

Nitric Oxide Generation from Aromatic *N*-Nitrosoareas at Ambient Temperature

Masayuki TANNO,*^a Shoko SUEYOSHI,^a Naoki MIYATA,^a and Shigeo NAKAGAWA^b

Division of Organic Chemistry, National Institute of Health Sciences,^a 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158, Japan and School of Pharmaceutical Sciences, Hokuriku University,^b 3-Ho Kanagawa-machi, Kanazawa 920-11, Japan. Received May 24, 1996; accepted June 24, 1996

Generation of nitric oxide (NO) from aromatic *N*-nitrosoareas 1–3 at ambient temperature without photo irradiation was confirmed by the trapping of NO as a nitrosyl complex of 5,10,15,20-tetraphenylporphyrinocobalt (II) and spectrophotometrically quantified by means of the Griess reaction using a newly designed test apparatus in which NO₂⁻ is generated from NO. 3,3-Dibenzyl-1-(4-tolyl)-1-nitrosoarene (1a) showed the greatest NO-generation ability among compounds 1–3 and *N*-acetyl-S-nitroso-DL-penicillamine (SNAP) in chloroform. A comparison of 1a and usual NO donors (NOC-18, NOR-4, SIN-1, SNAP, and SNP) in Krebs buffer was also made using the same test apparatus.

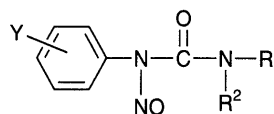
Key words nitric oxide; nitrosoarene; nitric oxide donor; Griess method; *N*-acetyl-S-nitroso-DL-penicillamine; tetraphenylporphyrin

While the photolytic decomposition of *N*-nitrosoacetamide¹⁾ or the thermal cleavage of the N–NO bond of *N*-nitrosodiphenylamine at high temperature^{2,3)} has been reported, little is known about the decomposition of aromatic *N*-nitrosoareas to generate nitric oxide (NO) at ambient temperature without photo irradiation. We have recently reported that the decomposition of 3,3-dialkyl-1-aryl-1-nitrosoarene (1) and 3-alkyl-1-aryl-1-nitrosoarene (2) (Fig. 1) produced 3,3-dialkyl- and 3-monoalkyl-1-(2-nitroaryl)ureas,^{4,5)} respectively, in CHCl₃ at 33 °C as N–NO bond-cleaved products. This reaction occurred even in the dark. The formation of the nitro compounds suggested NO generation in the reaction. Here we present evidence for NO generation from the *N*-nitroso compounds 1–3 at ambient temperature. Further, their NO-generating ability was confirmed using a convenient apparatus for NO determination.

Results and Discussion

We recently reported that an NO₃ complex of Fe^{III}-(salen)⁴⁾ was produced by the reaction of 3,3-dibenzyl-1-(4-tolyl)-1-nitrosoarene (1a) with the μ-oxo dimer of

N,N'-ethylenebis(salicylideneiminato)iron(III) ((Fe^{III}-(salen))₂O), in CHCl₃ at room temperature. The Fe^{III}-(NO₃)(salen) complex was identical with an authentic sample prepared from (Fe^{III}(salen))₂O and gaseous NO under aerobic conditions, and thus NO was assumed to be formed in the decomposition of 1a. However, the possibility of the formation of the Fe^{III}(NO₃)(salen)



- | | |
|---------------|---|
| 1a: Y = 4-Me | R ¹ = R ² = CH ₂ Ph |
| 1b: Y = 2-Me | R ¹ = R ² = CH ₂ Ph |
| 1c: Y = 4-Me | R ¹ = R ² = Et |
| 1d: Y = 2-Me | R ¹ = R ² = Et |
| 1e: Y = 4-Cl | R ¹ = R ² = Et |
| 1f: Y = 4-Ac | R ¹ = R ² = Et |
| 2a: Y = 4-Me | R ¹ = H, R ² = CH ₂ Ph |
| 2b: Y = 4-Me | R ¹ = H, R ² = Me |
| 2c: Y = 4-MeO | R ¹ = H, R ² = Me |
| 3: Y = 4-Me | R ¹ = R ² = H |

