Brief Articles

Preparation and Properties of S-Nitroso-L-Cysteine Ethyl Ester, an Intracellular Nitrosating Agent#

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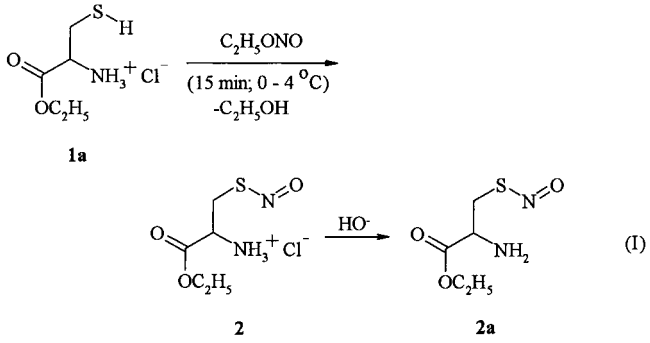
In this report, a protocol for the preparation of the hydrochloride of S-nitroso-L-cysteine ethyl ester (SNCEE·HCl; **2**) is presented. The synthesis of **2** has been targeted because S-nitroso-L-cysteine (SNC; **2b**), which is extensively used for trans-S-nitrosation of thiol-containing proteins, has a limited ability of crossing cellular membranes. The nitrosothiol **2** was prepared via direct S-nitrosation of the hydrochloride of L-cysteine ethyl ester (CEE·HCl; **1a**) with ethyl nitrite. **2** is relatively stable in crystal form and when neutralized to SNCEE (**2a**) in aqueous solutions treated with chelators of metal ions. Traces of metal ions, however, triggered the decomposition of **2a** to nitric oxide and a S-centered radical, which were detected by ESR spectrometry. In contrast to **2b**, **2a** is a lipophilic compound that was taken up by human neutrophils. The latter process was paralleled by inhibition of the NADPH oxidase-dependent generation of superoxide anion radicals, presumably via reaction(s) of intracellular trans-S-nitrosation. Intracellular accumulation of S-nitrosothiols was observed with **2a** but not with **2b**. It is expected that the use of **2a** will be advantageous when intracellular reactions of trans-S-nitrosation are to be studied.

Introduction

S-Nitrosothiols are signaling molecules that may act via direct exchange of their −NO function with thiol groups in low molecular weight compounds and thiol-containing proteins (trans-S-nitrosation). Reactions of trans-S-nitrosation are believed to trigger important physiological events such as neurotransmission,[1,2] activation of ion channels,[1,3] and intracellular signaling.[4,5] In model studies aimed to mimic the biochemistry of thiol-containing proteins, S-nitroso-L-cysteine (**2b**), S-nitrosoglutathione (**3**), and S-nitroso-*N*-acetylpenicillamine (**4**) are extensively used as specific S-nitrosating reagents. **2b** is an unstable compound that has not been isolated in crystal form. Nevertheless, when formed in situ, **2b** has a higher nitrosating potential than equimolar solutions of **3** or **4**.[3,6] **2b** is often used in experiments with intact cells. However, it is a hydrophilic compound that cannot efficiently cross phospholipid membranes, and its effects (or lack of effects) on the cellular homeostasis may partially reflect its extracellular decomposition to NO·, rather than formation of intracellular S-nitrosothiols.

Scheme 1



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In this report, we describe a method for preparation and isolation of the hydrochloride of S-nitroso-L-cysteine ethyl ester (**2**). The latter is relatively stable in crystal form and when neutralized to S-nitroso-L-cysteine ethyl ester (**2a)** in solutions containing chelators of transition metal ions. In contrast to **2b**, **2a** is a lipophilic compound that was taken up by human neutrophils. The latter process was paralleled by inhibition of the NADPH oxidase-dependent generation of superoxide anion radicals, presumably via reaction(s) of intracellular trans-S-nitrosation.[7]

Results and Discussion

The nitrosothiol **2** was prepared via direct S-nitrosation of **1a** (Scheme 1). Elemental analysis, UV/VIS, ^{1}H NMR, and ESR spectrometry were applied for confirma-

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Abbreviations: L-cysteine ethyl ester, CEE (**1**); L-cysteine ethyl ester hydrochloride, CEE.HCl (**1a**); S-nitroso-L-cysteine ethyl ester, SNCEE (**2a**); S-nitroso-L-cysteine ethyl ester hydrochloride, SNCEE-.HCl (**2**); S-nitroso-L-cysteine, SNC (**2b**); S-nitrosoglutathione, SNG (**3**); S-nitroso-*N*-acetylpenicillamine, SNAP (**4**); 5,5′-dimethyl-1-pyroline *N*-oxide, DMPO (**5**); EDTA, ethylenediaminetetracetic acid; 2,3-dihydroxybutane-1,4-dithiol, DTT (Cleland's reagent); *N*-formyl-methionyl-leucyl-phenylalanine, FMLP.

