Dendrimeric-Containing Nitronyl Nitroxides as Spin Traps for Nitric Oxide: Synthesis, Kinetic, and Stability Studies

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ABSTRACT: The in-vivo in-situ spin-trapping of NO• with iron chelates is limited in its ability to localize NO• at defined cellular compartments. Nitronyl nitroxides, such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline 3-oxide 1-oxyl, have been found to react with NO•. The resulting nitroxide, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolidine 1-oxyl, exhibits an EPR spectrum that is characteristically distinct from the parent nitroxide. However, nitronyl nitroxides are unstable in biologic milieu. This limits their ability to in vivo identify NO• at specific tissue compartments. Herein, we report on the preparation of a family of dendrimer-containing nitronyl nitroxides, which were developed to spin-trap NO•. Once synthesized, we determined their rate of reaction with NO• and their capacity to spin-trap NO• and estimated the stability of dendrimer-linked nitronyl nitroxides under various experimental paradigms. For the largest of the dendrimeric nitronyl nitroxides prepared, the reaction with NO• changed the EPR spectrum from one that was broad to that with a major component with narrow and defined spectral lines. These compounds promise to be excellent biological spin-trapping agents for NO•.

Introduction

Nitric oxide (NO•) is a free radical with a remarkably long lifetime in biological milieu.[1] Although this diminished rate of reaction toward many targets leads to a high degree of specificity, an essential prerequisite for a biologic second messenger, this low reactivity presents problems when attempting to identify this free radical in animal models in real time. In fact, only in the past several years has evidence surfaced, documenting the in-vivo generation of this free radical.[2] These in-vivo in-situ spin-trapping/EPR spectroscopic experiments entailed the reaction of NO•, secreted by activated murine macrophages, with iron chelates,[2] like the highly charged ferro-di(*N*-methyl-D-glucamine dithiocarbamate) [**1**] or the more hydrophilic ferro-di(*N*-(dithiocarboxy)sarcosine) [**2**] (Scheme 1). Despite the fact that these spin traps have successfully detected this free radical in mice in real time,[2] we continue to search for spin traps that may allow localization of NO• at defined sites. Further, in-vivo toxicity of these iron chelates remains a serious problem.

Recent studies, however, have suggested that nitronyl nitroxides, such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidizoline 3-oxide 1-oxyl [**5**], may be a viable alternative to iron chelates (Scheme 1).[3,4] Nitronyl nitroxide [**5**] reacts with NO• at reasonable rates.[3a] The resulting imino nitroxide, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazolidine 1-oxyl [**6**], exhibits an EPR spectrum characteristically unique to this latter nitroxide.[3,4] The chemical flexibility, offered by this class of spin traps for NO•, allows detection of this free radical at specific tissue sites. Excitement for these nitronyl nitroxides must, however, be tempered by the rapidity by which these spin-labels are bioreduced.[5]

Dendrimers with terminal functional groups have attracted much interest as models to incorporate a localized high concentration of a specific guest molecule.[6] On the basis of previous reported syntheses of nitroxide-linked dendrimers,[7] we have prepared a family of dendrimer-containing nitronyl nitroxides. With these compounds, we measured the rate of spin-trapping NO• and the capacity of this reaction. Further, we estimated the stability of dendrimer-linked nitronyl nitroxides under various experimental paradigms.

Experimental Section

Reagents. Hypoxanthine was purchased from Sigma Chemical Co. (St. Louis, MO). Superoxide dismutase, catalase, and xanthine oxidase were obtained from Boehringer Mannheim (Indianapolis, IN). All other reagents were purchased from commercial venders and were used without further purification.

DAB-Am-4-Dendrimer-(NH-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-Oxide [8]. To a solution of 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide [**5**] (prepared as reported,[3a] 0.5 g, 1.8 mmol) and *N*-hydroxysuccinimide (Aldrich Chemical Co., Milwaukee, WI, 0.21 g, 1.8 mmol) in $CHCl_3$ (50 mL) was added 1,3-dicyclohexylcarbodiimide (Aldrich, 0.4 g, 1.98 mmol). The reaction mixture was stirred for 1 day at room temperature, filtered, and evaporated in vacuo. The remaining residue was taken up in CH_2Cl_2 (10 mL), filtered, and evaporated in vacuo to

