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Formation of 2-Chloroinosine from Guanosine by Treatment of HNO_2 in the Presence of NaCl

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Abstract—We investigated the reaction of Guo with nitrous acid in the presence of NaCl. When 1 mM Guo was incubated with 100 mM $NaNO_2$ and 2 M NaCl in sodium acetate buffer at pH 3.2 and 37 °C, 2-chloroinosine (2-Cl-Ino) was produced in addition to oxanosine (Oxo) and xanthosine (Xao). The yield of 2-Cl-Ino was 0.033 mM at an incubation time of 2 h. Under the same reaction conditions, GMP and dGuo gave rise to the corresponding 2-chloro derivatives with comparable yields. All the 2-chloro derivatives were fairly stable ($t_{1/2} > 360$ h) at physiological pH and temperature. To elucidate the reaction mechanism of the chlorination, the diazoate derivative of Guo, a reaction intermediate of the Guo–HNO_2 system, was employed as a starting compound. When the diazoate was incubated with 2 M NaCl in a neutral solution, 2-Cl-Ino was produced in addition to Oxo and Xao. These results suggest that the 2-chloro derivatives can be produced from foodstuffs in the human stomach and may have potential importance as a carcinogen causing gastric cancer.

Introduction

Despite the long-standing and widespread reduction in the incidence and mortality rates, gastric cancer is still one of the most common neoplastic causes of death world-wide.[1] Based on the observation that gastric cancer mortality and stroke mortality were strongly correlated, a hypothesis was presented in 1969 that salt could be involved in the aetiology of gastric cancer.[2] Thereafter, a large number of epidemiological and laboratory studies relevant to the assessment of salt for carcinogenic potential were reported. Although many case control studies found a positive association between high salt intake and the stomach cancer incidence, the risk factor tends to show a great variability in different population.[3] Animal studies showed that salt had co-carcinogenic and promoting effects when administered with an N-nitrosoguanidine, a carcinogen.[4,5] Recently, Cohen and Roe have reviewed these findings and concluded that there is no reliable epidemiological evidence to indicate that dietary salt affects the incidence of gastric cancer.[6] Neither is there any laboratory evidence whatsoever to indicate that salt per se is a carcinogen.[6]

Another potential risk factor for gastric cancer is nitrite (NO_2^-). The reaction of NO_2^- with biomolecules has been well investigated. NO_2^- is protonated and results in nitrous acid (HNO_2) in the stomach. HNO_2 produces mutagenic N-nitroso compounds from secondary amines in foodstuff.[7] HNO_2 can also react directly with components of nucleic acids such as Guo, Ado, and Cyd, generating deamination products, that is xanthosine (Xao), inosine (Ino), and uridine (Urd), respectively.[8] Among these nucleosides, Guo reacts with HNO_2 more rapidly than Ado and Cyd[9] and gives rise to several products including oxanosine (Oxo) which is the second major product and potentially mutagenic.[10–12] In addition to direct ingestion of NO_2^- as a content of foodstuffs or a contaminant of drinking water, NO_2^- is generated in the saliva from ingested NO_3^- by nitrate-reducing microorganisms.[13,14] Since the amount of NO_2^- generated from NO_3^- in vivo is 2-fold greater than that ingested directly, dietary intake of NO_3^- can be important for gastric cancer. Many epidemiological and laboratory studies have been reported to assess carcinogenic potential of NO_3^- and NO_2^-.

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Case–control studies showed both positive and negative association between NO_3^- or NO_2^- and gastric cancer.[15] In certain animal studies, negative association were reported.[16,17] Therefore, although many efforts have been made to determine whether or not high intake of NO_3^- or NO_2^- is a cause of gastric cancer, it remains inconclusive.[15,18–20]

Recently, an ecological study which examined the individual importance of salt and NO_3^- intake was reported.[21] The analysis showed that the importance of NO_3^- in the stomach cancer mortality increased markedly with a high sodium level. However, there is no study on the molecular basis for the possible cooperative effect of HNO_2 and NaCl on carcinogenesis.

