

[Chem. Pharm. Bull.]
[28(6)1820—1826 (1980)]

N-Nitroso Compounds formed by the Reaction of Sulpyrine with Nitrite¹⁾

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(Received December 25, 1979)

The reaction of sulpyrine with nitrite under mild conditions similar to those of the human stomach gave three *N*-nitroso compounds. The main products were 4-(*N*-methyl-*N*-nitroso)aminoantipyrine (I) and 1-diketobutyryl-1-phenyl-2-methyl-2-nitrosohydrazide hydrate (III), and 1-acetyl-1-methyl-2-nitroso-2-phenylhydrazine (II) was formed as a minor product. The possible pathways of formation of I and III are discussed.

Keywords—formation of *N*-nitroso compounds; sulpyrine; nitrite; sodium nitrite; nitrosation; pyrazolone antipyretic; NMR

Some drugs containing secondary amino, tertiary amino, or *N,N*-dialkylamido groups can react with nitrous acid to give *N*-nitroso compounds under acidic conditions.³⁾ Among these drugs, aminopyrine gave dimethylnitrosamine on reaction with nitrous acid under mild conditions.⁴⁾ Since nitrites are contained in many kinds of foods and human saliva,⁵⁾ the oral administration of aminopyrine has been prohibited because of possible nitrosamine formation in the human stomach. Sulpyrine is a pyrazolone antipyretic that is permitted to be orally administered in Japan.

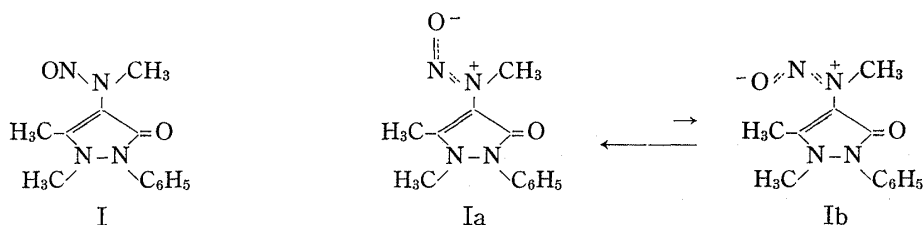
Scheunig and Ziebarth⁶⁾ reported that sulpyrine readily reacted with nitrite to give an *N*-nitroso compound in human gastric juice. Arisawa *et al.*⁷⁾ reported that the nitrosation products of sulpyrine showed mutagenicity for *Salmonella typhimurium*, while Degawa *et al.*⁸⁾ found that the ethyl acetate extract of the nitrosation products of sulpyrine showed no mutagenicity for the same strain. At present, the structures of the nitrosation products of sulpyrine have not been elucidated. We examined the nitrosation of sulpyrine under conditions similar to those of the human stomach and obtained three kinds of *N*-nitroso compounds. This paper describes the elucidation of the structures of these *N*-nitroso compounds and possible pathways for the formation of the two main products.

An aqueous solution of sulpyrine (0.57 mM) and sodium nitrite (1.09 mM) was adjusted to pH 2.0 with hydrochloric acid, and incubated at 37° for 30 min in the dark. The dichloro-