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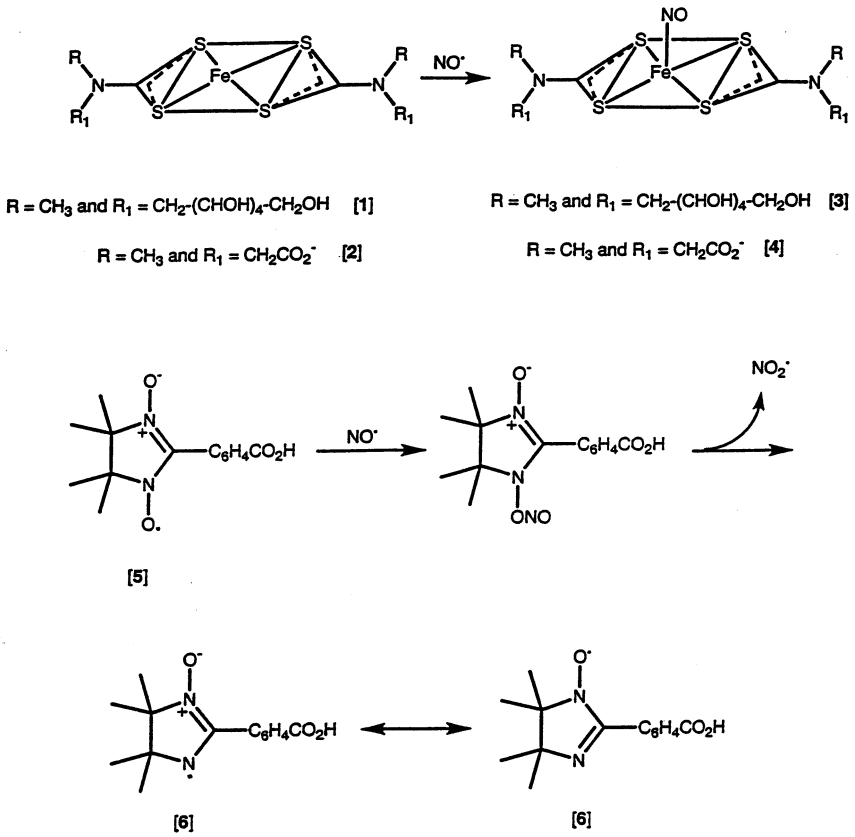
Scheme 1



dryness. The residual oil was passed through a chromatographic column containing silica gel (Aldrich, mesh 230−400) and eluted with chloroform:acetone (94:6). 2-{4-[(2,5-Dioxo-1-pyrrolidinyl)oxy]carboxyphenyl}-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide [**7**] was isolated as a deep blue solid (0.48 g, 72%); mp 156−160 °C (with decomposition). IR ($CHCl_3$): 1772, 1742, and 1698 (C=O), 1646 cm^{-1} (C=N−O). EPR: A_N [1,3] = 8.2 G.

A solution of DAB-Am-4-*N*,*N*,*N*′,*N*′-tetrakis(3-aminopropyl)-1,4-butanediamine (Aldrich, 0.22 g, 0.69 mmol) dissolved in CH_2Cl_2 (5 mL) was added to a solution of [**7**] (1.26 g, 3.3 mmol) dissolved in CH_2Cl_2 (50 mL). This reaction mixture was stirred at room temperature for 1 day. After evaporation to dryness, in vacuo, the remaining oil was purified by column chromatography using silica gel (Aldrich, mesh 230−400) and chloroform:acetone (94:6), which separated excess [**7**] from the reaction mixture. Changing the effluent to methanol:ammonium hydroxide (30%) (9:1), the desired dendrimer-containing nitroxide [**8**] was afforded as an oil, which solidified upon placement under high vacuum (0.45 g, 48%). IR ($CHCl_3$): 1647 cm^{-1} (C=N). A_N [1,3] = 8.2 G with broadening (Figure 1b).

General Procedure for the Preparation of Dendrimer-(NH-2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-Oxide)$_n$. Following the general method described above, other dendrimer-containing 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxides were prepared and purified by flash column chromatography using silica gel (mesh 230−400) and different effluent systems: (a) nitronyl nitroxide [**9**] from 1,4-diaminopropane (Aldrich), chloroform:methanol (9:1); (b) dendrimer-linked nitroxide [**10**] from TREN (tris(2-aminoethyl)amine) (Pressure Chemical Co., Pittsburgh, PA), chloroform:methanol (8:2); (c) dendrimer-linked nitroxide [**11**] from DAB-Am-8-(4,17-bis(3-aminopropyl)-8,13-bis[3-[bis(3-aminopropyl)amino]propyl]-4,8,13,17-tetraazaeicosane-1,20-diamine, Aldrich), methanol:ammonium hydroxide (30%) (98:2).

Kinetics of Spin-Trapping Nitric Oxide by Nitronyl Nitroxides [5, 8−11]. The apparent rate constant for the spin-trapping of NO• was estimated using anaerobic solutions of NO• (fixed at 10−28 μM, final concentration). These solutions were prepared by introducing NO• gas (99%, Matheson Gas Products, Baltimore, MD) into deionized H_2O, which had previously been passed through a glass column containing Chelex-100 ion-exchange resin (BioRad, Richmond, CA). The NO• solution was mixed with oxyhemoglobin (30 μM, final concentration) in the absence and presence of freshly prepared nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-containing nitronyl nitroxides [**8, 10, 11**] (varied from 0 to 200 μM). This reaction was conducted in sodium phosphate buffer (30 mM, pH 7.4). The concentration of NO• was estimated by its reaction with oxyhemoglobin using an extinction coefficient of 12 mM^{-1} cm^{-1} at 576 nm.[8]

Stability of Nitronyl Nitroxides [5], Dinitroxide [9], and Dendrimer-Containing Nitronyl Nitroxides [8, 10, 11]. The stability of nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-containing nitronyl nitroxides [**8, 10, 11**] in the presence of superoxide ($O_2^{\bullet -}$) (10 μM/min) and glutathione (100 μM) was evaluated and presented in Table 3. For a typical experiment, dendrimer-linked nitronyl nitroxide [**11**] (5 μM, final concentration) was added to a $O_2^{\bullet -}$ generating system consisting of xanthine/xanthine oxidase, pH 7.4. Addition of SOD (30 U/mL) confirmed that the loss of nitroxide was $O_2^{\bullet -}$ dependent. This reaction mixture was immediately added to an EPR flat quartz cell and introduced into the cavity of an EPR spectrometer (Varian Associates, model E-109, Palo Alto, CA). EPR spectra were recorded at room temperature. Identical studies were conducted with nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-containing nitronyl nitroxides [**8, 10**].