We report herein the formation of 2-chloroinosine (2-Cl-Ino) from Guo with HNO_2 in the presence of NaCl, and discuss the reaction mechanism of the chlorination based on the reactivity of a diazoate derivative of Guo.

Results

Guo (1 mM) was incubated with 100 mM $NaNO_2$ and 2 M NaCl in 3 M sodium acetate buffer at pH 3.2 and 37 °C. Figure 1 shows the reversed phase (RP)-HPLC chromatogram of the reaction mixture at the incubation time of 2 h. An unknown product eluted [retention time (t_R) = 11.6 min, λ_{max} = 256 nm] in addition to oxanosine (Oxo) (t_R = 10.5 min, λ_{max} = 245 and 287 nm), xanthosine (Xao) (t_R = 7.4 min, λ_{max} = 248 and 277 nm), and unreacted Guo in the RP-HPLC analysis. Formation of the product with t_R = 11.6 min was not observed in a similar reaction without NaCl. Figure 1 inset shows the UV spectrum of the product. The product was isolated by preparative RP-HPLC. Negative ion atmospheric pressure chemical ionization (APCI-) LC/MS of this product showed signals with m/z = 303 and 301 with relative intensities of 1:3. When authentic 2-chloroadenosine (2-Cl-Ado) was incubated with 100 mM $NaNO_2$ in 3 M acetate buffer (pH 3.7) at 37 °C for 24 h, a major peak with the same retention time (t_R = 11.6 min) and UV spectrum (λ_{max} = 256 nm) as the unknown product appeared in the RP-HPLC chromatogram. APCI-LC/MS analysis of the product formed from 2-Cl-Ado also showed signals with m/z = 303 and 301 (1:3). It has been reported that 2-chloro-2′-deoxyadenosine (2-Cl-dAdo) is deaminated by HNO_2 resulting in 2-chloro-2′-deoxyinosine (2-Cl-dIno).[22] Combining these data, we have concluded that the product is 2-chloroinosine (2-Cl-Ino) (Fig. 2). Figure 3 shows the time courses of the concentration changes in 2-Cl-Ino, Oxo, Xao, and Guo under the above reaction conditions. The yields of reaction products at the incubation time of 2 h were 3.3% 2-Cl-Ino, 13.4% Oxo, 68.9% Xao, and 9.7% unreacted Guo. The yield of 2-Cl-Ino was found to be proportional to the NaCl concentration: at 100 mM (plasma concentration) and 500 mM, the yields were 0.15 and 0.76%, respectively.

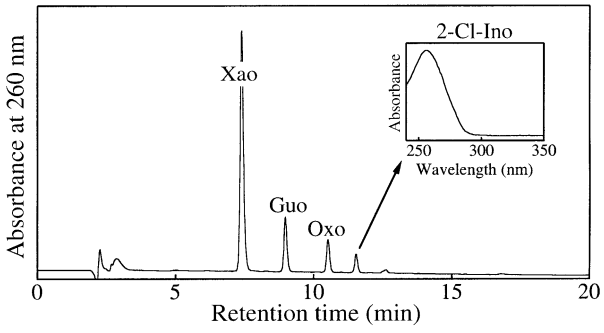

Figure 1. RP-HPLC chromatogram for the reaction mixture of Guo treated by HNO_2 in the presence of NaCl. Guo (1 mM) was incubated with 100 mM $NaNO_2$ and 2 M NaCl in 3 M sodium acetate buffer at pH 3.2 and 37 °C for 2 h. An HPLC system equipped with an ODS column (4.6×150 mm) was used. The eluent was 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) containing CH_3CN and the flow rate was 1 mL/min. The CH_3CN concentration was increased from 0 to 20% for 20 min in a linear gradient mode. Inset is an on-line detected UV spectrum of 2-Cl-Ino.