- 1) This work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August, 1979.
- 2) Location: 18-1, Kamiyoga 1-chome, Setagaya-ku, Tokyo 158, Japan.
- 3) a) W. Lijinsky, E. Conrad, and R.V.D. Bogart, *Nature* (London), **239**, 165 (1972); b) W. Lijinsky, *Cancer Res.*, **34**, 255 (1974); c) S.S. Mirvish, *Toxicol. Appl. Pharmacol.*, **31**, 325 (1975); d) G.S. Rao and G. Krishna, *J. Pharm. Sci.*, **64**, 1579 (1975).
- 4) S.S. Mirvish, B. Gold, M. Eagen, and S. Arnold, *Z. Krebsforsch.*, **82**, 259 (1974).
- 5) M. Harada, Y. Nakamura, and A. Tanimura, *J. Food Hyg. Soc. Japan*, **13**, 36 (1972); H. Ishiwata, P. Boriboon, Y. Nakamura, M. Harada, A. Tanimura, and M. Ishidate, *ibid.*, **16**, 19 (1975); B. Spiegelhalter, G. Eisenbrand, and R. Preussmann, *Food Cosmet. Toxicol.*, **14**, 545 (1976); S.R. Tannenbaum, M. Weisman, and D. Fett, *ibid.*, **14**, 549 (1976).
- 6) D. Ziebarth and G. Scheunig, "Environmental *N*-Nitroso Compounds: Analysis and Formation," IARC Scientific Publications No. 14, International Agency for Research on Cancer, Lyon, 1976, pp. 279—290; G. Scheunig and D. Ziebarth, *Pharmazie*, **33**, 722 (1978).
- 7) M. Arisawa, M. Fujiu, Y. Suhara, and H.B. Maruyama, *Mutation Res.*, **57**, 287 (1978).
- 8) M. Degawa, H. Watanabe, K. Masuko, and Y. Hashimoto, Abstracts of Papers, the 98th Annual Meeting of the Pharmaceutical Society of Japan, Okayama, April, 1978.

methane extract of the reaction mixture was presumed to contain three *N*-nitroso products, because the extract gave three spots on a developed thin-layer plate and the spots were detected not only under ultraviolet light but also on spraying with the Griess reagent followed by irradiation with UV light.⁹⁾ The products were provisionally named product 1, product 2 and product 3, and their *Rf* values were 0.43, 0.69 and 0.59, respectively with solvent system 4.

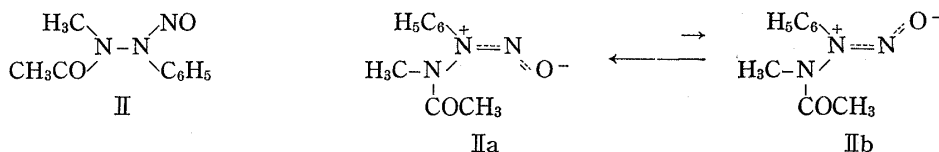
Product 1 and product 2 were isolated from the reaction mixture by preparative layer chromatography. Elemental analysis of product 1 gave a molecular formula of $C_{12}H_{14}N_4O_2$. The molecular ion was not detected by mass spectrometry, while a fragment of $C_{12}H_{14}N_3O$ (216.1266, calcd. 216.1136), which could arise by loss of the NO group, was detected. The IR spectrum showed the presence of a carbonyl group at 1653 cm^{-1} . The NMR spectrum showed five aromatic protons and three methyl groups. Another set of signals due to a conformational isomer was also observed (Fig. 1a). These results suggested that the structure of product 1 was 4-(*N*-methyl-*N*-nitroso)aminoantipyrene (I).



The *N*-nitrosamino group seems to be in a virtually planar conformation¹⁰⁾ because of the partial double bond character of its formally single N-N linkage, and two isomeric conformations are thus possible. Conformers of *N*-nitroso compounds can generally be distinguished by means of NMR spectroscopy. Karabatsos and Taller¹¹⁾ reported that aliphatic protons resonate at higher field when *syn* to the nitroso oxygen than when *anti*, except when they lie in or very near to the plane defined by the N-N=O group. Consequently, the major conformer of I should have the *anti* structure Ia.

Finally, the structure of I was confirmed by synthesis. Treatment of 4-methylaminoantipyrene (MAA) with sodium nitrite and hydrochloric acid gave 4-(*N*-methyl-*N*-nitroso)-aminoantipyrene (I), which was identical with product 1 obtained by the nitrosation of sulpyrine.

Product 2 was analyzed as $C_9H_{11}N_3O_2$, and the mass spectrum suggested a fragment of $C_9H_{11}N_2O$ (163.0871, calcd. 163.0873) due to loss of the NO group. The IR and NMR spectra showed the presence of a carbonyl group at 1687 cm^{-1} , two methyl groups and five aromatic protons. These results suggested that the structure of product 2 was 1-acetyl-1-methyl-2-nitroso-2-phenylhydrazine (II). The NMR spectra indicated that the major conformer of II had the structure IIa (Fig. 1b). In measurement of the NMR spectrum of II, when the solvent was changed from CDCl_3 to methanol- d_4 , the peak strength was increased at 2.31 and 3.20 δ (solvent effect). This showed that the peaks at 2.31 and 3.20 δ were attributable to CO-CH₃ and N-CH₃ of the conformer IIb, respectively.



