agree with the observation of Singer et al.[31] Protonation of

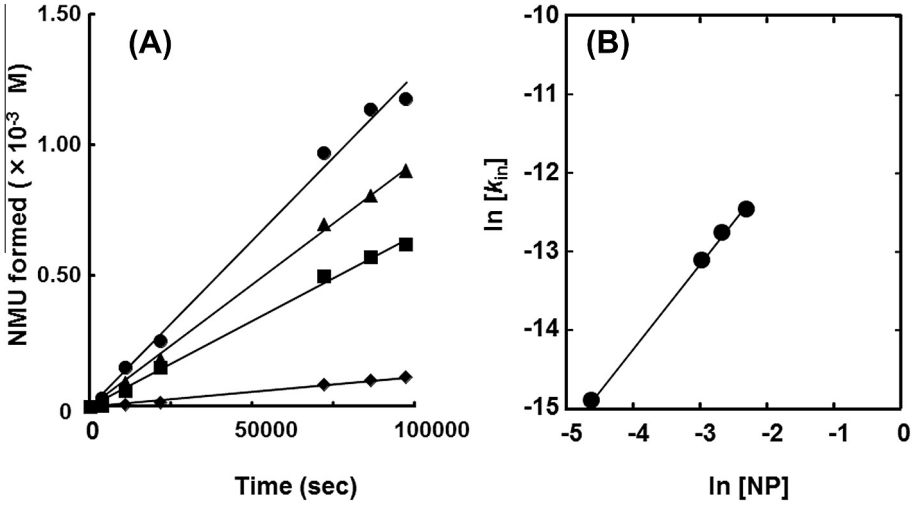


Figure 5. Effect of NP concentration on NMU formation by transnitrosation of NP. (A) NMU formation from NP at several concentrations and *N*-methylurea in the presence of TTU. Reaction conditions were as follows: NP [0.01 M (♦), $y = 1.14\ x \times 10^{-6}$ ($R^2 = 0.99$); 0.05 M (■), $y = 6.58\ x \times 10^{-6}$ ($R^2 = 1.00$); 0.07 M (▲), $y = 9.37\ x \times 10^{-6}$ ($R^2 = 1.00$); 0.1 M (●), $y = 12.8\ x \times 10^{-6}$ ($R^2 = 0.99$)]; *N*-methylurea, 0.05 M; TTU, 0.01 M; pH 2.0; 37 °C. (B) Plot of ln [k_{in}] versus ln [NP]. Reaction conditions were as follows; NP, 0.01–0.1 M; *N*-methylurea, 0.05 M; TTU, 0.01 M; pH 2.0; 37 °C; $y = 1.08\ x$ ($R^2 = 1.00$).

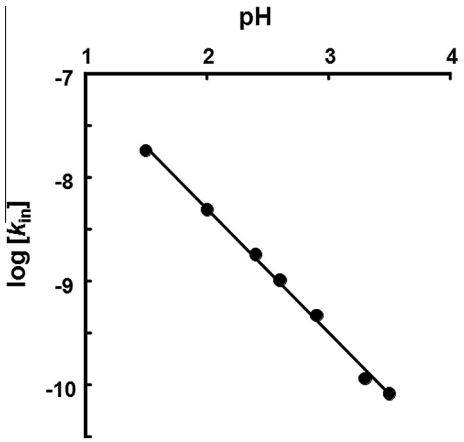


Figure 6. pH profile for the TTU-catalyzed reaction of NP with *N*-methylurea. Reaction conditions were as follows: NP, 0.05 M; *N*-methylurea, 0.05 M; TTU, 0.01 M; 37 °C.

nitrogen atom attaching the nitroso group and protonation of oxygen atom are present at an equilibrium process. In addition, the amino nitrogen atom has been reported to be protonated predominantly below pH 2.[37] The initial step underwent necessarily N protonation to take place the transnitrosation of *N*-nitrosamines.[31] Ohwada et al. demonstrated that alicyclic nitrosamines formed nitrosonium ion ($^{+}$NO) upon acid-catalyzed heterolytic cleavage of the N–NO bonds.[38] The observed kinetics suggest a mechanism by which $^{+}$NO is transferred directly from the protonated NP to the thiourea without being released as nitric oxide, nitrous acid or covalent nitrosyl chloride, and finally is transferred to *N*-methylurea to form NMU.