Fig. 1. Structures of *N*-Nitrosoarenes

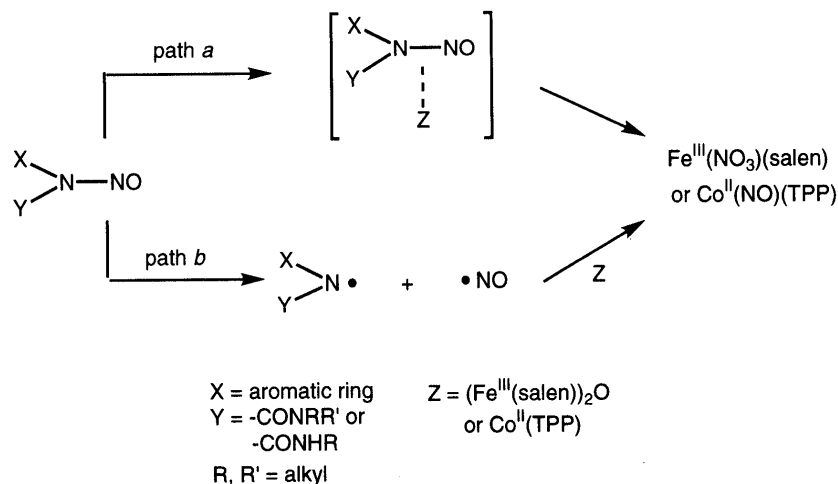


Chart 1. Possible Pathways for Production of NO- or NO₃-Complex by the Reaction of Aromatic *N*-Nitrosoarene with Metal Complex in CHCl₃

* To whom correspondence should be addressed.

complex by the direct reaction of **1a** with $(\text{Fe}^{\text{III}}(\text{salen}))_2\text{O}$ *via* path *a* in Chart 1 could not be ruled out. Consequently, we tried to confirm the NO generation by removing and trapping with a metal porphyrin the NO gas from the reaction solution after the decomposition of the *N*-nitroso compound *via* path *b*.

Confirmation of NO Liberation from *N*-Aryl-*N*-nitroso Compounds A stable nitrosyl complex has been produced by the reaction of NO with a metal porphyrin.^{6,7)} Therefore, we tried to trap NO generated from various *N*-nitrosoareas (**1a**—**d**, **2a**) shown in Fig. 1 and *N*-acetyl-*S*-nitroso-DL-penicillamine (SNAP)^{8a)} using 5,10,15,20-tetraphenylporphyrinatocobalt(II) ($\text{Co}^{\text{II}}(\text{TPP})$). Each of the nitrosoareas or SNAP was dissolved in CCl_4 or CHCl_3 and the solution was allowed to stand at 33 °C. The NO gas thus produced by thermal self-decomposition was led to a CHCl_3 solution of $\text{Co}^{\text{II}}(\text{TPP})$ bubbled through with argon for 5 h. The nitrosyl porphyrinatocobalt(II) ($\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$) resulting from the decomposition of compounds **1a**, **b** was separated from the starting material $\text{Co}^{\text{II}}(\text{TPP})$ by taking advantage of the difference in their solubility in CHCl_3 . Further purification was done by column chromatography on silica gel with CHCl_3 . The IR

spectrum of the $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$ complex in CHCl_3 showed a strong characteristic absorption at around 1685 cm^{-1} due to NO coordinated with the central metal (Fig. 2a).^{6,7)} As shown in Table 1, the absorption intensity of the coordinated NO in the IR spectra of nitrosyl complexes obtained from **1c**, **d**, **2a** and SNAP was weak compared with those obtained from **1a**, **b**. Thus, the NO generated from the compounds **1c**, **d**, **2a** and SNAP was insufficient to produce the nitrosyl complex; therefore, the purification of the complex from the impure nitrosyl complex containing a relatively large amount of unreacted $\text{Co}^{\text{II}}(\text{TPP})$ was difficult.