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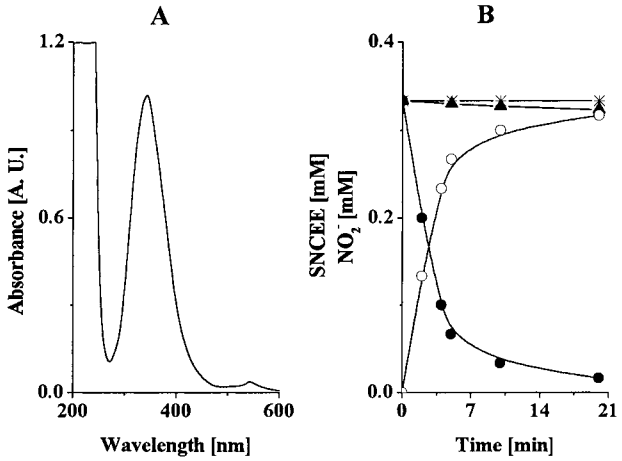


Figure 1. Spectral characteristics and stability of **2a**. **A.** Electronic absorption spectrum of **2** (1 mM) in methanol. **B.** Effects of metal ion chelators on the kinetics of **2a** decomposition. Changes in the concentration of **2a** were followed spectrophotometrically at 343 nm. All experiments were carried out in PBS (20 °C): closed circles, **2a**; stars, plus EDTA (0.1 mM); triangles, **2a** in Chelex 100-treated PBS; and open circles, accumulation of NO_2^- in PBS containing **2a** (0.33 mM).

tion of the structure of **2**. As determined by HPLC-EC, SNCEE·HCl contained less than 1% **1a** (data not shown). In buffered aqueous solutions (pH = 7.4), the decomposition of **2a** was paralleled by accumulation of equimolar amounts of NO_2^- (Figure 1B), while DTT (10 mM) quantitatively reduced the S-nitrosothiol (0.2 mM) back to **1** (data not shown).

Figure 1A depicts the electronic absorption spectrum of **2** in methanol ($\epsilon_{343} = 1019$ and $\epsilon_{544} = 36$ $M^{-1}\cdot cm^{-1}$, respectively; **3** and **4** have ϵ_{335} values of 922 and 519$M^{-1}\cdot cm^{-1}$, respectively); no spectral changes were observed within a period of 30 min (20 °C). In EDTA-containing or Chelex 100-treated PBS, **2a** was relatively stable (Figure 1B). In contrast to **2b**, **2a** could be quantitatively extracted with ethyl acetate from buffered aqueous solutions (pH = 7.4; **2b** was not detected in ethyl acetate extracts), suggesting that it is sufficiently nonpolar to overcome hydrophobic barriers such as cellular membranes. In the absence of EDTA, or when PBS not treated with Chelex 100 was used, **2a** rapidly decomposed with accumulation of equimolar concentrations of NO_2^- (Figure 1B). The latter suggests that PBS contained traces of transition metal ions that could catalyze the homolytic fission of the −S−N=O function of **2a** to NO· and L-cysteine thiyl radical (Scheme 2).[8] In support of this assumption, an addition of Fe^{2+} and L-cysteine into a solution of **2a** led to the appearance of the ESR spectrum of [Fe(cysteine)$_2$-

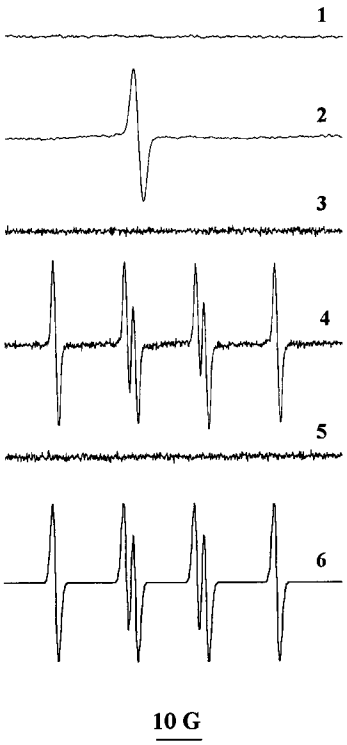
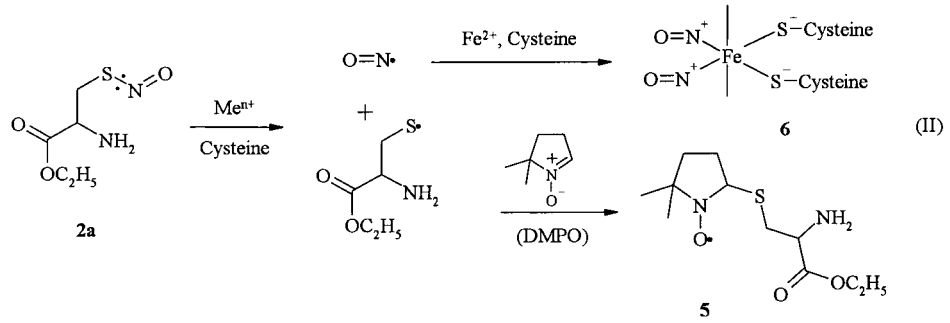