9) N.P. Sen, D.C. Smith, L. Schwinghamer, and J.J. Marleau, *J. Assoc. Offic. Anal. Chem.*, **52**, 47 (1969).

10) W. Lijinsky, L. Keefer, and J. Loo, *Tetrahedron*, **26**, 5137 (1970).

11) G.J. Karabatsos and R.A. Taller, *J. Am. Chem. Soc.*, **86**, 4373 (1964).

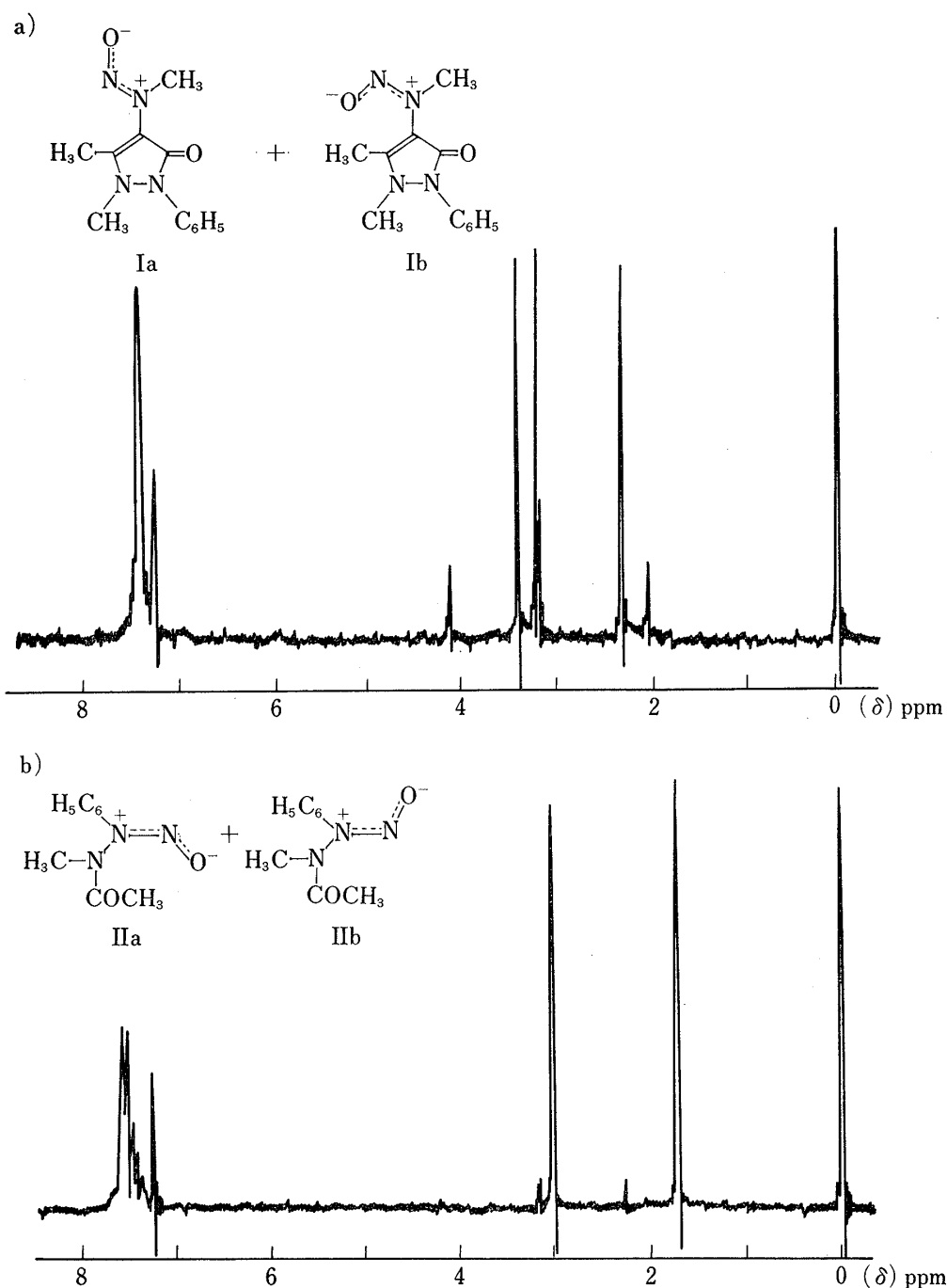


Fig. 1. NMR Spectra of Product 1 and Product 2

In order to confirm the structure, compound II was synthesized by the nitrosation of 1-acetyl-1-methyl-2-phenylhydrazine (AMPH) with sodium nitrite in acetic acid; the authentic 1-acetyl-1-methyl-2-nitroso-2-phenylhydrazine (II) was identical with product 2.