The formation of $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$ was also characterized by the visible spectrum. The absorption maxima of the nitrosyl complex were at 414 and 538 nm in CHCl_3 (Fig. 2b), in accordance with the spectral data of an authentic sample.⁷⁾

Detection Method of NO Generation Several NO detection systems have already been reported, for example: the chemiluminescence method using ozone⁹⁾ or luminol/ H_2O_2 ,¹⁰⁾ the colorimetric assay,¹¹⁾ the electrode method,¹²⁾ the ESR method,^{8b,13)} the oxyhemoglobin

Table 1. NO-Generating Ability^{a)} of Aromatic *N*-Nitrosoareas and SNAP, and Absorption Intensity of $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$ Complex^{b)}

Y-C ₆ H ₄ -N(NO)-X			NO-Generating ability mm	Absorption intensity at 1685 cm ⁻¹
Compd.	Y	X		
1a	4-Me	CON(CH ₂ Ph) ₂	4.79 (8.6)	s
1b	2-Me	CON(CH ₂ Ph) ₂	2.12 (3.9)	s
1c	4-Me	CON(Et) ₂	0.35 (0.6)	w
1d	2-Me	CON(Et) ₂	0.22 (0.4) ^{c)}	w
1e	4-Cl	CON(Et) ₂	0.24 (0.4)	—
1f	4-Ac	CON(Et) ₂	1.08 (2.0)	—
2a	4-Me	CONHCH ₂ Ph	1.06 (1.9)	w
2b	4-Me	CONHMe	1.07 (1.9)	—
3	4-Me	CONH ₂	0.90 (1.6)	—
SNAP			0.55 (1.0)	w

a) Measured in CHCl_3 at 37 °C for 2 h. Concentration of each *N*-nitrosoare was $1\text{--}10 \times 10^{-3}\text{ M}$. Detection as NO_2^- using the Griess method. The values are amounts of NO_2^- generated *via* NO from 100 mm of the compound and those in parenthesis are the ratio to SNAP. b) The IR absorption intensity in CHCl_3 is indicated as follows: s, strong absorption of isolated $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$; w, weak band of the mixture of $\text{Co}^{\text{II}}(\text{TPP})$ and $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$. c) Data for 4 h.

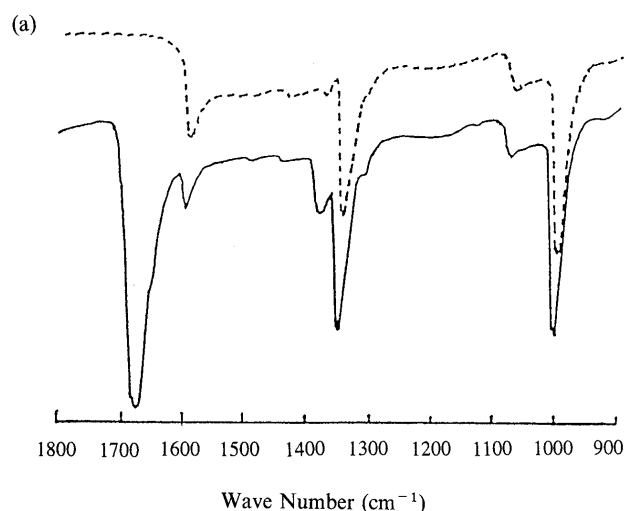


Fig. 2a. IR Spectra of $\text{Co}^{\text{II}}(\text{TPP})$ (Broken Line) and $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$ (Solid Line) in CHCl_3

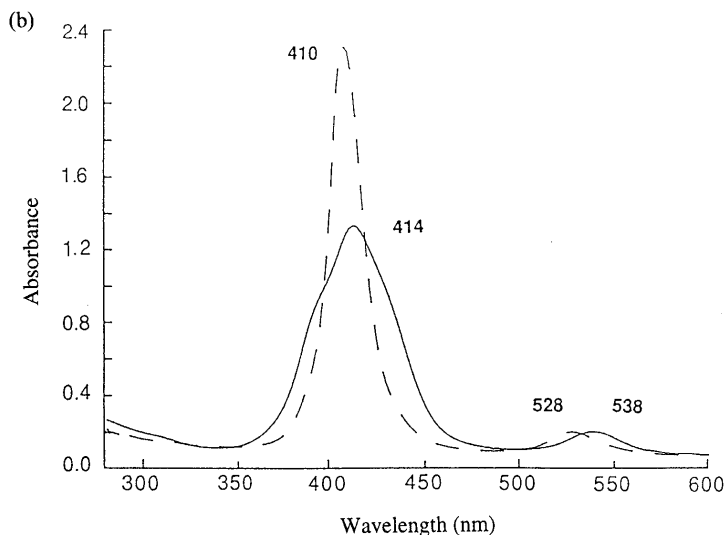


Fig. 2b. Visible Spectra of $\text{Co}^{\text{II}}(\text{TPP})$ (Broken Line) and $\text{Co}^{\text{II}}(\text{NO})(\text{TPP})$ (Solid Line) in CHCl_3 (Concentration: $1.0 \times 10^{-5}\text{ M}$)

