Nitrosation of *N*-Methyl Derivatives of Uric Acid and Their Transnitrosation Ability to *N*-Acetylcysteine

Toshinori SUZUKI,*[,a] Hiroshi YAMAMOTO,[a] and Wolfgang PFLEIDERER[b]

[a] *School of Pharmacy, Shujitsu University; Okayama 703–8516, Japan: and* [b] *Fachbereich Chemie, Universität Konstanz; Postfach 5560, D-78457 Konstanz, Germany.*

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When 1,3-dimethyluric acid was treated with a nitric oxide donor, diethylamine NONOate, in an aerobic neutral solution and the reaction was analyzed by HPLC, 1,3-dimethyluric acid was consumed to yield a nitrosated derivative, which decomposed with a half-life of 17.9 min at pH 7.4 and 37 °C. When 1,3,7-trimethyluric acid was treated with diethylamine NONOate, no consumption of 1,3,7-trimethyluric acid was observed. However, in the reaction of *N*-acetylcysteine with diethylamine NONOate, the yield of *N*-acetyl-*S*-nitrosocysteine increased by the addition of 1,3,7-trimethyluric acid as well as 1,3-dimethyluric acid. For 1,3,7,9-tetramethyluric acid, no consumption in the reaction with diethylamine NONOate and no effect on the *S*-nitrosation were observed. These results suggest that 1,3-dimethyluric and 1,3,7-trimethyluric acids are both nitrosated by diethylamine NONOate on the nitrogen atom of their oxoimidazole ring, although the half-life of the nitrosated 1,3,7-trimethyluric acid is too short to detect by HPLC. Consequently, these two acids can act as vehicles of nitric oxide.

Key words 1,3-dimethyluric acid; 1,3,7-trimethyluric acid; *N*-acetylcysteine; nitrosation; nitric oxide

Nitric oxide (NO) is an inorganic gas synthesized from L-arginine by the enzyme nitric oxide synthase in various types of cells.[1)] NO is involved in numerous biological functions, including vasodilation, neurotransmission, and inflammation.[2)] NO is a radical, demonstrating both cytotoxic and cytoprotective properties.[3)] Although the reactivity of NO *per se* is relatively low, NO is converted to a reactive nitrosating reagent, dinitrogen trioxide (N_2O_3), in the presence of O_2.[4)] N_2O_3 can react with amino and imino groups in various biological molecules resulting in corresponding *N*-nitroso compounds, many of which are mutagenic and carcinogenic since they can act as alkylation reagents to form DNA-adducts.[5)] N_2O_3 can also react with thiols such as free cysteine and cysteine residues of peptides or proteins resulting in *S*-nitroso compounds, nitrosothiols.[6,7)] Nitrosothiols are relatively stable and can release NO when they encounter transition metals or other reducing agents.[8—10)] Therefore, it can be seen that thiols act as an NO buffering system, controlling the intra- and extracellular activities of NO. We have recently reported the identification and characterization of a reaction product of uric acid (UA) with NO.[11,12)] When UA was treated with NO gas in a neutral solution under aerobic conditions, UA was consumed, yielding an unknown product. The product was identified as a nitrosated UA (NO-UA) from mass spectrometric data, although the position of the nitroso group on the molecule was not determined. NO-UA was unstable and decomposed with a half-life of 2.2 min at pH 7.4 and 37 °C. The incubation of NO-UA with glutathione resulted in the formation of *S*-nitrosoglutathione. NO-UA was also formed in the reaction with an NO donor, diethylamine NONOate (DEA-NO). NO-UA was detected in human serum and urine by *in vitro* treatment with DEA-NO. In the reactions of glutathione and *N*-acetycysteine (AcCys) with DEA-NO, the addition of UA caused increases in the yields of *S*-nitrosoglutathione and *N*-acety-*S*-nitrosocysteine (NO-AcCys), respectively. These results indicate that UA can readily react with NO, thereby generating a nitroso derivative which, in turn, can effectively transfer the nitroso group to thiols. Several UA derivatives methylated on nitrogen atoms exist in nature. 1,3-Dimethyluric acid (DiMUA, Fig. 1) is a metabolite of 1,3-dimethylxanthine (known as theophylline) formed by the C-8 hydroxylation. 1,3,7-Trimethyluric acid (TriMUA, Fig. 1) is a metabolite of 1,3,7-trimethylxanthine (well known as caffeine) formed by the C-8 hydroxylation. 1,3,7,9-Tetramethyluric acid (TetraMUA, Fig. 1), known as theacrine, is a major purine alkaloid in the leaves of an unusual Chinese tea kucha.[13)] In the present study, we investigated the reactions of DiMUA, TriMUA, and TetraMUA with DEA-NO and the effects of these compounds on the nitrosation of AcCys by DEA-NO.