GMP was also treated with HNO_2 and NaCl under similar reaction conditions and the reaction was monitored by RP-HPLC. GMP produced 2-chloroinosine 5′-monophosphate (2-Cl-IMP) (t_R = 10.3 min, λ_{max} = 256 nm) in addition to oxanosine 5′-monophosphate (OMP) (t_R = 9.1 min, λ_{max} = 245 and 287 nm) and xanthosine 5′-monophosphate (XMP) (t_R = 6.3 min, λ_{max} = 249 and 276 nm). Identification of each product was achieved by alkaline phosphatase digestion, which dephosphorylated 2-Cl-IMP, OMP, and XMP to 2-Cl-Ino, Oxo, and Xao, respectively. The yields of reaction products at the incubation time of 2 h were 3.8% 2-Cl-IMP, 15.5% OMP, 86.1% XMP, and 2.4% unreacted GMP. Their yields were essentially comparable to those for Guo.

A similar reaction was performed with dGuo. dGuo produced 2-Cl-dIno (t_R = 12.9 min, λ_{max} = 256 nm) in addition to 2′-deoxyoxanosine (dOxo) (t_R = 12.1 min, λ_{max} = 246 and 287 nm), 2′-deoxyxanthosine (dXao)

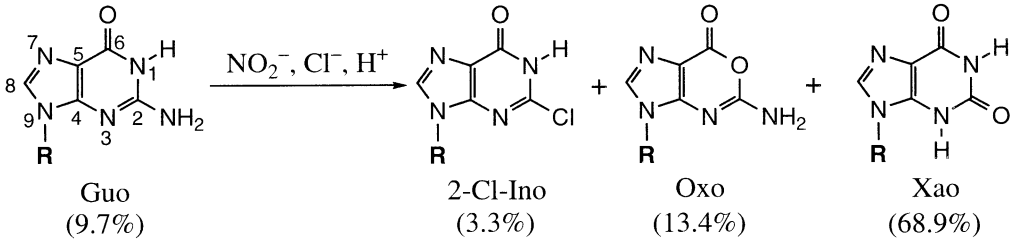

Figure 2. Reaction products of Guo treated by HNO_2 in the presence of NaCl. The numbers in the parentheses denote percentage yields of products at the reaction time of 2 h. R = ribose.

($t_R = 8.4$ min, $\lambda_{max} = 248$ and 277 nm), and xanthine (Xan) ($t_R = 6.3$ min, $\lambda_{max} = 267$ nm). Identification of 2-Cl-dIno was achieved by negative ion APCI-LC/MS analysis, which showed peaks of $m/z = 287$ and 285 with relative intensities of 1:3. RP-HPLC analysis also revealed that HNO_2-treated 2-Cl-dAdo gave a peak with the same t_R and UV spectrum of the product formed from dGuo. The yields of reaction products at the incubation time of 2 h were 3.4% 2-Cl-dIno, 12.6% dOxo, 43.2% dXao and 30.0% Xan with 10.4% unreacted dGuo.

To examine the stability of the 2-chloro derivatives, purified 2-Cl-Ino, 2-Cl-IMP, and 2-Cl-dIno (all 25 μM) were incubated in 100 mM sodium phosphate buffer under physiological conditions (pH 7.4, 37 °C) up to 92 h and analyzed by RP-HPLC. Although 2-Cl-IMP underwent dephosphorylation slowly resulting in 2-Cl-Ino exclusively ($t_{1/2} = 360$ h), 2-Cl-Ino and 2-Cl-dIno remained intact after 92 h (data not shown).