oxidation method,¹⁴⁾ and the GC-MS detection assay.¹⁵⁾ The NO detection in our experiment was performed by means of the Griess method using the test apparatus shown in Fig. 3. The advantages of this method are that it is simple and convenient for the treatment of many samples and that it can handle both aqueous and organic media. The apparatus is composed of two vessels separated by a polyvinylidene fluoride film,¹⁶⁾ which has the physical property of passing only gaseous molecules. A test solution was placed in vessel *a* and kept at a constant temperature. The evolved NO was detected in vessel *b* by the Griess reagent (sulfanilic acid and *N*-(1-naphthyl)-ethylenediamine). In this system, the NO generated in vessel *a* was oxidized to NO₂ by atmospheric oxygen and passed into vessel *b* through the film. It was then converted to NO₂⁻ in an aqueous solution of the Griess reagent.¹⁷⁾ Finally, NO₂⁻ was converted to an azo compound which showed absorption at 546 nm. The observed values represent the amount of NO₂⁻ generated *via* the NO evolved from the compounds, evaluated by comparison with a standard curve for known amounts of NaNO₂.

Determination of the NO-Generating Ability The NO-generating ability of 3,3-dialkyl-1-aryl-1-nitrosoureas (**1a–f**), 3-monoalkyl-1-aryl-1-nitrosoureas (**2a,b**), or 1-aryl-1-nitrosourea (**3**) was evaluated using this system. A CHCl₃ solution of one of the *N*-nitrosoureas **1–3** or SNAP was kept in vessel *a* at 37 °C for 2 h, and the amount of NO₂⁻ in vessel *b* was determined by using visible spectroscopy. The results are shown in Table 1. The aromatic *N*-nitrosoureas, except for **1c–e**, were excellent NO donors compared with SNAP,^{8b)} which is known to generate NO. The conjugation between the phenyl ring and ureido-*N*¹ in the aromatic *N*-nitrosourea should be the principal requirement for the radical cleavage of the N–NO bond. Under the same conditions, NO generation from aliphatic *N*-nitrosoureas could not be observed because there was no such conjugation.¹⁸⁾

In these aromatic *N*-nitrosoureas, the NO-generating ability was influenced by the steric effect in addition to the conjugation. Compound **1a** having dibenzyl groups at ureido-*N*³ showed the greatest ability among the tested 3,3-dialkyl derivatives (**1a–f**). Disruption of the N–NO bond of **1a** with its bulky dibenzyl groups is likely to be accelerated by steric hindrance. The NO-generating ability of the *N*³-dialkyl-substituted *N*-nitrosoureas (**1a–d**)

appreciably changed with the size of the ureido-*N*³ substituents; compound **1a,b** (*R*¹=*R*²=benzyl) showed greater ability than compounds **1c,d** (*R*¹=*R*²=ethyl).

The conjugation of the C¹–N¹ bond of *ortho*-tolyl derivatives **1b,d** may be less effective compared with that of *para*-tolyl derivatives **1a,c** because of twisting of the C¹–N¹ bond owing to the hindrance of the neighboring nitroso group by the *ortho*-methyl group. The NO-generating ability of **1a** and **1c** is actually larger than that of **1b** and **1d**, respectively.