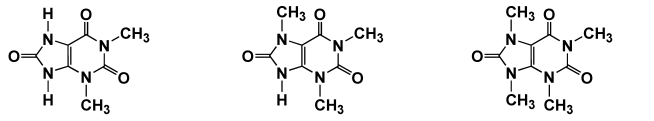

Fig. 1. Structures of 1,3-Dimethyluric Acid (DiMUA), 1,3,7-Trimethyluric Acid (TriMUA), and 1,3,7,9-Tetramethyluric Acid (TetraMUA)

Results

DiMUA (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C, and the reaction was monitored by reversed phase HPLC (RP-HPLC). Figure 2 is the RP-HPLC chromatogram of the reaction mixture at the reaction time of 10 min. In addition to the starting DiMUA peak (retention time t_R=6.2 min, λ_{max}=294 nm), a single peak due to a product **1** (t_R=8.6 min) showing a UV spectrum with λ_{max}=298 and 380 nm (Fig. 2, inset) appeared in the chromatogram. The product was isolated and subjected to mass spectrometry. Negative-ion electrospray ionization time of flight mass spectrometry (ESI-TOF/MS) of **1** showed signals at *m*/*z* 224 for a molecular ion $[M-H]^-$, which was 29 mass units higher than that of DiMUA, and at *m*/*z* 194 attributable to a denitrosated fragment ion $[M-NO-H]^-$ as shown in Fig. 3. High-resolution

* To whom correspondence should be addressed. e-mail: tsuzuki@shujitsu.ac.jp

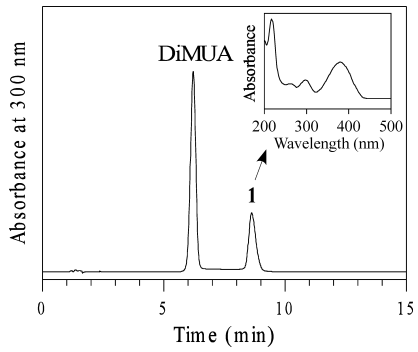

Fig. 2. RP-HPLC Chromatograph of a Reaction Solution of DiMUA with DEA-NO Detected at 300 nm

The inset is the on-line UV spectrum of **1**. DiMUA (300 μM) and DEA-NO (300 μM) were incubated in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 10 min. The sample was injected into RP-HPLC immediately after the reaction. The high performance liquid chromatography (HPLC) system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. For RP-HPLC, an Inertsil ODS-3 octadecylsilane column of 4.6×100 mm and particle size 5 μm (GL Science, Tokyo) was used. The eluent was 20 mM ammonium acetate buffer (pH 7.0) containing 10% methanol. The column temperature was 20 °C and the flow rate was 1.0 ml/min.

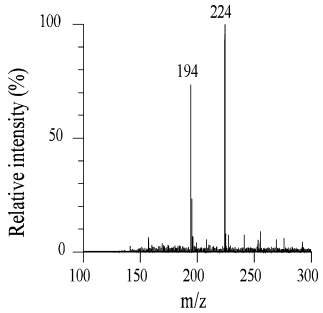

Fig. 3. Negative Ion Electrospray Ionization TOF (Time of Flight) Mass Spectrometry (ESI-TOF/MS, MicroTOF, Bruker) Spectrum of **1**

The sample isolated by RP-HPLC including 20 mM ammonium acetate and 10% methanol stored at 0 °C was directly infused into the MS system using a syringe pump.