Product 3 could not be isolated by preparative layer chromatography because of its instability. It was obtained as a powder in 70% yield by treating sulpyrine with sodium nitrite at 0°, both at high concentrations. The behavior of product 3 in thin-layer chromatography using solvent systems 1, 2, 3 and 4, coincided with that of the hydrate of 1-diketobutyryl-1-phenyl-2-methyl-2-nitrosohydrazide (DPMN, III). Since this compound has already been reported as a nitrosation product of aminopyrine,⁴⁾ it was prepared from aminopyrine by the

reported method;⁴⁾ the authentic 1-diketobutyryl-1-phenyl-2-methyl-2-nitrosohydrazide was identical with product 3.

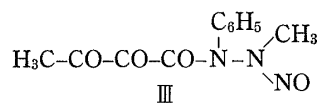
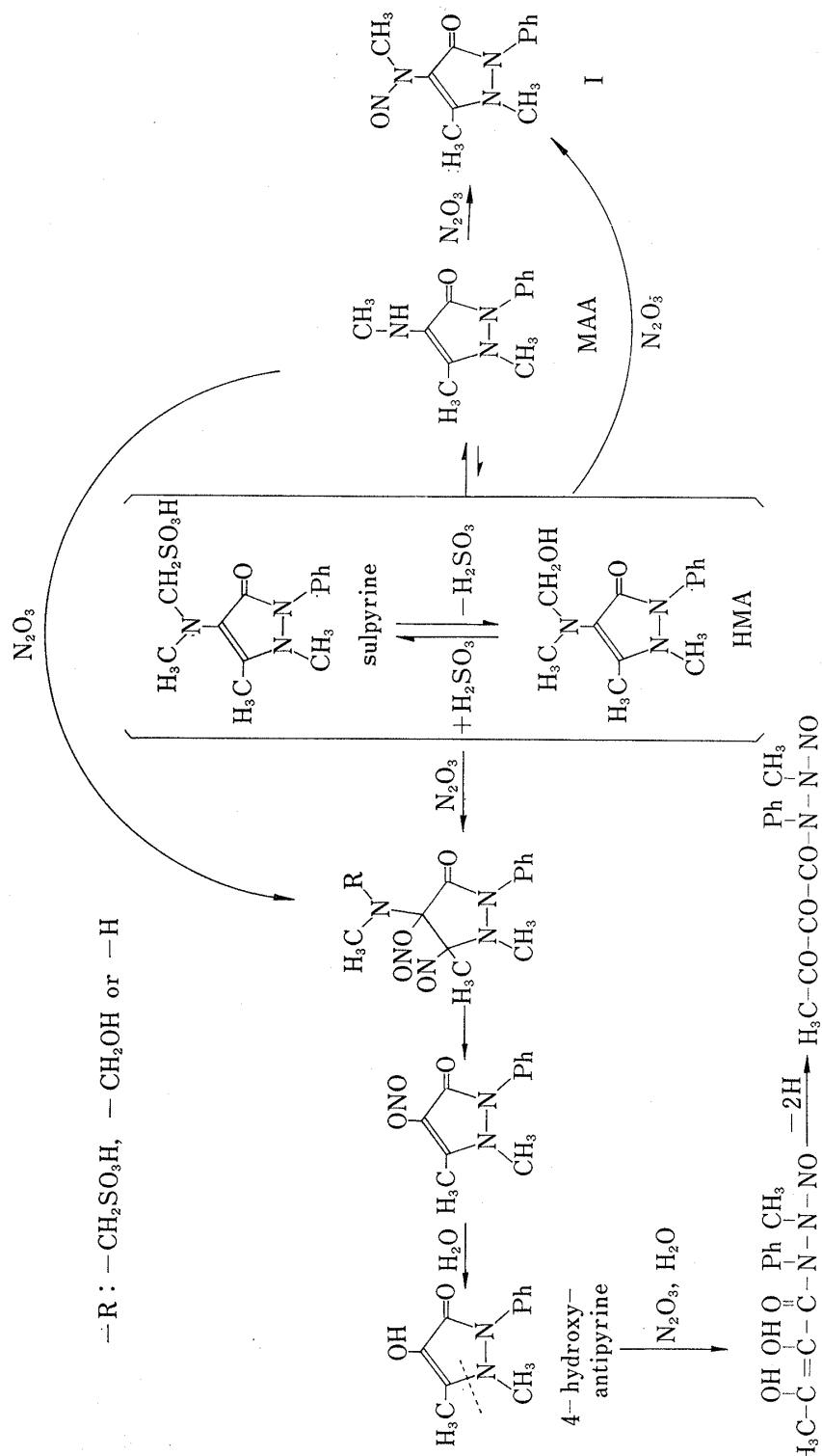