Superoxide Production. Xanthine oxidase was added to a sodium phosphate buffer, pH. 7.4, containing xanthine such that the initial rate of $O_2^{\bullet -}$ generation was 10 μM/min. The superoxide flux was estimated as the SOD-inhibitable reduction of ferricytochrome *c*, monitored at 550 nm spectrophotometrically as previously described.[9] Initial rate of $O_2^{\bullet -}$ production was calculated using an extinction coefficient of 21 mM^{-1} cm^{-1}.

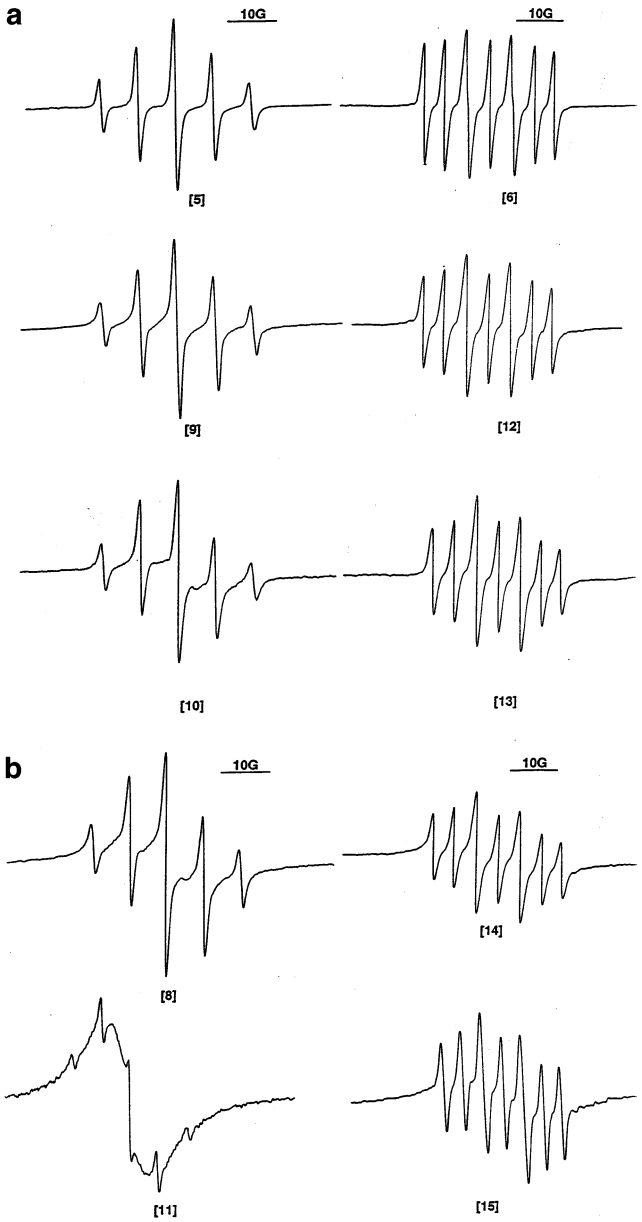


Figure 1. (a) Representative EPR spectra of nitronyl nitroxide [**5**] (5 μM), dinitroxide [**9**] (5 μM), and dendrimer-linked nitronyl nitroxides [**10**] (5 μM) in sodium phosphate buffer (30 mM, pH 7.4). After reaction with NO$^\bullet$ (at 2 μM/min, from the hydrolysis of SPER-NO), the EPR spectra of imidazolidinoxyls [**6, 12, 13**] were obtained. EPR spectra were continually recorded. EPR spectra shown were at 40 min, independent of whether SPER-NO was added. Receiver gain for nitroxides [**5, 6, 9, 10, 12, 13**] was 4×10^3 using a Varian Associates model E-109 spectrometer. (b) Representative EPR spectra of dendrimer-linked nitroxides [**8**] (5 μM) in sodium phosphate buffer (30 mM, pH 7.4). After reaction with NO$^\bullet$ (at 2 μM/min, from the hydrolysis of SPER-NO), the EPR spectrum of imidazolidinoxyls [**14**] was recorded. Dendrimer-linked nitronyl nitroxide [**11**] was dissolved in sodium phosphate buffer (30 mM, pH 7.4 containing DMSO, 3.6% final concentration) prior to reaction with NO$^\bullet$, at a flux of 2 μM/min from the hydrolysis of SPER-NO. EPR spectra were continually recorded. EPR spectra shown were 40 min, independent of whether SPER-NO was added. Receiver gain for nitroxides [**8, 14**] was 4×10^3, and for nitroxide [**11, 15**] it was 5×10^4. EPR spectra were obtained using s Varian Associates model E-109 spectrometer.