To elucidate the reaction mechanism for the formation of 2-Cl-Ino in the Guo–HNO_2–NaCl system, a diazoate derivative of Guo (Guo-diazoate) was used. The RP-HPLC fraction containing the Guo-diazoate was prepared as described previously.[23] The RP-HPLC fraction of the Guo-diazoate (20 μL) was directly dropped into 2.5 M NaCl solution containing 500 mM phosphate buffer of (pH 7.4, 80 μL). The final NaCl concentration of the reaction mixture is 2 M. The reaction mixture was incubated at 37 °C for 1 h and analyzed by RP-HPLC. As shown in Figure 4, 2-Cl-Ino was produced in addition to Oxo and Xao in the Guo-diazoate–NaCl system. The relative yield of the products was 2-Cl-Ino–Oxo–Xao = 1:3.5:13.9. The ratio was similar to that for the Guo–HNO_2–NaCl system (2-Cl-Ino–Oxo–Xao = 1:4.0:20.7) (Fig. 5).

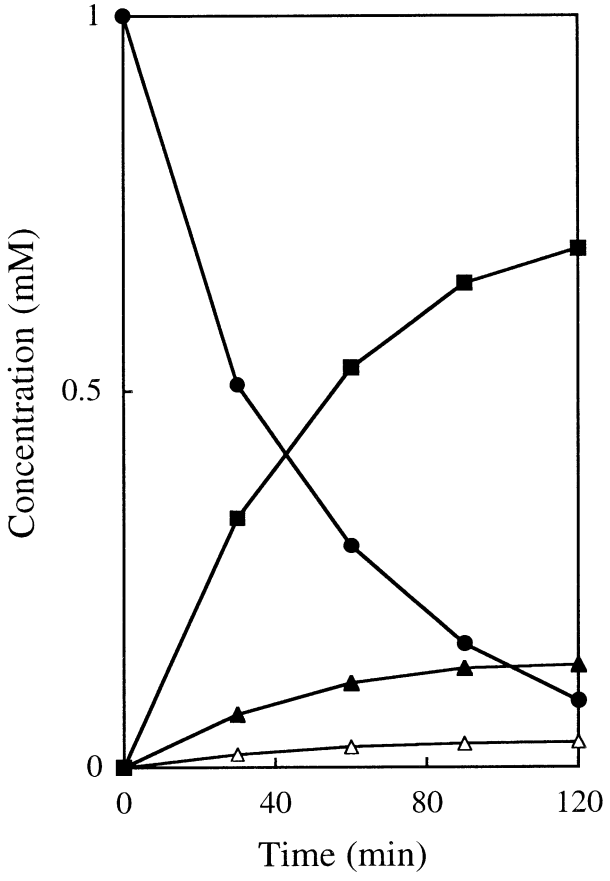

Figure 3. The time courses of formation of 2-Cl-Ino (open triangle), Oxo (closed triangle), Xao (closed square) and conversion of Guo (closed circle) when Guo (1 mM) was incubated with 100 mM $NaNO_2$ and 2 M NaCl in 3 M sodium acetate buffer at pH 3.2 and 37 °C. The concentration was determined by RP-HPLC analysis.

Discussion

In the present study, it was shown that 2-Cl-Ino was produced by the incubation of Guo with $NaNO_2$ and NaCl under mild acidic conditions. Similarly, 2-Cl-IMP and 2-Cl-dIno were produced from GMP and dGuo, respectively. The materials required for the reactions (nucleic acid components, $NaNO_2$, and NaCl) can be supplied by ingested foods. In addition, the saliva supplies additional NO_2^- at levels ranging from several to several hundreds mg/L[13,14] and the secreted gastric acid solution contains about 160 mM of hydrochloric acid.[24] These suggest that 2-chloro derivatives can be formed in the human stomach. Since 2-chloro derivatives were fairly stable under physiological conditions, it would remain intact in the intestines and be absorbed. If the absorbed 2-chloro derivatives are converted to the deoxyribonucleoside triphosphate by salvage and/or de novo pathways of purine nucleotide biosynthesis and are incorporated into DNA by DNA polymerase, it may have an important role in mutagenic or lethal events in cells. Inosine 5′-monophosphate dehydrogenase (IMPDH), which catalyzes the oxidation of IMP to XMP, plays an important role in the purine metabolic pathways. Recently, it has been shown that human type II IMPDH catalyzes dehalogenation of 2-Cl-IMP resulting in XMP and Cl^-.[25] Since the turnover rate of 2-Cl-IMP was one-third of that observed for IMP, 2-Cl-IMP was a reasonably good substrate of IMPDH. The IMPDH was not inhibited irreversibly by 2-Cl-IMP. The report on IMPDH implies that the 2-chloro