Figure 2. ESR spectra of **6** and **5** formed in solutions of **2a**. All ESR spectra were recorded after 2 min of incubation in PBS (20 °C). Spectrum (**1**), cysteine (0.5 mM), and $Fe(NH_4)_2(SO_4)_2$ (0.1 mM); spectrum (**2**), plus **2** (1 mM); spectrum (**3**), DMPO (0.1 M); spectrum (**4**), DMPO plus **2** (5 mM); spectrum (**5**), DMPO plus EDTA (0.1 mM) plus **2**; and spectrum (**6**), computer simulation of the ESR spectra of DMPO/cysteine thiyl radical adduct.

$(NO^+)_2$] (Scheme 2, **6;** Figure 2.2),[9,10] suggesting that iron and cysteine catalyzed the decomposition of **2a** to nitric oxide. No ESR spectrum was observed if the reaction was carried out in the presence of EDTA (1 mM; data not shown). When 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO; Scheme 2) was added into PBS containing **2a**, the appearance of a six-line ESR spectrum with hyperfine structure (in G) of $A_N = 14.45$ and $A_H = 16.23$ was observed that allows the assignment of the adduct as that formed by addition of L-cysteinyl thiyl radical to DMPO (Scheme 2, **5**; Figure 2.4).[11] An addition of EDTA fully prevented the formation of **5** (Figure 2.5), which supports the notion that metal ion catalysis is required for the homolytic fission of the nitrosothiol. Figure 2.6 represents a computer simulation of the ESR spectrum of DMPO/L-cysteinyl thiyl radical adduct.[11]

With regard to the potential of **2a** to accumulate in

Scheme 2



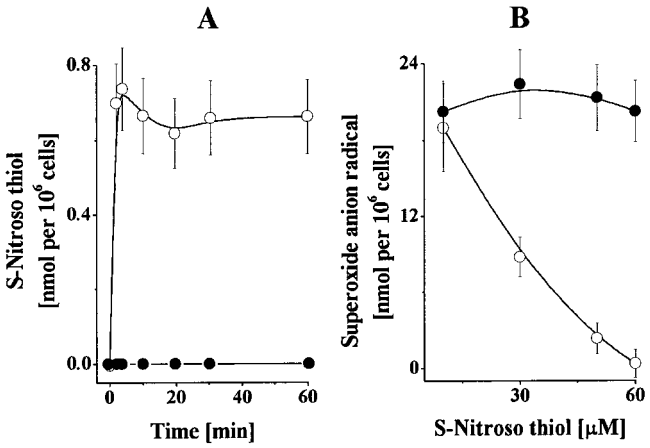


Figure 3. Effects of **2b** and **2a** on NADPH oxidase activity and intracellular S-nitrosothiols content in human neutrophils. **A.** Kinetics of **2b**- and **2a**-dependent accumulation of S-nitrosothiols in human neutrophils. Neutrophils (5×10^6 per mL) were incubated (for up to 1 h) in PBS with either **2b** (0.3 mM; filled circle) or **2a** (0.3 mM; open circle). At the end of the incubation, the cells were separated from the incubation solution, and the intracellular content of S-nitrosothiols was determined as described in the Experimental Section. Results are from four independent experiments. **B.** Effects of **2b** and **2a** on $O_2^{\cdot -}$ generation by human neutrophils. Before activation with FMLP (10^{-7} M), neutrophils (1.25×10^6 per mL) were incubated in PBS, plus ferri-cytochrome *c* for 5 min with varying concentrations of either **2b** (filled circle) or **2a** (open circle). Subsequently, the cells were spun down, the supernatant was collected, and its absorption at 550 nm was determined. The amount of $O_2^{\cdot -}$ was calculated as described in ref 7. Results are from four independent experiments.