***g* Value Determination for Nitronyl Nitroxides.** All EPR spectra (Varian Associates, model E-12, Palo Alto, CA) were recorded at room temperature using a frequency counter (Hewlett-Packard 5342A). DPPH (1,1′-diphenyl-2-picrylhydrazyl) was used as a marker of *g* value = 2.0036. The sample volume was adjusted to make the EPR signal height of DPPH the same as a 5 μM aqueous solution of each of the nitronyl nitroxides tested. DPPH was sandwiched between 5 mm square pieces of Scotch tape, which in turn was taped to the EPR quart flat cell. This cell was then fitted into the cavity of the EPR spectrometer. Spectrometer parameters were microwave power, 10 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 0.3 s; sweep width, 100 G; and number of points, 512.

Table 1. MALDI−TOF Mass Spectrometry of Nitronyl Nitroxide-Linked Dendrimers

		average mass [g/mol]	
nitronyl nitroxides	molecular formula	calcd	measd
9	$C_{31}H_{40}N_6O_6$	592.7	588.4
10	$C_{48}H_{63}N_{10}O_9$	924.0	922.8
8	$C_{72}H_{100}N_{14}O_{12}$	1353.6	1352.3
11	$C_{152}H_{216}N_{30}O_{24}$	2847.5	2844.2

Table 2. Rate Constants for the Spin-Trapping of Nitric Oxide

spin trap	rate constant, $\times 10^3$ M^{-1} s^{-1}	experimental conditions
[**5**][a]	161 ± 48	anaerobic solution of NO$^\bullet$ gas in H_2O added to sodium phosphate buffer, pH 7.4
[**9**][a]	191 ± 48	anaerobic solution of NO$^\bullet$ gas in H_2O added to sodium phosphate buffer, pH 7.4
[**10**][a]	179 ± 42	anaerobic solution of NO$^\bullet$ gas in H_2O added to sodium phosphate buffer, pH 7.4
[**8**][a]	242 ± 16	anaerobic solution of NO$^\bullet$ gas in H_2O added to sodium phosphate buffer, pH 7.4
[**11**][a]	40.2 ± 5.2	anaerobic solution of NO$^\bullet$ gas in H_2O/DMSO (0.1%) added to sodium phosphate buffer, pH 7.4
[**5**][b]	10.1	sodium phosphate buffered solutions of NO$^\bullet$, pH 7.4

[a] [**5, 9, 8, 10, 11**] = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide, dinitroxide, and dendrimer-linked nitronyl nitroxides; apparent rate constants using nitronyl nitroxides as competitive inhibitors for oxyhemoglobin.[12] [b] [**5**] = 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide; absolute rate constants (data taken from ref 3a).

Table 3. Stability of Dendrimer-Containing Nitronyl Nitroxides in the Presence of Reduced Glutathione and Superoxide[a]

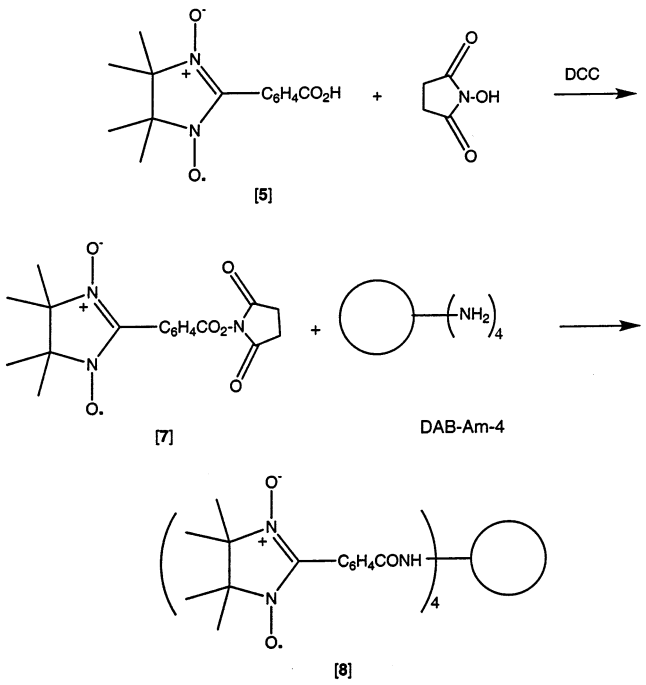
spin trap	half-life (min)[a]	half-life (min)[b]
[**5**]	20 ± 2.0	10 ± 0.7
[**9**]	13 ± 2.5	19 ± 1.1
[**10**]	stable[c]	37 ± 2.8
[**8**]	9 ± 1.4	17 ± 2.3
[**11**]	15 ± 1.8	182 ± 6.6

[a] The half-life of dendrimer-containing nitronyl nitroxides (5 μM) in the presence of reduced glutathione (100 μM), potassium phosphate buffer, (30 mM, pH 7.4) was determined. In the absence of glutathione, each of the nitronyl nitroxides were stable for at least 80 min. Data presented was the average of three independent experiments. [b] The half-life of nitronyl nitroxides (5 μM) exposed to a $O_2^{\bullet-}$ generating system consisting of xanthine and xanthine oxidase. The rate of $O_2^{\bullet-}$ production was 10 μM/min. Data presented was the average of three independent experiments. [c] The term stable refers to the fact that the EPR spectrum for dendrimer-linked nitronyl nitroxide [**10**] was unchanged for over 30 min.