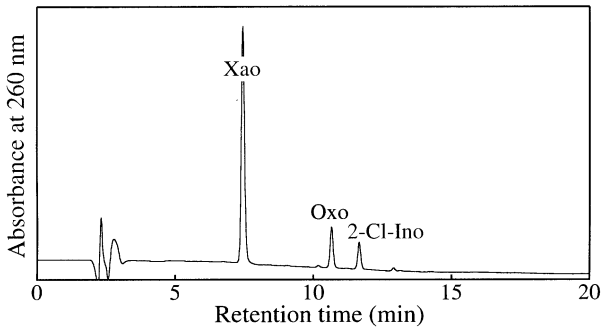

Figure 4. RP-HPLC chromatogram for the reaction mixture of the diazoate derivative of Guo incubated in the presence of NaCl. The RP-HPLC fraction containing the diazoate (20 μL) was directly dropped into 2.5 M NaCl solution (80 μL) containing 500 mM phosphate buffer (pH 7.4) and incubated at 37 °C for 1 h. RP-HPLC conditions were the same as those described in Figure 1.

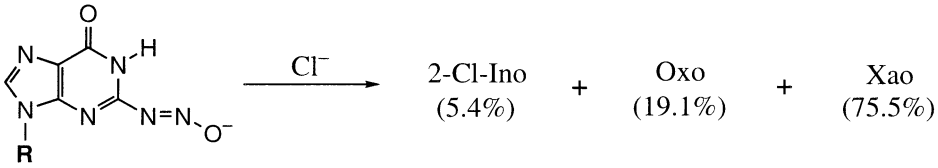

Figure 5. Reaction products formed from the diazoate derivative of Guo (Guo-diazoate) in the presence of NaCl. The numbers in the parentheses denote relative yields of products.

derivatives may exist in human bodies and human cells have a protection system to the 2-chloro derivatives.

The reaction of protonated NO_2^- (i.e., HNO_2) is complicated.[26,27] Protonated NO_2^- generates reactive nitrogen species such as N_2O_3 and NO^+, both of which nitrosate amines. A nitrosated primary amine is converted to hydroxylated compounds (R–OH) via several intermediates such as diazohydroxide (R–N=N–OH), diazoate (R–N=N–O^-), diazonium (R–N≡N^+), and alkyl cation (R^+). The reaction between chloride (Cl^-) and the intermediates from Guo can result in 2-Cl-Ino. In addition to N_2O_3 and NO^+, nitrosyl chloride (NOCl), another reactive nitrogen species, could be produced when NO_2^- is protonated in the presence of Cl^-. NOCl nitrosates amines and chlorinates aromatic compounds. Moreover, it is reported that in concentrated hydrochloric acid, NO_2^- facilitates the formation of Cl_2, which can also chlorinate aromatic compounds. The reaction between these reactive chlorinating species and Guo can lead to the formation of 2-Cl-Ino. Recently we have isolated an intermediate formed in the reaction of Guo with HNO_2 by RP-HPLC.[23] The intermediate was identified as a diazoate derivative of Guo with a half-life of 5.6 min at pH 7.0 and 20 °C. This intermediate turned out to be very useful to study the reaction mechanism of the Guo–HNO_2 system since various reaction conditions could be employed. In the present study, when the diazoate derivative was incubated with NaCl in neutral solution, 2-Cl-Ino was produced. This result clearly shows that 2-Cl-Ino is formed by the reaction between chloride anion (Cl^-) and the cation formed from the diazonium derivative or its precursor (diazonium derivative).