intact cells, the effects of **2b** and **2a** on the activity of NADPH oxidase in intact human neutrophils were compared. Recent studies have suggested that the protective function of S-nitrosothiols in inflammatory processes may reflect their ability to reversibly inactivate the NADPH oxidase, an event that suppresses superoxide anion radical production and subsequent cellular injury.[12,13] The NADPH oxidase is a multicomponent complex that is composed of several membrane-bound proteins, including cytochrome b558 and three cytosolic regulatory proteins (p47phox, p67phox, and Rac1/Rac2). This multiprotein complex is assembled within the plasma membrane after neutrophil stimulation. **3** has been shown to act before the assembly of the NADPH oxidase complex and to suppress the production of $O_2^{\cdot -}$ without affecting its redox centers or binding site of NADPH. However, **3** efficiently inhibited superoxide anion radical production only in electropermeabilized neutrophils, most likely due to the inability of intact neutrophils to transport this compound.[7]

As shown in Figure 3B, a pretreatment of neutrophils with **2a** reduced in a concentration-dependent manner the FMLP-induced production of superoxide anion radical. To minimize the extracellular decomposition of the S-nitrosothiols, EDTA was included in the incubation medium. In contrast to **2a**, **2b** did not affect the ability of the NADPH oxidase system to produce superoxide anion radical (Figure 3B). Since **2a** accumulated in neutrophils as evident by the intracellular production of S-nitrosothiols (Figure 3A), it is likely that reactions of intracellular trans-S-nitrosation were responsible for the decreased production of superoxide anion radical by **2a**. At least three pathways of biotransformation of **2a** in intact cells could be envisioned: (i) esterase-dependent hydrolysis of **2a** to **2b**, (ii) rapid exchange of the −NO function of **2a** with thiol-containing biomolecules (trans-S-nitrosation), and/or (iii) intracellular metal ion-dependent decomposition of **2a** to nitric oxide.

In conclusion, a protocol for the preparation and isolation of **2a** has been developed. The structure of this compound has been targeted as **2b** has limited potential of crossing cellular membranes. Due to its lipophilicity, **2a** accumulated in intact neutrophils and affected the activity of the NADPH oxidase. The protocol for ethyl nitrite-dependent S-nitrosation of **2b** could be extended to the preparation of the ethyl esters of **3**[14] and **4**. It is expected that the use of lipophilic S-nitrosothiols will be helpful when intracellular reactions of trans-S-nitrosation are to be studied.

Experimental Procedures

Reagents. All reagents were of analytical grade and purchased from Sigma Chem. Co. (St Louis, MO). Phosphate Buffered Saline (PBS; pH = 7.4) was purchased from Bio-Whittaker, Co. (Walkersville, MD). Ethyl nitrite was prepared as described previously.[15] *During the preparation of **2**, care must be exercised in handling solutions containing ethyl nitrite (bp 16.5 °C); inhalation of its vapor may cause severe headache and heart excitation. The preparation must therefore be conducted in an efficient fume cupboard.*

Chemistry. UV/VIS spectra of **2** (Supporting Information) were recorded on an UV160U Shimadzu spectrophotometer (Kyoto, Japan). ^{1}H NMR (200 MHz) analysis of **1a** and **2** was carried out in Numare Spectralab, Inc. (Berkeley Heights, NJ) on a Brucker AC200 spectrometer. Chemical shifts were recorded in parts per million (ppm) downfield from tetramethylsilane. ESR measurements (Supporting Information) were performed on a Bruker ECS106 spectrometer with 50 kHz magnetic field modulation at room temperature (25° C). All experiments were carried out in phosphate buffer (0.1 M; pH 7.4). For spin trapping of ·NO, ferrous ammonium sulfate and L-cysteine were used. S-centered radicals were spin trapped with DMPO. ESR spectra simulations were made using a program created by Philip D. Morse II and Richard Reiter (EPR Simulation System 2.01, Scientific Software Services, IL). The hyperfine splitting constants (in G) used for simulation of the spectrum of DMPO/L-cysteine thiyl radical were as follows: $A_N = 15.3$; $A_H = 17.2$.[11]