ESI-TOF/MS (negative) of **1** showed m/z=224.0421 for the deprotonated molecular ion, which agreed with the theoretical molecular mass for $C_7H_6N_5O_4^-$ composition (m/z= 224.0425) within 3 ppm. Based on these data, **1** was identified as a nitrosated DiMUA (NO-DiMUA), although the position of the nitroso group on the molecule remains to be determined. Figure 4A shows the time course of the concentrations of formed NO-DiMUA and unreacted DiMUA when DiMUA (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C. The NO-DiMUA concentration increased up to 10 min and then decreased, while the DiMUA concentration decreased up to 10 min and then stayed constant. Figure 4B shows the DEA-NO dose dependence of the DiMUA/DEA-NO reaction at the reaction time of 10 min. The formed NO-DiMUA concentration increased with increasing DEA-NO dose. Figure 4C shows the DiMUA dose dependence of the DiMUA/DEA-NO reaction. The NO-DiMUA concentration increased with increasing DiMUA dose. However, the increase of the NO-DiMUA concentration was small above 300 μM of DiMUA. To evaluate the stability of NO-DiMUA, NO-DiMUA (*ca.* 8 μM) isolated by RP-HPLC including 2 mM ammonium acetate, 1% methanol, and 100 mM potassium phosphate was incubated at pH 7.4 and 37 °C, and the reaction was monitored by RP-HPLC. Figure 5 shows the time course of the change of NO-DiMUA concentration with its fitted line assuming first-order kinetics. The rate constant (k_1) was 6.44×10^{-4} s^{-1} and the half-life was 17.9 min. To reveal the effect of DiMUA on the nitrosation reaction of a thiol by an NO donor, AcCys (300 μM) was treated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C in the absence or presence of DiMUA (300 μM) and the concentrations were monitored by RP-HPLC. Figure 6A shows the time course of the concentration changes of NO-AcCys when AcCys is incubated with DEA-NO. The concentration of NO-AcCys increased up to 5 min and then reached a plateau. When AcCys was incubated with DEA-

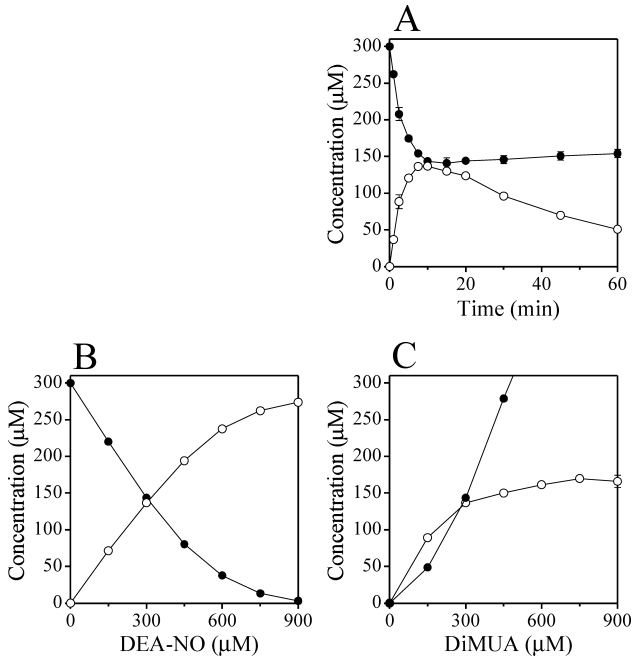

Fig. 4. The Concentration Changes in Formed NO-DiMUA (Open Circle) and Unreacted DiMUA (Closed Circle) on the Reaction of DiMUA with DEA-NO

(A) The time course of the reaction when DiMUA (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 0—60 min. (B) DEA-NO dose dependence (0—900 μM) of the reaction at the reaction time of 10 min. All other conditions are the same as in (A). (C) DiMUA dose dependence (0—900 μM) of the reaction at the reaction time of 10 min. All other conditions are the same as in (A). The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