Singer et al. have proposed that carcinogenic *N*-nitrosamines formed by the transnitrosation of non-carcinogenic *N*-nitrosamines under acidic conditions.[32] In the present study, we demonstrated that NMU formed in the presence of TU was mutagenic in *Salmonella typhimurium* TA1535. The extract derived from the reaction of NP and *N*-methylurea without accelerator showed no mutagenicity. This indicates that the accelerator was essential for the NMU formation by transnitrosation. Many ureas and carbamates are used as medicines and pesticides[39] and can be easily nitrosated to become a possible source of endogenous carcinogens.

4. Conclusion

We demonstrated that the reaction of NP and *N*-methylurea with thioureas under acidic conditions formed NMU that was identical to authentic NMU according to instrument data. The fraction containing NMU was mutagenic in *Salmonella typhimurium* TA1535. Although NMU formation rate was very small via transnitrosation of NP, NMU is highly mutagenic and carcinogenic in rodents. Our data showed first time that mutagenic NMU was formed via transnitrosation by non-mutagenic *N*-nitrosamines under acidic conditions such as in the stomach.

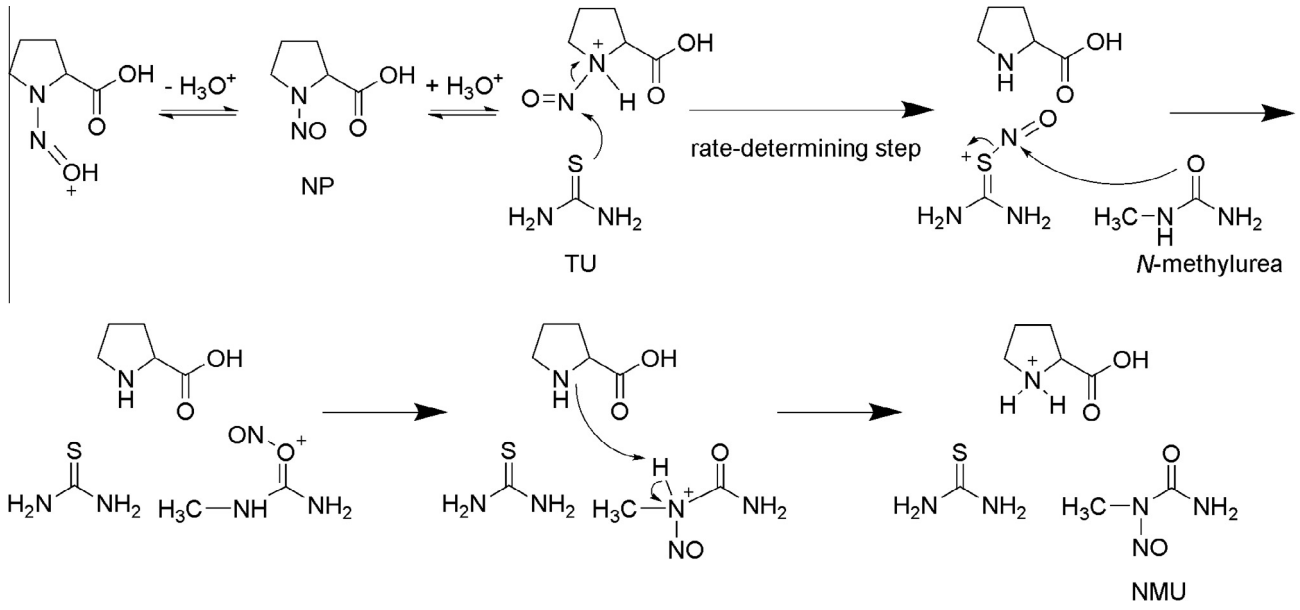
5. Materials and methods

5.1. Chemicals

L-Proline was obtained from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Bacto agar and bacto nutrient broth were purchased from Becton Dickinson Microbiology System (Sparks, USA). Sodium ammonium hydrogen phosphate tetrahydrate was obtained from Merck (Darmstadt, Germany). Other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan). NP was prepared and purified by recrystallization from chloroform using the method of Lijinsky et al. (mp 106 °C).[40]