In the case of the *N*³-monoalkyl compounds **2** (*R*¹=H, *R*²=benzyl or methyl) and *N*³-dihydrogen compound **3** (*R*¹=*R*²=H), the NO-generating ability was high, despite the reduced steric hindrance compared with that of **1c,d**, as shown in Table 1. The variation of the *N*³-alkyl size from benzyl to methyl in compounds **2a,b** did not cause a remarkable change, and a similar relationship seems to hold for compounds **2** and **3**. One reason for the relatively high NO generation of compounds **2a,b** compared with **1c,d** is thought to be that when the steric hindrance of the *N*³-alkyl group and the nitroso group is small, the stability of N–NO bond decreases in the order of the ureido-*N*¹ nitrogen basicity owing to the substituent effect of the *N*³ nitrogen: *N*³-dialkyl (**1**)>*N*³-monoalkyl-monohydrogen (**2**)>*N*³-dihydrogen (**3**). The presence of an electron-attracting group at the *para* position of the phenyl ring is also effective for NO generation. Compound **1f** having an acetyl group showed increased NO-generating ability owing to the decreased basicity of the *N*¹ nitrogen.

Consequently, N–NO bond radical cleavage appears to be controlled by the resonance effect of the substituents on the aromatic ring. The steric hindrance due to the ureido-*N*³ disubstituents in the *N*-nitrosoureas **1** should promote the N–NO bond radical cleavage controlled by the conjugation of the benzene ring π electrons.

A Comparison of NO-Generating Ability among Several NO Donors A comparison of the NO-generating ability of **1a** and the usual NO donors^{8b,c,19)} (NOC-18, NOR-4, SIN-1, SNP, and SNAP) was performed in a Krebs buffer at 37 °C for 2 h using the same apparatus. Compound **1a**, NOR-4, and SNAP were reacted in the buffer containing 10% dimethyl sulfoxide (DMSO). These results are summarized in Table 2. The aromatic *N*-nitrosourea **1a** is a good candidate for an NO donor; however, some modification of **1a** to improve its solubility in water would

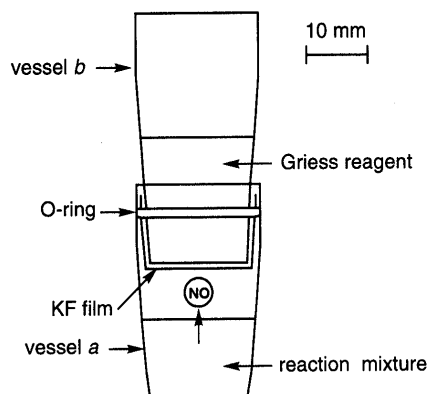


Fig. 3. Apparatus Used for the Evaluation of NO-Generating Ability

Table 2. NO-Generating Ability^{a)} of Aromatic *N*-Nitrosourea (**1a**) and Usual NO Donors

NO donor ¹⁹⁾	NO-Generating ability (mm)
1a ^{b)}	0.45
NOC-18	0.95
NOR-4 ^{b)}	0.11 ^{d)}
SIN-1	0.38
SNP	0.04
SNP ^{c)}	0.71
SNAP ^{b)}	0.50

a) Each compound (1.6×10^{-3} M) was reacted in Krebs buffer at 37 °C for 2 h and NO was detected as NO₂⁻ using the Griess method. The values are amounts of NO₂⁻ generated from 100 mm. b) Reaction in the mixture of DMSO–Krebs buffer (1:9). c) Reaction in H₂O (pH 6.4). d) Data for 3 h.

be desirable.

The use of our test apparatus for investigating NO generation ability allows us to distinguish NO and O_2^- . Compound **1a** and SNAP show similar NO-generating ability in Krebs buffer and the NO-generating ability of sodium pentacyanonitrosylferrate dihydrate (SNP) varies remarkably with the pH value. The ability shown in Table 2 is lower than the reported NO-generating ability^{8b)} of these donors obtained by the ESR method using 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (carboxy-PTIO) as an NO trapping agent. This is because the NO_x (NO, NO_2 , NO_2^- , etc.) produced from the NO donors may remain in the buffer solution or the space between vessels *a* and *b*. However, this novel method using a simple apparatus is advantageous for the separation and detection of NO (or NO_2) gas from the reaction mixture.

Experimental

Infrared (IR) spectra were recorded on a JASCO A-102 spectrometer. Electronic spectra (UV) were recorded on a Hewlett Packard 8452A spectrophotometer.