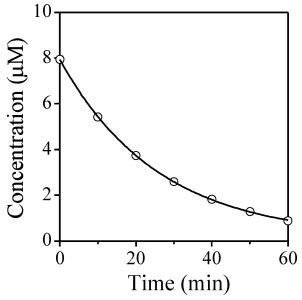

Fig. 5. Time Course of the Change of Concentration of NO-DiMUA (Open Circle) with Its Fitted Line Assuming First-Order Kinetics

NO-DiMUA isolated by RP-HPLC including 2 mM ammonium acetate, 1% methanol, and 100 mM potassium phosphate was incubated at pH 7.4 and 37 °C. The first-order rate constant was calculated using the nonlinear least-squares fitting algorithm in Igor Pro (WaveMetrics). The rate constant (k_1) was 6.44×10^{-4} s^{-1} and the half-life was 17.9 min.

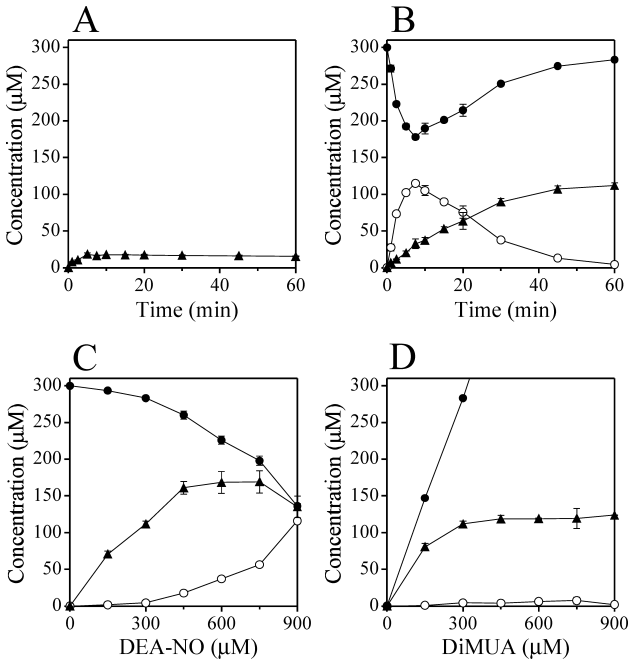

Fig. 6. (A) The Concentration Changes in Formed NO-AcCys (Closed Triangle) on the Reaction of AcCys with DEA-NO

AcCys (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 0—60 min.

(B) The Concentration Changes in Formed NO-DiMUA (Open Circle) and NO-AcCys (Closed Triangle) and Unreacted DiMUA (Closed Circle) on the Reaction of DiMUA and AcCys with DEA-NO

DiMUA (300 μM) and AcCys (300 μM) were incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 0—60 min.

(C) DEA-NO Dose Dependence (0—900 μM) of the DiMUA/AcCys/DEA-NO Reaction at the Reaction Time of 60 min

All other conditions are the same as in (B).

(D) DiMUA Dose Dependence (0—900 μM) of the DiMUA/AcCys/DEA-NO Reaction at the Reaction Time of 60 min

All other conditions are the same as in (B). The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

NO in the presence of DiMUA, the concentration of NO-DiMUA increased and the concentration of DiMUA decreased in the initial stage similar to the reaction without AcCys (Fig. 4A) as shown in Fig. 6B. The concentration of NO-AcCys then gradually increased with decreasing NO-DiMUA. Simultaneously, the DiMUA concentration increased, but the recovery of DiMUA was not complete even when NO-DiMUA had mostly disappeared at 60 min. At the reaction time of 60 min, the concentration of NO-AcCys was 7.4-fold greater than that in the reaction without DiMUA (Fig. 6A). Figure 6C shows the DEA-NO dose dependence of the DiMUA/AcCys/DEA-NO reaction at the reaction time of 60 min. The NO-AcCys concentration increased with increasing DEA-NO dose up to 450 μM DEA-NO, while NO-DiMUA increased with increasing DEA-NO dose especially above 450 μM DEA-NO. Figure 6D shows the DiMUA dose dependence of the reaction. The NO-AcCys concentration increased up to 300 μM DiMUA and reached a plateau, while the NO-DiMUA concentration stayed low. To evaluate the reaction of NO-DiMUA with AcCys, NO-DiMUA (*ca.* 8 μM) isolated by RP-HPLC was incubated with 300 μM AcCys in 100 mM potassium phosphate at pH 7.4 and 37 °C. Figure 7 shows the time course of the change of NO-DiMUA concentration with its fitted line assuming first-order kinetics. The