Preparation of 2. The nitrosothiol **2** was prepared in methanol (5 mL) by direct S-nitrosation of **1a** (1 g) with ethyl nitrite (0.4 mL). At the end of the incubation (15 min at 0−4 °C), the reaction solution was rotor evaporated (37 °C) to dryness, and the red crystals formed were recrystallized from acetone to afford an yield of 0.8−0.85 g of **2**. When kept on KOH in open vials (4 °C), **2** was stable for at least 3 weeks. However, at room temperature **2** decomposed within a few hours. ^{1}H NMR spectrum of a solution of **1a** in CD_3OD (Supporting Information): δ 1.33 (triplet, 3H, C**H**$_3$), 3.15 (doublet, 2H, C**H**$_2$S), 4.25−4.4 (multiplet, 3H, NC**H** and C**H**$_2$O). ^{1}H NMR spectrum of a solution of **2** in CD_3OD (Supporting Information): δ 1.28 (triplet, 3H, C**H**$_3$), 4.2−4.4 (pentet, 4H, C**H**$_2$S and C**H**$_2$O), 4.53 (triplet, 1H, NC**H**). In CD_3OD, the ^{1}H NMR spectra of CH_3CH_2OD formed via an H^+-dependent hydrolysis of **2** could be observed (δ 1.33, triplet, C**H**$_3$; 3.35, quartet, C**H**$_2$O). Elemental analysis: found (%) C, 28.21; H, 5.37; N, 12.25; S, 15.04. $C_5H_{11}ClN_2O_3S$ requires (%) C, 27.98; H, 5.16; N, 13.05; S, 14,94 (Supporting Information).

Determination of Intracellular S-Nitrosothiols and NO_2^-.[16] After preincubation with either **2b** or **2**, human neutrophils were centrifuged (11 000g × 20 s) through a layer of PBS (1 mL) containing sucrose (10%), NaCl (0.9 M), and EDTA (1 mM). The top fluid and intermediate sucrose layers were immediately removed, and the cell pellets were treated

at pH 7.4 with a lysis solution consisting of Triton X-100 (0.1%), 2,3-diaminonaphthalene (0.5 mM), and $HgCl_2$ (0.4 mM). The S-nitrosothiol/Hg^{2+}-depended conversion of 2,3-diaminonaphthalene to naphthaltriazole in the cell lysate was monitored fluorimetrically with a Perkin-Elmer fluorometer LS 50B (excitation wavelength - 375 nm; emission wavelength - 450 nm; slit - 5 nm). The concentration of S-nitrosothiol(s) in the samples was deduced by standardizing the 450 nm fluorescence with consecutive additions of **2** (0−10 μM). The concentration of NO_2^- was determined via the same protocol except that $HgCl_2$ was excluded from the reaction solution, and the reactions were carried out at pH ≤ 3.

HPLC Determination of 1 and 2a.[17] HPLC was performed with a Shimadzu liquid chromatograph. Separation was achieved with a C-18 reverse phase column (Nova Pak, 4.6 mm × 15 cm, Waters Associates, Inc., Milford, MA). The mobile phase was saturated with helium and contained 10 mM lithium perchlorate and 40% (v/v) methanol. All HPLC analyses were conducted at a flow rate of 0.5 mL per min. Electrochemical detection of **1** and **2a** was carried out at + 0.75 V with an ESA 5200A Coulochem detector (ESA, Inc., Chelmsford, MA) equipped with a working electrode made of porous graphite (EC cell 5014B). A 0.020 mL injection loop was used for all experiments. The retention time of **1** and **2a** under these experimental conditions was 4.9 and 6.8 min, respectively.

Neutrophil Isolation and Measurement of Neutrophil-Dependent Generation of $O_2^{\cdot-}$. Human neutrophils were obtained from the heparinized venous blood of healthy volunteers by Ficoll-Hypaque centrifugation, dextran sedimentation, and hypotonic lysis. Generation of $O_2^{\cdot-}$ by neutrophils activated with the physiological receptor agonist n-formyl-methionyl-leucyl-phenylalanine (FMLP) was monitored via reduction of cytochrome C in the presence or absence of superoxide dismutase.[7] Briefly, FMLP-activated neutrophils (1.25×10^6 per mL) were combined with a solution of horse heart ferricytochrome C (type III; 0.9 mg per ml) and cytochalasin B (5 μg per ml) in EGTA (0.1 mM)-containing PBS; the cells were spun down, the supernatant was collected, and its absorption at 550 nm determined. The amount of $O_2^{\cdot-}$ released by the neutrophils was estimated by the increase of A_{550} as described previously.[7] Cell death was assessed by release of cytoplasmic lactate dehydrogenase (LDH); in all experiments, the cell viability was higher than 95%.

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Supporting Information Available: Tables containing the UV/VIS, ^1H NMR, and ESR spectral data of **1a**, **2**, **2a, 5,** and **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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