Results and Discussion

Synthesis. In the preparation of dendrimer-containing nitronyl nitroxides, a substituent on the aromatic

Scheme 2

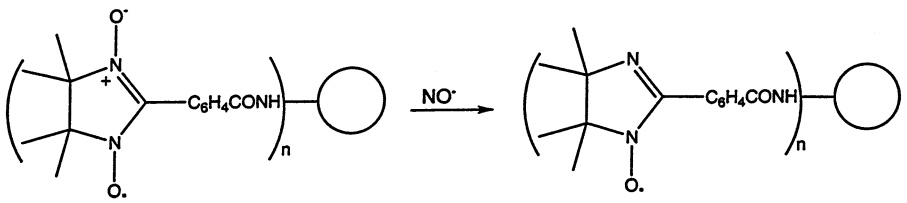


ring of 2-phenyl-4,4,5,5-tetramethylimidazolidine 1-oxyl was used to link the imidazolidine to various dendrimers. For these initial studies, we activated 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide [**5**] through its reaction with *N*-hydroxysuccinimide in the presence of 1,3-dicyclohexylcarbodiimide (Scheme 2).[7b] The resulting activated ester [**7**] was combined with poly(propyleneimine tetraamine) dendrimers (DAB-Am)$_n$ where, for instance, $n = 4$, yielding the corresponding dendrimer-linked nitronyl nitroxide [**8**] (Scheme 2). In a similar fashion other dendrimer-containing nitronyl nitroxides were prepared using tris(2-aminoethyl)amine and poly(propyleneimine octaamine) dendrimer (DAB-Am)$_n$ where $n = 8$ as well as 1,3-diaminopropane (Scheme 3). The resulting dinitroxide [**9**] and dendrimer-linked 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxides [**8, 10, 11**] were purified by flash column chromatography using silica gel and various combinations of polar solvents as effluents. For the dinitroxide [**9**] and dendrimer-containing nitronyl nitroxides [**8, 10**], the solvent mixture of chloroform:methanol (9:1) and chloroform:methanol (8:2), respectively, was found to be ideal. In the case of dendrimer-containing nitronyl nitroxide [**11**], a more polar solvent mixture was required, methanol:ammonium hydroxide (30%) (98:2) to isolate the desired product, nitroxide [**11**]. The identification of the dendrimer-containing nitronyl nitroxides was confirmed using matrix-assisted laser desorption and ionization−time-of-flight (MALDI−TOF) mass spectrometry using 2,4,6-trihydroxyacetophenone and α-cyano-4-hydroxycinnamic acid as matrices (Table 1). In each case a small deviation from calculated mass (*m*/*z*) values has been found. Further, the MALDI−TOF mass spectra showed fragmentation due to repetitive loss of oxygen.

EPR Spectroscopy. The EPR spectrum of nitronyl nitroxide [**5**] is composed of five lines in the ratio of 1:2:3:2:1, from the coupling of two identical nitrogen atoms (nuclear spin $I = 1$) to the electron spin (Figure 1a). The nitrogen isotropic hyperfine coupling constant A_N [1,3] = 8.2 G is about half of that found with simple dialkyl nitroxides such as 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolinidinoxyl.[3a,4] In the presence of NO•, nitronyl nitroxide [**5**] was converted to the corresponding imidazolidine 1-oxyl [**6**], as demonstrated by its characteristic seven-line EPR spectrum, A_N [1] = 9.8 G and A_N [3] = 4.9 G (Figure 1a). The identity of the nitrogen atoms has been lost by the elimination of the oxygen at one of the nitrogen atoms.[3a] As the number of nitroxides linked to dendrimers increased, from $n = 3$ to $n = 4$ and to $n = 8$ active site attachments, there appeared a broadened peak that overlaps the very visible five-line EPR spectrum of typical nitronyl nitroxides (Figure 1b). However, only a small percent of the doubly integrated EPR spectrum derives from the narrow component. In contrast, for dendrimer-linked pyrrolinidinoxyls, the narrow nitroxide triplet of typical pyrrolinidinoxyls with an $A_N = 15$ G was lost by attachment of as few as four of these nitroxides to the dendrimer.[7] The *g* values for dendrimer-linked nitroxides [**10, 11**] were 2.0066 and 2.0065, respectively.

Reaction of dinitroxide [**9**] and dendrimer-containing nitronyl nitroxides [**8, 10, 11**] with NO• leads to the corresponding imidazolidinoxyls [**12**−**15**] (Figure 1). Of particular interest is the finding of a shift in the EPR spectrum from a near exclusive broad-lined of that found for dendrimer-containing nitroxide [**11**] to the seven-lined imidiazolidininoxyl [**15**] with a substantial narrow component. The narrow component seen in [**15**] represents as much as 25% of the EPR spectral intensity. This increase in the narrow component can be qualitatively understood by noting that this change in the EPR spectrum results from the loss of an oxygen atom from the nitronyl nitroxide moiety of dendrimer-containing nitroxide [**11**]. This should reduce the steric hindrance of the imidiazolidininoxyls in [**15**] relative to the nitronyl nitroxides in [**11**] and allow rapid spinning of the former, thereby narrowing the EPR spectral lines. Work is underway to better understand this phenomenon. This narrowing of the EPR spectrum of imidazolidinoxyls will make it easier to identify NO• at cellular fluxes, as has been observed with LPS-stimulated RAW macrophages

Scheme 3



using 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidizoline 3-oxide 1-oxyl [**5**].[5b] Further, we suggest that this spectral property will be important in the in-vivo in-situ measurement of $NO^{\bullet}$ using low-frequency EPR spectroscopy.