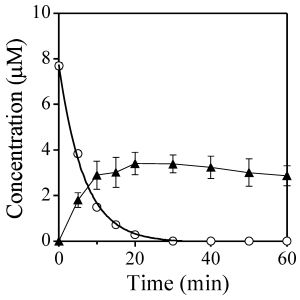

Fig. 7. Time Course of the Changes of Concentration of NO-DiMUA (Open Circle) with Its Fitted Line Assuming First-Order Kinetics and Formed NO-AcCys (Closed Triangle)

NO-DiMUA isolated by RP-HPLC including 2 mM ammonium acetate, 1% methanol, and 100 mM potassium phosphate was incubated with 300 μM AcCys at pH 7.4 and 37 °C. The first-order rate constant was calculated using the nonlinear least-squares fitting algorithm in Igor Pro. The rate constant (k_1) was 2.51×10^{-3} s^{-1} and the half-life was 4.6 min.

rate constant (k_1) was 2.51×10^{-3} s^{-1} and the half-life was 4.6 min. NO-AcCys was formed with the decrease of NO-DiMUA, although the yield of NO-AcCys was about 50% of consumed NO-DiMUA. As a control, similar experiments were performed for 1,3-dimethylxanthine (theophylline), but no reactions were observed (data not shown).

Similar experiments were performed for TriMUA. TriMUA (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 10 min, and the reaction was monitored by RP-HPLC. However, the concentration of TriMUA did not change and no product peak was observed (data not shown). Figure 8A shows the time course of the concentrations of TriMUA on the reaction of TriMUA with DEA-NO. The TriMUA concentration did not change up to 60 min. Figure 8B shows the time course of the concentration changes of TriMUA and NO-AcCys when AcCys (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C in the presence of TriMUA (300 μM). The concentrations of NO-AcCys increased more rapidly than those in the reaction in the presence of DiMUA (Fig. 6B). At the reaction time of 60 min, the concentration of NO-AcCys was 6.6-fold higher than that in the reaction in the absence of TriMUA (Fig. 6A). Figure 8C shows the DEA-NO dose dependence of the TriMUA/AcCys/DEA-NO reaction at the reaction time of 10 min. NO-AcCys increased with increasing DEA-NO dose, while TriMUA was not consumed. Figure 8D shows the TriMUA dose dependence of the reaction. The NO-AcCys concentration increased up to 300 μM TriMUA dose then reached a plateau. As a control, similar experiments were done for 1,3,7-trimethylxanthine (caffeine), but no reactions were observed (data not shown).

In the case of TetraMUA, its concentration did not change and no product peak was observed when 300 μM TetraMUA was incubated with 300 μM DEA-NO in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C. The concentration of NO-AcCys did not change by addition of 300 μM TetraMUA in the reaction mixture of 300 μM AcCys and 300 μM DEA-NO at pH 7.4 and 37 °C.

To clarify the contribution of oxygen molecules dissolved in the reaction mixture on the nitrosation reactions, the reactions were performed under Ar gas bubbling. Table 1 shows the concentrations of DiMUA or TriMUA and formed NO-