Conclusion

It has been found that 2-Cl-Ino, 2-Cl-IMP and 2-Cl-dIno are formed from Guo, GMP and dGuo, respectively, by HNO_2 treatment in the presence of NaCl. The intermediates involved in the reaction have been elucidated as the diazoate derivatives which are generated from the starting compounds by HNO_2. These results suggest that the 2-chloro derivatives can be produced in the human stomach and may play unique role in carcinogenesis.

Experimental

Materials

Guo was obtained from Kohjin (Tokyo, Japan), and dGuo from Wako Pure Chemicals (Osaka, Japan). GMP, 2-chloroadenosine (2-Cl-Ado), 2-chloro-2′-deoxyadenosine (2-Cl-dAdo), and xanthosine (Xao) were purchased from Sigma (St. Louis, MO, USA). Authentic oxanosine (Oxo), 2′-deoxyoxanosine (dOxo), and 2′-deoxyxanthosine (dXao) were prepared from Guo and dGuo by HNO_2 treatment, respectively, as reported previously.[10,23] All other chemicals of reagent grade were purchased from Wako Pure Chemicals or Nacalai Tesque (Osaka, Japan), and used without further purification. Water was purified with a Millipore Milli-QII deionizer.

RP-HPLC conditions

The HPLC system consisted of Tosoh DP-8020 pumps and a PX-8020 system controller. On-line UV spectra were obtained with a Tosoh PD-8020 UV–vis photodiode-array detector. For reversed phase (RP-) HPLC, a YMC-Pack ODS-A octadecylsilane column (4.6×150 mm, particle size 5 μm) was used. The eluent was 100 mM triethylammonium acetate (TEAA) buffer (pH 7.0) containing CH_3CN. The CH_3CN concentration was increased from 0 to 20% for 20 min in a linear gradient mode. HPLC analysis was performed at room temperature with a flow rate of 1 mL/min.

Spectrometric measurements

Negative-ion atmospheric pressure chemical ionization (APCI-) LC mass spectra were obtained by a Hitachi M-2000 MS system. The sample was directly injected into the MS system by an HPLC pump without separation columns. The following LC/MS conditions were used: eluent, 100% CH_3CN (isocratic); flow rate, 1 mL/min; vaporization temperature, 300 °C; desolvation temperature, 340 °C; drift voltage, −195 V.

Quantitative procedures

The concentrations of products were evaluated from integrated peak areas of the HPLC chromatogram and the molar extinction coefficients at 260 nm. The ε_{260} values of 1.18×10^4 M^{-1} cm^{-1} for 2-Cl-Ino, 2-Cl-IMP, and 2-Cl-dIno, 5.1×10^3 M^{-1} cm^{-1} for Oxo, OMP, and dOxo, 7.8×10^3 M^{-1} cm^{-1} for Xao, XMP, and dXao,

8.5×10^3 M^{-1} cm^{-1} for Xan, and 1.15×10^4 M^{-1} cm^{-1} for Guo, GMP, and dGuo were used.[28–30] In the quantitative analysis of the reaction products, the initial RP-HPLC peak area was used as a standard.

Enzymatic digestion of nucleotides

The solution of 2-Cl-IMP, OMP, and XMP was incubated in 0.5 mL of 100 mM Tris–HCl buffer (pH 7.0) containing alkaline phosphatase (1 unit) at 37 °C for 4 h. Aliquots of the solution were analyzed by RP-HPLC. The chromatograms of the digested 2-Cl-IMP, OMP, and XMP showed the peaks of the corresponding nucleosides (2-Cl-Ino, Oxo, and Xao, respectively).