Chart 1 shows possible pathways for the formation of the two main products, I and III.



III
Chart 1

According to Yoshioka *et al.*,¹²⁾ sulpyrine is hydrolyzed to produce 4-(*N*-hydroxymethyl-*N*-methyl)aminoantipyrine (HMA), and an equilibrium is attained between them immediately after sulpyrine is dissolved at a pH below 3 (Chart 1). Sulpyrine and/or HMA are then slowly hydrolyzed to 4-methylaminoantipyrine (MAA), and the reverse reaction is negligible in the pH range below 3.

As shown in Fig. 2, the bulk of MAA was immediately converted to I by reaction with sodium nitrite at pH 2.0 and 37°. We next examined the relation between the hydrolysis of sulpyrine and the formation of I at pH 2.0 and 37° (Fig. 3). The fact that the yield of I increased with the progress of hydrolysis of sulpyrine, indicates that I is formed *via* MAA from sulpyrine. However, I was formed in about 40% yield when sulpyrine reacted with sodium nitrite without preincubation of the sulpyrine solution, though from the rate of sulpyrine hydrolysis it could be estimated that little MAA had been produced. This suggests the possibility that I is formed directly from sulpyrine and/or HMA as well as *via* MAA. Conversely, if the hydrolysis rate from sulpyrine to MAA were fast enough and the whole of I were formed *via* MAA, the yield of I should increase much more than that shown in Fig. 3 on increasing the preincubation time from 0 to 5 min, or from 0 to 15 min.

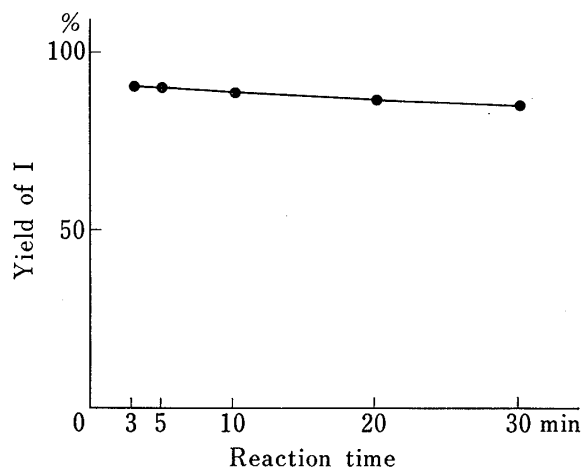


Fig. 2. Formation of I from MAA and Nitrite at pH 2.0 and 37°

The concentrations of MAA and nitrite were 3 and 8 mM, respectively.