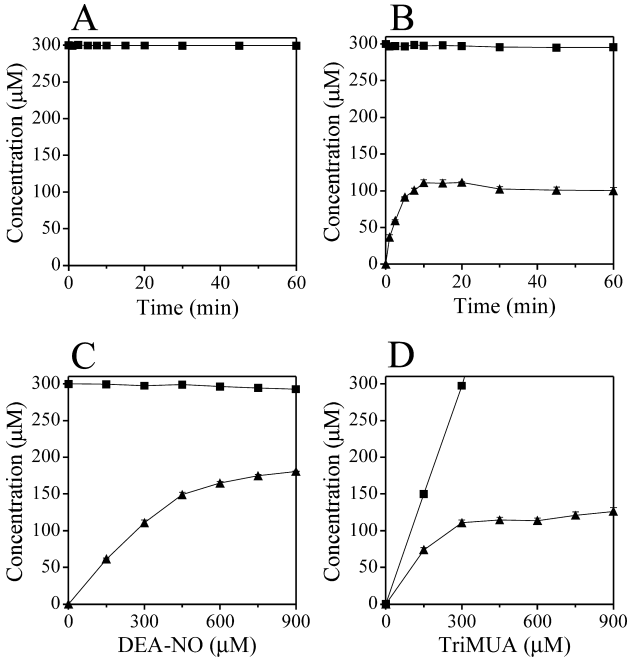

Fig. 8. (A) The Concentration Changes in Formed TriMUA (Closed Square) on the Reaction of TriMUA with DEA-NO

TriMUA (300 μM) was incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 0—60 min.

(B) The Concentration Changes in Formed NO-AcCys (Closed Triangle) and Unreacted DiMUA (Closed Square) on the Reaction of TriMUA and AcCys with DEA-NO

TriMUA (300 μM) and AcCys (300 μM) were incubated with DEA-NO (300 μM) in potassium phosphate buffer (100 mM) at pH 7.4 and 37 °C for 0—60 min.

(C) DEA-NO Dose Dependence (0—900 μM) of the TriMUA/AcCys/DEA-NO Reaction at the Reaction Time of 10 min

All other conditions are the same as in (B).

(D) TriMUA Dose Dependence (0—900 μM) of the TriMUA/AcCys/DEA-NO reaction at the reaction time of 10 min

All other conditions are the same as in (B). The concentrations were determined by RP-HPLC analysis. Means±S.D. (n=3) are presented.

Table 1. Effects of Ar Bubbling on the Nitrosation Reaction Systems[a)]

Reaction systems	Ar bubbling	DiMUA or TriMUA (μM)	NO-DiMUA (μM)	NO-AcCys (μM)
AcCys/DEA-NO	−			17.7±0.1
	+			2.4±0.2
DiMUA/DEA-NO	−	143.7±0.1	136.7±1.0	
	+	299.8±1.1	1.4±0.2	
DiMUA/AcCys/DEA-NO	−	189.6±7.3	105.0±6.8	37.3±3.7
	+	299.9±0.6	0.7±0.6	2.8±0.7
TriMUA/DEA-NO	−	299.5±1.6		
	+	300.2±0.4		
TriMUA/AcCys/DEA-NO	−	297.4±1.5		110.9±4.0
	+	300.2±0.4		4.4±0.7

a) Substrates (AcCys, DiMUA, DiMUA/AcCys, TriMUA, or TriMUA/AcCys: 300 μM each) with 100 mM potassium phosphate buffer (pH 7.4) were incubated with DEA-NO (300 μM) with or without Ar bubbling in a glass tube. Ar bubbling: The mixture (990 μl) of the substrate and buffer was bubbled by Ar gas with a flow rate of 10 ml/min at 37 °C for 10 min. Then DEA-NO solution (30 μM, 10 μl) was added into the mixture and incubated at 37 °C for 10 min with the Ar bubbling. Immediately after the reaction, the samples were injected into the RP-HPLC system to determine the concentrations. Means±S.D. (n=3) are shown.

DiMUA and NO-AcCys with and without Ar bubbling. In all the systems, Ar bubbling largely depressed the formation of NO-DiMUA and NO-AcCys, showing that oxygen is essential to the nitrosation of the present study.