5.2. Experimental

Melting points were measured on a Yanagimoto microapparatus and were uncorrected. The FAB-MS data were obtained with a JEOL JMS-700 mass spectrometer. The NMR experiments were performed with JEOL JNM-LA400 using tetramethylsilane as an internal standard. HPLC was performed using a Shimadzu LC-6A system [SPD-6AV UV/vis spectrometric detector]. TLC was performed on precoated Kieselgel 60F_{254} (Merck), and spots were visualized under UV light. Column chromatography was performed on silica gel 60 (0.063–0.200 mm, Merck).



Scheme 1. Proposed transnitrosation mechanism of NP under acidic conditions.

5.3. Identification of NMU from reaction mixture of NP and MU in the presence of TU

Each solution of *N*-methylurea [2.07 g (28 mmol)/28 mL) and TU [0.85 g (11 mmol)/35 mL] in 0.1 M phosphate solution was adjusted to pH 1.5 by adding 0.1 M HCl, and NP [4.0 g (28 mmol)/28 mL) was dissolved in acetonitrile. The *N*-methylurea solution and the NP solution were mixed, and the reaction started by addition of the thioureas solution. The reaction proceeded at 37 °C for 4 h. The reaction mixture was extracted three times with chloroform, dried over anhydrous sodium sulfate, filtered, and the organic solvent evaporated to produce a tan yellow solid. The crude product was fractionated on silica gel column chromatography [*n*-hexane/diethyl ether/dichloromethane = 4:3:2, UV 254 nm] to yield 1.9 mg (yield: 0.09%) of a white solid. mp 123 °C [lit. 126 °C (decomp)[41]]. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 3.19 (s, 3H, N—CH_3). IR (neat) cm^{-1}: 1722 (C=O), 1417 (N—N=O). The retention time by HPLC, R_f value on TLC, ^{1}H NMR and IR spectra of the authentic NMU was identical to that of the isolated compound.

5.4. Bacterial mutation assay

A residue from the chloroform extract of the reaction mixture was obtained as described above and was assayed according to the Ames method with a plate-incorporation protocol.[42,43] *Salmonella typhimurium* TA1535 was kindly provided by Professor B.N. Ames (University of California, Berkeley, USA).

The fraction containing NMU was diluted with dimethyl sulfoxide. Solutions (each 50 μL in dimethyl sulfoxide) with varying concentrations of the NMU fraction were each added to a test tube, and 0.1 M sodium phosphate buffer (pH 7.4, 0.5 mL) and a culture of the *Salmonella typhimurium* TA1535 (0.1 mL) were added to the tube and thoroughly mixed. Top agar (2 mL) was added, and the mixture was poured onto a minimal-glucose agar plate. The revertant colonies were counted after incubating at 37 °C for 44 h. Each sample was assayed using duplicate plates twice in separate experiments.

5.5. The conditions of NP and *N*-methylurea reaction in the presence of thioureas under acidic conditions

N-Methylurea (0.05 M, 0.07 M, 0.1 M), a thiourea (0.005 M, 0.01 M,0.02 M, 0.05 M) and NP (0.01 M,0.05 M, 0.07, 0.1 M) were mixed and adjusted to the desired pH by adding of 0.1 M HCl. The reactions were started by adding thioureas, and the incubation was allowed to proceed at 37 °C for various times, after which aliquots were taken and ethyl acetate was added to each to remove sulfur formed. The mixtures were centrifuged (3000 rpm) for 10 min, and the supernatants were analyzed by HPLC [column; Lichrosorb RP-18 (10 μm, 4.6 × 250 mm); eluent, acetonitrile/H_2O (7:3); flow rate, 0.5 mL/min; detection, 254 nm]. The extraction efficacy for NMU by ethyl acetate was 87.5%.

The kinetics of the reactions was followed by monitoring the formation of the NMU peak by HPLC. The reaction rate for the appearance of NMU was obtained from the initial rate method because the NMU formation rate was very small (below 5%).[33,34] A plot of ln[k_{in}] versus ln[compound] yields an order of the reaction from the slope and a reaction rate from *y*-intercept as ln*k*.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2015.04.058.

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