Kinetics. The rate constant for the reaction of $NO^{\bullet}$ with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidizoline-1-oxyl 3-oxide [**5**] is given in Table 2. In aerobic aqueous solutions, $NO^{\bullet}$ combines with O_2 according to the following:

$$NO^{\bullet} + {}^1/_2O_2 \rightarrow NO_2 \quad (1)$$

$$NO_2 + NO^{\bullet} \rightarrow N_2O_3 \quad (2)$$

$$N_2O_3 + H_2O \rightarrow 2H^+ + 2NO_2^- \quad (3)$$

in which $-d[NO^{\bullet}]/dt = k[O_2][NO^{\bullet}]^2$. Thus, the reaction is second order in $NO^{\bullet}$ and first order in O_2.[1,11] To simplify the kinetic model, we used low concentrations of $NO^{\bullet}$ added to an anaerobic solution of nitronyl nitroxide [**5**]. The concentration of nitronyl nitroxide [**5**] was set sufficiently high to trap essentially all of the $NO^{\bullet}$ in the absence of oxyhemoglobin.[8] In the presence of oxyhemoglobin $Hb(Fe^{2+})O_2$ there are two competing reactions:

$$NO^{\bullet} + \text{nitroxide [5]} \rightarrow \text{nitroxide [6]} \quad (4)$$

$$NO^{\bullet} + Hb(Fe^{2+})O_2 \rightarrow Hb(Fe^{3+}) + NO_3^- \quad (5)$$

Thus, from eqs 4 and 5, the rate of $NO^{\bullet}$ elimination can be expressed as

$$V = -d[NO^{\bullet}]/dt = k_{app}[\mathbf{5}][NO^{\bullet}] + k_{Hb}[Hb(Fe^{2+})O_2][NO^{\bullet}] \quad (6)$$

In the absence of nitronyl nitroxide [**5**], eq 6 can be described as

$$v = -d[NO^{\bullet}]/dt = d[Hb(Fe^{3+})]/dt = k_{Hb}[Hb(Fe^{2+})O_2][NO^{\bullet}] \quad (7)$$

By dividing eq 7 into eq 6 and rearranging the terms, competing reactions can be represented as

$$V/v = 1 + k_{app}\{\text{nitroxide [5]}\}/k_{Hb}[Hb(Fe^{+2})O_2] \quad (8)$$

As the initial rate of spin-trapping is first-order with respect to $[NO^{\bullet}]$, $k_{app}/k_{Hb}[Hb(Fe^{2+})O_2]$ becomes the constant, k' at fixed $[Hb(Fe^{2+})O_2]$. Then, eq 8 can be simplified to

$$V/v = 1 + k'\{\text{nitroxide[5]}\} \quad (9)$$

By plotting V/v vs {nitroxide[**5**]}, k' can be determined.[12b] From this, the k_{app} for the spin-trapping of $NO^{\bullet}$ by nitronyl nitroxide [**5**] was calculated (Table 2).

Several different experimental designs were used to determine the apparent rate constant for spin-trapping $NO^{\bullet}$ by nitronyl nitroxide [**5**]. As described above, the concentration of $[Hb(Fe^{2+})O_2]$ was held constant, and the concentration of nitronyl nitroxide [**5**] was varied. In this case, spectrophotometry was used to obtain measurements by taking advantage of the absorbance of $[Hb(Fe^{2+})O_2]$.[8] We also used an alternative approach by fixing the concentration of nitronyl nitroxide [**5**], while the concentration of $[Hb(Fe^{2+})O_2]$ was varied. Here, the ratio of the peak height of the first line in the EPR spectra of nitronyl nitroxide [**5**] and imino nitroxide [**6**] was calculated. For dendrimer-linked nitroxides, however, only spectrophotometric assays were used, as the broadened EPR spectra of these compounds made accurate estimates of peak heights difficult. From these experimental designs, the apparent rate constants for nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-linked nitronyl nitroxides [**8, 10, 11**] were obtained and are presented in Table 2. We were puzzled as to why the rate constant for the spin-trapping of $NO^{\bullet}$ by dendrimer-containing nitronyl nitroxide [**11**] was significantly different than those rate constants attained for nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-linked nitronyl nitroxides [**8, 10**]. All control experiments, including those that examined the reaction of $NO^{\bullet}$ with DMSO, did not result in any insightful theory as to why differing rate constants were found for the reaction of $NO^{\bullet}$ with dendrimer-linked nitronyl nitroxide [**11**]. Nevertheless, we are currently exploring possible explanations.