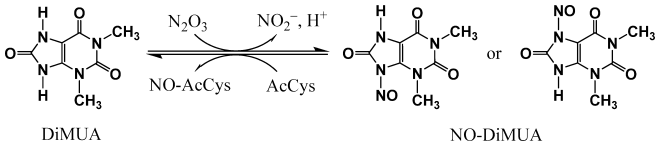

Fig. 9. Possible Reaction Scheme of the DiMUA/AcCys/DEA-NO System

Discussion

Under aerobic conditions, DiMUA reacted with DEA-NO forming a nitrosated compound (NO-DiMUA) at neutral pH. Since Ar bubbling in the reaction mixture largely depressed the formation of NO-DiMUA (Table 1), the reactive species for the nitrosation in the system is presumed to be N_2O_3. As shown in Fig. 4, when 300 μM DiMUA was incubated with 300 μM DEA-NO at pH 7.4 and 37 °C for 10 min, the yield of DiMUA was 136.8 μM while 156.3 μM of DiMUA was consumed. Thus, the yield of NO-DiMUA was 88% relative to the consumed DiMUA. DEA-NO, the NO-donor, has been reported to decompose with a half-life of 2 min in 100 mM phosphate buffer at pH 7.4 and 37 °C.[14)] Almost all (*ca.* 97%) DEA-NO can therefore be assumed to have decomposed by the reaction time of 10 min under the present reaction conditions. Thus, the yield of NO-DiMUA relative to the consumed DEA-NO was *ca.* 30%. Incubation of isolated NO-DiMUA with AcCys generated NO-AcCys at neutral pH. As shown in Fig. 7, the yield of NO-AcCys relative to the consumed NO-DiMUA was *ca.* 50%. The decomposition rate of NO-DiMUA with AcCys was greater than that in the absence of AcCys (Figs. 5, 7). Thus, NO-DiMUA would directly pass the NO group to AcCys *via* transnitrosation forming NO-AcCys. A possible reaction scheme of NO-DiMUA and NO-AcCys formation in the DiMUA/AcCys/DEA-NO system under aerobic conditions is shown in Fig. 9.

DiMUA, similar to UA, has a five-membered ring, an imidazolone, in its structure, while two amido groups on the pyrimidine ring of DiMUA are methylated. DiMUA reacted with the NO donor to generate an unstable nitrosated derivative, thus accelerating *S*-nitrosation of AcCys by the NO donor similar to UA. 1,3-Dimethylxanthine, which has an imidazole ring and the same pyrimidine ring as that of DiMUA, showed no reaction with the NO donor and no effect on the *S*-nitrosation. Thus, the imidazolone ring should be essential to the nitrosation reactions, suggesting that either N-7 or N-9 on DiMUA is likely to be the nitrosated site. On the other hand, TriMUA has another five-membered ring, an *N*-methylated imidazolone, while two amido groups on the pyrimidine ring are both methylated as in the case of DiMUA. When TriMUA was treated with the NO donor, neither consumption of TriMUA nor any product was observed. However, TriMUA did accelerate *S*-nitrosation of AcCys with the NO donor in a manner similar to UA and DiMUA. These results suggest that TriMUA was in fact also nitrosated with the NO donor and then transfered the nitroso group to AcCys. The half-life of the nitrosated TriMUA is presumed to be too short to be detected by the HPLC analysis. Trimethylxanthine, which has an *N*-methylated imidazole ring and the same pyrimidine ring as TriMUA, showed no reaction with the NO donor and no effect on the *S*-nitrosation.

Therefore, the *N*-methylated oxoimidazole ring of TriMUA could be subjected to a rapid, transient nitrosation most likely at N-9. This is supported by the results on TetraMUA in which no reaction with the NO donor and no effect on the *S*-nitrosation were observed. The half-life of NO-DiMUA at pH 7.4 and 37 °C (17.9 min) was longer than that of NO-UA (2.2 min). It has been reported that *N'*-methylations of *N*-methyl-*N*-nitrosourea cause increased stability at neutral pH, since base-catalyzed hydrolysis is predominant for the decomposition reactions.[15] NO-UA and NO-DiMUA behave similarly to *N*-methyl-*N*-nitrosourea and its methyl derivatives. However, NO-TriMUA was unstable under the present neutral conditions. NO-UA and NO-DiMUA can possess an enolate form on their amide groups. Indeed, UA and DiMUA exist mainly as their enolate forms releasing H^+ at N3 or N9 under neutral conditions with pK_a values of 5.3—6.2.[16—18] If NO-UA and NO-DiMUA exist in their enolate forms at pH 7.4 and if their decomposition reactions are base-catalyzed hydrolysis, the reactions would be slower than those in the neutral keto forms. On the other hand, NO-TriMUA cannot possess an enolate form, since NO-TriMUA has no hydrogen atom to release as H^+ on its structure. This may be a possible explanation of the reduced stability of NO-TriMUA.