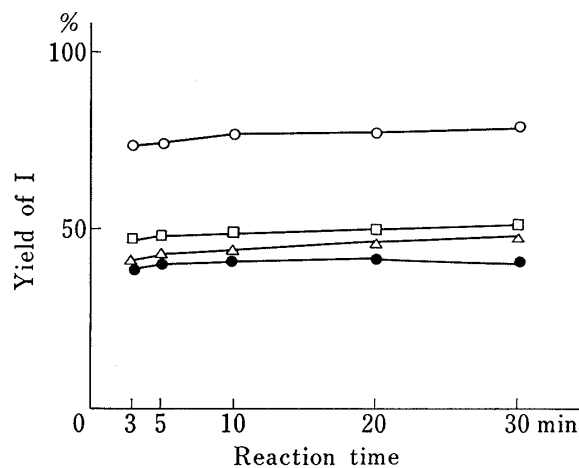


Fig. 3. Relation between the Formation of I and the Hydrolysis of Sulpyrine

The sulpyrine solution was preincubated at pH 2.0 and 37° at a concentration of 6 mM for 0 (—●—), 5 (—△—), 15 (—□—) and 180 (—○—) min in order to hydrolyze sulpyrine. Immediately after the preincubation, nitrite solution (pH 2.0 and 37°) was added to the sulpyrine solution, and the mixture was kept at 37° for the indicated reaction period. At the time of reaction, the concentration of sulpyrine was 3 mM and that of nitrite was 8 mM.

Sulpyrine and HMA have enamine structures similar to that of aminopyrine. Enamines are subject to electrophilic attack. Mirvish *et al.*⁴⁾ proposed for the nitrosation of aminopyrine that the first step to form III and dimethylnitrosamine was the addition of dinitrogen trioxide (N_2O_3) to the double bond of aminopyrine. It seems likely that sulpyrine and/or HMA react with nitrite to form III by the same mechanism as aminopyrine.

MAA and I also have enamine structures. Thus, MAA or I (3 mM) was allowed to react with sodium nitrite (8 mM) at pH 2.0 and 37° for 1 hr. It was confirmed by thin-layer chromatography that the reaction mixture of MAA and sodium nitrite contained III as a minor product in addition to the main product I, while compound I gave only a trace of III on reaction with sodium nitrite.

12) S. Yoshioka, H. Ogata, T. Shibazaki, and T. Inoue, *Chem. Pharm. Bull.*, **25**, 484 (1977).

As the yield of II is slight in the reaction of sulpyrine with sodium nitrite, the pathway for the formation of II was not pursued in this paper.

Compounds I and III showed mutagenic activity for *Salmonella typhimurium* TA100, but not for TA98, while II was not mutagenic for either strain. Compound I required metabolic activation, while III did not. Details of the mutagenicity of nitrosation products of sulpyrine and related compounds will be reported in the near future.

Experimental

Apparatus—All melting points were obtained on a Yanagimoto melting point determination apparatus. They are uncorrected. UV spectra were measured with a Shimadzu UV-200 spectrophotometer. IR spectra were taken using Jasco DS-403G and Jasco A-102 spectrophotometers. NMR spectra were recorded on a Varian EM-360 spectrometer (60 MHz) using tetramethylsilane as an internal standard. The chemical shifts of various compounds are given in δ units. Mass spectra were recorded on JEOL JMS-01SG-02 machine at 70 eV chamber voltage on a direct inlet system.

Materials—The sulpyrine and aminopyrine used were of J.P.IX grade. MAA was prepared according to the procedure reported by Ono *et al.*¹³⁾ AMPH was synthesized from 1-acetyl-2-phenylhydrazine according to the method reported by Stühmer and Elbrächter.¹⁴⁾ DPMN was prepared from aminopyrine by the method of Mirvish *et al.*⁴⁾

Griess Reagent⁹⁾—One percent sulfanilic acid solution and 0.1% *N*-1-naphthylethylenediamine dihydrochloride solution were prepared in 30% acetic acid, stored at 4° and mixed just before use in a ratio of 1:1.