Stability. Nitronyl nitroxide [**5**], dinitroxide [**9**], and dendrimer-linked nitronyl nitroxides [**8, 10, 11**] were stable for at least 80 min in chelexed potassium phosphate buffer (30 mM, pH 7.4) (Table 3). However, after 24 h in the buffer, dendrimer-linked nitronyl nitroxides [**8, 10, 11**] began to lose nitroxide groups from the dendrimer, leading to an EPR spectrum characteristic of nitronyl nitroxide [**5**]. This loss of nitroxides from the dendrimer was greatest with dendrimer-linked nitronyl nitroxide [**11**] at 43%, for dendrimer-linked nitronyl nitroxide [**8**] at 22% and dendrimer-linked nitronyl nitroxide [**10**] at 5%. Interestingly, the EPR spectrum of dinitroxide [**9**] did not change over this 24 h period, suggesting, as in the case of nitronyl nitroxide [**5**], that dinitroxide [**9**] is stable in phosphate buffer at pH 7.4. While the mechanism remains undefined, perhaps crowding of the nitroxides in the dendrimeric molecule, which cannot occur with either nitronyl nitroxide [**5**] or dinitroxide [**9**], was responsible for the degradation of dendrimer-linked nitronyl nitroxides [**8, 10, 11**]. Of importance from an experimental perspective is the requirement that only freshly prepared aqueous solutions of dendrimer-linked nitronyl nitroxides should be used in studies measuring $NO^{\bullet}$ production.

Except for nitroxide [**10**], each of the nitroxides [**5, 9, 8, 11**] was unstable in the presence of reduced glutathione (100 μM) (Table 3). Thus, in intracellular environments where levels of glutathione are high (in the millimolar range, depending on cell type) nitroxide [**10**] would be the best of these dendrimer-linked nitronyl nitroxides at detecting nitric oxide synthase produced $NO^{\bullet}$.

Purified nitric oxide synthase, independent of the isozyme, has been reported to generate $O_2^{\bullet -}$, even in the presence of L-arginine.[13] In light of this, it was important to determine the stability of the dendrimer-linked nitroxides in the presence of a continued flux of $O_2^{\bullet -}$ (Table 3). For these experiments, the metabolism of xanthine by xanthine oxidase was used as a source of $O_2^{\bullet -}$ (generated at a rate of 10 μM/min) to which nitroxides [**5**, **8**−**11**] were subjected. Inclusion of SOD (30 U/mL) confirmed that the loss of EPR spectra was attributed to the effects of $O_2^{\bullet -}$ on each nitroxide. Not unexpectedly, the stability of the dendrimer-linked

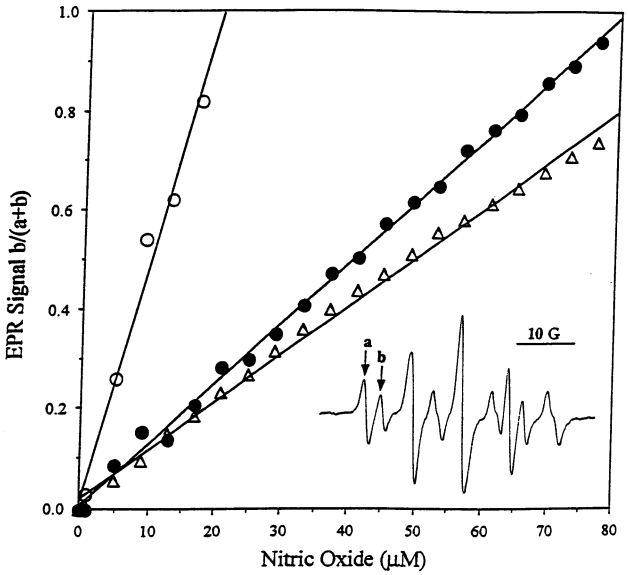


Figure 2. Plot of the EPR spectral peak height ratio vs concentration of NO•. Nitric oxide was generated from SPER-NO at a NO• flux of 1 μM/min, added to either nitronyl nitroxide [**5**] (5 μM, open circles), dendrimer-linked nitronyl nitroxide [**8**] (5 μM, solid circles), or nitronyl nitroxide [**5**] (20 μM, triangles).

nitroxides enhanced as the number of nitroxide groups attached to the dendrimer was increased. Of interest was the finding that dendrimer-linked nitronyl nitroxide [**10**] was considerably more resistant to $O_2^{\bullet-}$-mediated reduction than would be predicted based on dinitroxide [**9**] and dendrimer-linked nitroxide [**8**]. Further, dendrimer-linked nitroxide [**11**] exhibited a half-life of over 3 h. This finding was rather surprising and suggests that nitroxide [**11**] may be completely resistant to bioreduction in the harsh environment of activated phagocytes, where NO• and $O_2^{\bullet-}$ are likewise produced.

NO• Spin-Trapping Capacity. The ratio of the conversion to dendrimer-containing nitronyl nitroxides to imidazolidinoxyls was estimated as a function of NO• concentration. For these experiments, the concentration of nitroxides [**5, 8**−**11**] was fixed at 5 μM, and the capacity to spin-trap NO• was estimated as a function of NO• concentration. As the rate constants for the spin-trapping of NO• are similar (Table 2), one would expect differences in the capacity to reflect the number of nitroxides attached to a specific dendrimer. To test this theory, we compared the capacity of nitronyl nitroxide [**5**] to dendrimer-linked nitronyl nitroxide [**8**]. At equal molar concentrations, there was, as expected, approximately a 4-fold increase in NO• spin-trapping capacity for dendrimer-linked nitroxide [**8**] as compared to nitronyl nitroxide [**5**] (Figure 2). When the concentration of nitroxide [**5**] was increased 4-fold, there was no difference in the capacity to spin-trap NO• between nitroxide [**5**] and dendrimer-linked nitroxide [**8**] (Figure 2).