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References and Notes

1. Parkin, D. M.; Pisani, P.; Ferlay, J. *Int. J. Cancer* **1993**, *54*, 594.
2. Joossens, J. V.; Geboer, J. *Nutr. Cancer* **1981**, *2*, 250.
3. Kono, S.; Hirohata, T. *Cancer Causes Control* **1996**, *7*, 41.
4. Takahashi, M.; Kokubo, T.; Furukawa, F.; Kurokawa, Y.; Tatematsu, M.; Hayashi, Y. *Gann* **1983**, *74*, 28.
5. Takahashi, M.; Kokubo, T.; Furukawa, F.; Kurokawa, Y.; Hayashi, Y. *Gann* **1984**, *75*, 494.
6. Cohen, A. J.; Roe, F. J. C. *Food Chem. Toxicol.* **1997**, *35*, 271.
7. Correa, P. *Cancer Res.* **1988**, *48*, 3554.
8. Schuster, H.; Schramm, G. *Z. Naturforsch.* **1958**, *13b*, 697.
9. Shapiro, R.; Pohl, S. H. *Biochemistry* **1968**, *7*, 448.
10. Suzuki, T.; Yamaoka, R.; Nishi, M.; Ide, H.; Makino, K. *J. Am. Chem. Soc.* **1996**, *118*, 2515.
11. Suzuki, T.; Matsumura, Y.; Ide, H.; Kanaori, K.; Tajima, K.; Makino, K. *Biochemistry* **1997**, *36*, 8013.
12. Suzuki, T.; Yoshida, M.; Yamada, M.; Ide, H.; Kobayashi, M.; Kanaori, K.; Tajima, K.; Makino, K. *Biochemistry* **1998**, *37*, 11592.
13. Tannenbaum, S. R.; Sinskey, A. J.; Weisman, M.; Bishop, W. *J. Natl. Cancer Inst.* **1974**, *53*, 79.
14. Tannenbaum, S. R.; Weisman, M.; Fett, D. *Food Cosmet. Toxicol.* **1976**, *14*, 549.
15. Forman, D. *Cancer Surv.* **1989**, *8*, 443.
16. Maekawa, A.; Ogiu, T.; Onodera, H.; Furuta, K.; Matsuoka, C.; Ohno, Y.; Odashima, S. *Food Chem. Toxicol.* **1982**, *20*, 25.
17. Inai, K.; Aoki, Y.; Tokuoka, S. *Gann* **1979**, *70*, 203.
18. Forman, D.; Al-Dabbagh, S.; Doll, R. *Nature* **1985**, *313*, 620.
19. Mirvish, S. S. *Nature* **1985**, *315*, 461.
20. Tannenbaum, S. R.; Correa, P. *Nature* **1985**, *317*, 675.
21. Joossens, J. V.; Hill, M. J.; Elliott, P.; Stamler, R.; Stamler, J.; Lesaffre, E.; Dyer, A.; Nichols, R.; Kesteloot, H. *Int. J. Epidemiol.* **1996**, *25*, 494.
22. Venner, H. *Chem. Ber.* **1960**, *93*, 140.
23. Suzuki, T.; Ide, H.; Yamada, M.; Endo, N.; Kanaori, K.; Tajima, K.; Morii, T.; Makino, K. *Nucleic Acids Res.* **2000**, *28*, 544.
24. Guyton, A. C. *Textbook of Medical Physiology*; W. B. Saunders: Philadelphia, 1991; pp 709–725.
25. Antonino, L. C.; Wu, J. C. *Biochemistry* **1994**, *33*, 1753.
26. Zollinger, H. *Diazo Chemistry I—Aromatic and Heteroaromatic Compounds*; VCH: Weinheim, 1994; pp 39–64.
27. Williams, D. L. H. *Nitrosation*; Cambridge University Press: Cambridge, 1988; pp 10–16.
28. Kato, K.; Yagisawa, N.; Shimada, N.; Hamada, M.; Takita, T.; Maeda, K.; Umezawa, H. *J. Antibiot.* **1984**, *37*, 941.
29. Sambrook, J.; Fritsch, E. F.; Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Plainview, NY, 1989.
30. Moschel, R. C.; Keefer, L. K. *Tetrahedron Lett.* **1989**, *30*, 1467.