Thin-Layer Chromatography (TLC)—TLC plates were prepared in a conventional manner with a 250 μ layer of silica gel 60 HF₂₅₄ (Merck). Chromatography was carried out in the dark using the following solvent systems.

System 1); methyl acetate-*n*-hexane (3:1)

System 2); *n*-hexane-diethyl ether-CH₂Cl₂ (4:3:2)

System 3); methyl acetate-iso-propanol-ammonia water (28%) (9:7:1)

System 4); CHCl₃-dioxane (3:1).

The chromatograms were visualized under UV light (254 nm), and also by spraying Griess reagent, then irradiating the plates under UV light (without a filter) for 2–5 min.^{3c,14)}

Reaction of Sulpyrine with Nitrite *in Vitro*—An aqueous solution containing sulpyrine (200 ppm, 0.57 mm) and NaNO₂ (50 ppm of NO₂⁻, 1.09 mm) was adjusted to pH 2.0 with conc. HCl and incubated at 37° for 30 min in the dark. The reaction mixture was extracted with CH₂Cl₂. The extract was concentrated under reduced pressure, and TLC was carried out using solvent systems 1–4.

Isolation of Product 1—One gram of sulpyrine (2.85 mmol) was reacted with 500 mg of NaNO₂ (7.25 mmol) in 1 l of an aqueous solution at pH 2.0 and 37° for 2 hr in the dark. After the reaction, a large excess of sulfamic acid was added to decompose nitrite and product 3. The mixture was extracted with CH₂Cl₂. The extract was washed with water, and dried over anhyd. Na₂SO₄. The solvent was evaporated off under reduced pressure to leave an oil. Product 1 was separated from the residual oil by preparative layer chromatography (PLC) using precoated PLC plates (Merck, silica gel 60 F₂₅₄) and solvent system 1, and was eluted with CH₂Cl₂. After removal of the silica gel by centrifugation and filtration, the CH₂Cl₂ was evaporated off under reduced pressure to leave a pale yellow solid, 223 mg (32%). This was recrystallized from diethyl ether to give pale yellow crystals, mp 99°. *Anal.* Calcd for C₁₂H₁₄N₄O₂: C, 58.53; H, 5.73; N, 22.75. Found: C, 58.68; H, 5.75; N, 22.88. Mass spectrum: the parent ion was not detected; fragment at *m/e*: 216 (loss of NO). IR (KBr) cm⁻¹: 1653 (C=O). UV (CH₂Cl₂) nm (ϵ): 283 (11300). NMR (CDCl₃) δ : 2.30 and 2.02 (total 3H, s, C-CH₃, *anti* and *syn*), 3.23 and 3.20 (total 3H, s, -N(C₆H₅)N(CH₃)-, *anti* and *syn*), 3.41 and 4.14 (total 3H, s, N(NO)-CH₃, *anti* and *syn*), 7.44 (5H, m, aromatic H).

Isolation of Product 2—Product 2 was isolated in the same way as product 1. In this case, solvent system 2 was used for PLC. The fraction containing product 2 gave an orange solid, 7 mg (1.3%). This was recrystallized from a mixture of diethyl ether and petroleum ether to give yellow crystals, mp 74°. *Anal.* Calcd for C₉H₁₁N₃O₂: C, 55.95; H, 5.74; N, 21.75. Found: C, 55.95; H, 5.72; N, 21.90. Mass spectrum: the parent ion was not detected; fragment at *m/e*: 163 (loss of NO). IR (KBr) cm⁻¹: 1687 (C=O). UV (CH₂Cl₂) nm (ϵ): 296 (5300). NMR (CDCl₃) δ : 1.73 and 2.31 (total 3H, s, CO-CH₃, *syn* and *anti*), 3.06 and 3.20 (total 3H, s, N-CH₃, *syn* and *anti*), 7.30–7.80 (5H, m, aromatic H).