Caffeine (1,3,7-trimethylxanthine) is one of the most frequently consumed alkaloids and occurs in many plants.[19,20] A high concentration of caffeine is present in coffee, green tea, black tea, cocoa, and cola beverages. Caffeine is catabolized *via* removal of the methyl groups and hydroxylation at C-8 position in humans forming several metabolites including DiMUA and TriMUA as minor components.[21] Excretion of DiMUA and TriMUA in the urine has been reported to increase following oral ingestion of caffeine by healthy adults.[22] Theophylline (1,3-dimethylxanthine) is a nonspecific inhibitor of phosphodiesterases and is extensively used as a drug to treat asthma and COPD.[23] Theophylline is also a metabolite of caffeine in humans and is metabolized by liver microsomal enzymes generating DiMUA, which accounts for *ca.* 50% of the total theophylline clearance.[24—26] Excretion of DiMUA in the urine is also increased after an oral ingestion of theophylline.[27] In addition, DiMUA and TriMUA are generated by reactions of reactive oxygen species such as hydroxyl radicals with theophylline and caffeine, respectively, *via* the C-8 hydroxylation.[28,29] Intake of caffeine and theophylline would therefore increase intra- and intercellular concentrations of DiMUA and TriMUA in humans.

In conclusion, the present study suggests that both DiMUA and TriMUA react with NO, forming their *N*-nitroso derivatives which can act as vehicles of NO to thiols. Especially, TriMUA acts as a good catalyst for the *S*-nitrosation without consumption. Since DiMUA and TriMUA are the metabolites of caffeine and theophylline, their intake or administration may affect the fate of NO generated in humans.

Experimental

Materials 1,3-Dimethyuric acid (DiMUA), 1,3,7-trimethyuric acid (TriMUA), and *N*-acetylcystein (AcCys) were obtained from Sigma (MO, U.S.A.). 1,3,7,9-Tetramethyuric acid (TetraMUA) was from Pfaltz & Bauer (CT, U.S.A.). Diethylamine NONOate (DEA-NO) was purchased from Cayman Chemical (MI. U.S.A.). All other chemicals of reagent grade were purchased from Sigma, Aldrich (WI, U.S.A.), Cica (Tokyo), and Nacalai Tesque (Osaka, Japan), and used without further purification. Water was purified with a Millipore Milli-Q deionizer.

HPLC and MS Conditions The high performance liquid chromatography (HPLC) system consisted of Shimadzu LC-10ADvp pumps and an SCL-10Avp system controller. On-line UV spectra were obtained with a Shimadzu SPD-M10Avp UV–vis photodiode-array detector. Absorbance spectra were collected from 200 to 500 nm. For the reversed phase HPLC (RP-HPLC), an Inertsil ODS-3 octadecylsilane column of 4.6×100 mm and particle size 5 μm (GL Science, Tokyo) was used. The eluent was 20 mM ammonium acetate buffer (pH 7.0) with 10% methanol. The column temperature was 20 °C and the flow rate was 1.0 ml/min. The electrospray ionization time of flight mass spectrometry (ESI-TOF/MS) measurements were performed on a MicroTOF spectrometer (Bruker, Bremen, Germany) in negative mode. The sample isolated by RP-HPLC was directly infused into the MS system by a syringe pump without a column.

Quantitative Procedures The concentrations of all the compounds in the reaction mixture were evaluated from integrated peak areas on HPLC chromatograms compared with those of authentic standard solutions. The concentration of NO-DiMUA was determined assuming that NO-DiMUA was converted into DiMUA exclusively when NO-DiMUA isolated by RP-HPLC was incubated with 13 mM AcCys at 37 °C for 15 min. NO-AcCys was synthesized from AcCys by bubbling a mixture of NO/O_2 through an AcCys solution using the reported procedures.[30] The detection wavelength was 300 nm. All the reaction mixtures were analyzed by RP-HPLC immediately after the reactions.

References and Notes

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