Materials Aromatic *N*-nitrosoareas (**1**–**3**) were prepared according to the methods described in our previous papers.^{4,5)} 3,3-Diethyl-1-(4-acetylphenyl)-1-nitrosoarea (**1f**) was similarly prepared. Pale yellow needles, mp 61.5–62.5 °C (dec.) (from ether–hexane). IR ($CHCl_3$) cm^{-1} : 1700 (C=O), 1598 (ring). *Anal.* Calcd for $C_{13}H_{17}N_3O_3$: C, 59.30; H, 6.51; N, 15.96. Found: C, 59.45; H, 6.56; N, 16.14.

SNP and $Co^{II}(TPP)$ were purchased from Wako Pure Chemical Co., Ltd. (Osaka). (*E*)-*N*-4-Ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenyl nicotinamide (NOR-4), 1-hydroxy-2-oxo-3,3-bis(3-aminoethyl)-1-triazene (NOC-18), and 3-morpholinisodnonimine hydrochloride (SIN-1) were purchased from Dojindo Co., Ltd. (Kumamoto). SNAP was purchased from Alexis Co., Ltd. (California). Nitric oxide (containing 90% nitrogen) was purchased from Nihon Sanso Chemical Co., Ltd. (Tokyo).

Trapping of NO Generated from the *N*-Nitrosoareas by $Co^{II}(TPP)$ NO generation from the *N*-nitroso compounds **1**–**3** was confirmed by the formation of $Co^{II}(NO)(TPP)$. In a typical experiment, 3,3-dibenzyl-1-(4-tolyl)-1-nitrosoarea **1a** (30 mg, 8.35×10^{-2} mmol) was dissolved in CCl_4 (4 ml), and the solution was kept at 33 °C for 5 h under argon. The resulting gaseous NO was bubbled into a dry CH_2Cl_2 (2 ml) solution of $Co^{II}(TPP)$ (20 mg, 2.98×10^{-2} mmol) via a constant stream of argon. The CH_2Cl_2 solution was evaporated under reduced pressure, and the residue was re-dissolved in a small amount of $CHCl_3$. The solution was filtered and the filtrate was evaporated to dryness under reduced pressure. The extraction procedure with $CHCl_3$ was repeated several times, and the $Co^{II}(NO)(TPP)$ complex was purified by column chromatography on silica gel with $CHCl_3$ (Table 1 and Figs. 2a and 2b). Dull purple-tinged grayish powder. IR ($CHCl_3$) cm^{-1} : 1685 (NO).⁶⁾ UV-visible $\lambda_{max}^{CHCl_3}$ nm (ϵ): 414 (230000), 538 (16000).⁷⁾ *Anal.* Calcd for $C_{44}H_{28}CoN_5O$: C, 75.32; H, 4.02; N, 9.98. Found: C, 74.53; H, 3.97; N, 9.89.

Determination of NO Generated from *N*-Nitrosoareas The NO-generating ability of the *N*-nitroso compounds **1**–**3** was evaluated in $CHCl_3$ using the described test apparatus (Fig. 3). It consists of two vessels separated by a polyvinylidene fluoride film (KF film),¹⁶⁾ which has the physical property of passing only gaseous molecules. A $CHCl_3$ solution (2 ml) of one of the *N*-nitroso compounds or SNAP (1–10 mg) was placed in vessel *a* (10 i.d. \times 45 mm), and kept at 37 °C for 2 h. The generated NO was detected in vessel *b* (10 i.d. \times 35 mm) by means of the Griess reagent (a mixture of sulfanilic acid and *N*-(1-naphthyl)ethylenediamine). The amount of NO_2^- was determined by comparison with a standard curve prepared by using known amounts of $NaNO_2$, an absorption intensity at 546 nm. The result are shown in Table 1 as the amount of NO_2^- generated via NO from 100 mM *N*-nitroso compounds or SNAP.

Comparison of NO Generation by the NO Donors Comparison of NO generation between **1a** and the usual NO donors^{8b,c,19)} was done by a method similar to that previously described. The Krebs buffer solution

(2 ml) of **1a** (1 mg) or one of the usual NO donors (1–10 mg) was kept at 37 °C for 2 h in the test apparatus. However, **1a**, SNAP and NOR-4 were first dissolved in 0.2 ml of cold DMSO and then diluted with the Krebs buffer (1.8 ml), respectively. The results are shown as the amount of NO_2^- in Table 2.

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