Synthesis of 4-(*N*-Methyl-*N*-nitroso)aminoantipyrine (I)—A solution of 1.38 g (20 mmol) of NaNO₂ in 10 ml of water was added dropwise to a solution of 2.46 g (10 mmol) of MAA in 100 ml of 2N HCl, with

13) S. Ono, R. Onishi, and K. Kawamura, *Yakugaku Zasshi*, **86**, 11 (1966).

14) W. Stühmer and E.A. Elbrächter, *Arch. Pharm. Ber. Dtsch. Pharm. Ges.*, **285**, 161 (1952).

gentle stirring in an ice bath, over a period of 10 min. The reaction vessel was stoppered and left to stand for 1 hr in the ice bath. After the addition of 5 g of sulfamic acid, the reaction mixture was extracted with CH_2Cl_2 , and the CH_2Cl_2 layer was washed with water and dried over anhyd. Na_2SO_4 . After removal of the solvent, the residual oil was crystallized from diethyl ether to give I, mp 99° , in 70% yield, as pale yellow crystals. The physicochemical data for I agreed completely with those for product 1.

Synthesis of 1-Acetyl-1-methyl-2-nitroso-2-phenylhydrazine (II)—A solution of 5.48 g (80 mmol) of NaNO_2 in 15 ml of water was added dropwise to a mixture of 3.28 g (20 mmol) of AMPH in 25 ml of glacial acetic acid, with gentle stirring at about 10° , and the mixture was stirred for 2 hr, keeping the temperature below 10° . The reaction mixture was then poured into 250 ml of ice-cold water. The orange-yellow precipitate that separated was collected, washed with water and dried, and then recrystallized from a mixture of diethyl ether and petroleum ether to give II, mp 74° , in 55% yield, as yellow crystals. The physicochemical data for II coincided with those for product 2.

Synthesis of 1-Diketobutyl-1-phenyl-2-methyl-2-nitrosohydrazide Hydrate (III) from Sulpyrine—Conc. HCl (60 ml) was slowly added to 200 ml of an aqueous solution containing 7.03 g (20 mmol) of sulpyrine and 5.52 g (80 mmol) of NaNO_2 at 0° . After 2 hr, the precipitate of III was collected by filtration, washed with cold water, and recrystallized from diethyl ether to give unstable, pale yellow crystals, mp $90\text{--}92^\circ$, in 70% yield. *Anal.* Calcd for $\text{C}_{11}\text{H}_{11}\text{N}_3\text{O}_4 \cdot \text{H}_2\text{O}$: C, 49.43; H, 4.86; N, 15.73. Found: C, 49.45; H, 4.84; N, 15.57. Mass spectrum: the parent ion was not detected; fragment at m/e : 219 (loss of NO). IR (KBr) cm^{-1} : 1732 (C=O), 1673 (2C=O's). UV (EtOH) nm (ϵ): 370 (101). NMR (CDCl_3) δ : 2.28 and 2.37 (total 3H, s, CO-CH₃, *anti* and *syn*), 3.15 and 3.98 (total 3H, s, N-CH₃, *anti* and *syn*), 5.3 (br, s, H_2O , exchangeable with D_2O), 7.44 (5H, m, aromatic H).

Determination of 4-(N-Methyl-N-nitroso)aminoantipyrine (I) formed by the Reaction of Sulpyrine or MAA with Nitrite—Sulpyrine or MAA was reacted with nitrite at 37° and pH 2.0. Aliquots of the reaction mixture (25 ml) were withdrawn at appropriate intervals, and extracted with 25 ml of CHCl_3 after the addition of a large excess of sulfamic acid. An aliquot (200 μl) of the CHCl_3 layer was spotted as a streak on a TLC plate (5×20 cm). The plate was developed with solvent system 1. The band of I was scraped off and eluted with MeOH. Compound I in the eluate was determined by measurement of the optical density at 269 nm.

Acknowledgement The authors wish to thank Dr. I. Tomita of Shizuoka College of Pharmacy for encouragement and useful discussions. This work was supported in part by a Grant-in-Aid for Cancer Research (52-9) from the Ministry of Health and Welfare, Japan.