EPR Spectra of Dendrimer-Containing Nitronyl Nitroxides and Imidazolidinoxyls. The EPR spectrum of dendrimer-containing nitronyl nitroxide [**11**] shown in Figure 1b is consistent with less than 1% spectral intensity contained in the narrow component. While mass spectral data of [**11**] (Table 2) are supportive of one compound, it is conceivable that the 1% may be monomer, detached from the dendrimer during dissolution in sodium phosphate buffer (30 mM at pH 7.4, containing DMSO 3.6%, final concentration). Reaction of dendrimer-containing nitronyl nitroxide [**11**] with NO•, affording imidazolidinoxyl [**15**], resulted in the EPR spectrum shown in Figure 1b with a much larger fraction of the spectrum contained in the doubly integrated intensity of the narrow lines, approximately 25%. Initially, we suspected that this larger fraction might be due to monomeric nitroxide that detached upon treatment of dendrimer-linked nitronyl [**11**] with NO•. To test this possibility, nitroxide [**11**] was incubated with sufficient NO• so that the resultant EPR spectrum corresponded to that of nitroxide [**15**]. Then, mass spectral analysis of the reaction mixture was determined. To our surprise, we observed a mass spectral line corresponding to imidazolidinoxyl [**15**]. This finding suggests that the seven-line EPR spectrum typical of these imidazolidinoxyls[4] does not arise from hydrolysis of dendrimer-attached nitronyl nitroxides. But rather, we suspect that elimination of oxygen atoms from dendrimer-linked nitronyl nitroxide [**11**], for instance, after attack by NO• that resulted in $NO_2^{\bullet}$, may free the imidazolidinoxyl [**15**] to rotationally average and narrow the EPR spectrum (Figure 1b, Scheme 3). This large fraction of the spectral intensity due to a narrow component is very favorable for spin-trapping NO• under a variety of experimental paradigms, particularly for the in-vivo detection of NO•. A large number of nitronyl nitroxides can be delivered via a dendrimer to the in-vivo site of NO• formation with very low spectral amplitude per nitronyl nitroxide due to the dipolar broadening. Spin-trapping of NO• then generates a narrow and highly measurable seven-line EPR spectrum corresponding to imidazolidinoxyl.

Conclusion

Herein, we report on the first synthesis of dendrimer-linked nitronyl nitroxides as spin-traps for NO•. Unexpectedly, the unique EPR spectrum of nitronyl nitroxides was still observable, although small, within the broaden spectrum of these nitroxide-attached dendrimers. As the number of nitronyl nitroxides attached to the dendrimer increased, however, the singular five-line EPR spectrum was less apparent, ~ 1% spectral intensity for dendrimer-linked nitronyl nitroxide [**11**]. Surprisingly, after reaction with NO•, the seven-line EPR spectrum of imidazolinoxyls was clearly visible, up to 25% spectral intensity for imidazolinoxyl [**15**]. The rate constants for the spin-trapping of NO• by the dendrimer-linked nitronyl nitroxides were similar to nitronyl nitroxide [**5**], except for dendrimer-linked nitronyl nitroxide [**11**]. One possible explanation for the smaller rate constant may be that as we increase the size of the dendrimer, packing of the nitronyl nitroxide groups retarded the rate of their reaction with NO•. We are currently investigating this possibility. Finally, the increased capacity of these dendrimer-linked nitronyl nitroxides may make it possible to effectively detect NO• in vivo and in real time. These studies are currently ongoing.

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References and Notes

(1) Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. *J. Biol. Chem.* **1994**, *269*, 5881.

(2) (a) Komarov, A.; Mattson, D.; Jones, M. M.; Singh, P. K.; Lai, C.-S. *Biochem. Biophys. Res. Commun.* **1993**, *195*, 1191. (b) Lai, C.-S.; Komoarov, A. M. *FEBS Lett.* **1994**, *345*, 120. (c) Yoshimura, T.; Yokoyama, H.; Fujii, S.; Takayama, F.; Oikawa, K.; Kamada, H. *Nat. Biotechnol.* **1996**, *14*, 992. (d) Fujii, H.; Koscielniak, J.; Berliner, L. J. *Magn. Reson. Med.* **1997**, *38*, 565.

(3) (a) Akaike, T.; Yoshida, M.; Miyamoto, Y.; Sato, K.; Kohno, M.; Sasamoto, K.; Miyazaki, K.; Ueda, S.; Maeda, H. *Biochemistry* **1993**, *32*, 827. (b) Joseph, J.; Kalyanaraman, B.; Hyde, J. S. *Biochem. Biophys. Res. Commun.* **1993**, *192*, 926. (c) Woldman, Y. Y.; Khramtsov, V. V.; Grigor'ev, I. A.; Kiriljuk, I. A.; Utepbergenov, D. I. *Biochem. Biophys. Res. Commun.* **1994**, *202*, 195. (d) Konorev, E. A.; Tarpey, M. M.; Joseph, J.; Baker, J. E.; Kalyanaraman, B. *Free Radical Biol. Med.* **1995**, *18*, 169. (e) Pieper, G. M.; Siebeneich, W. *J. Pharmacol. Exp. Ther.* **1997**, *283*, 138.

(4) Ullman, E. F.; Osiecki, J. H.; Boocock, D. G. B.; Darcy, R. *J. Am. Chem. Soc.* **1972**, *94*, 7049.

(5) (a) Griffeth, L. K.; Rosen, G. M.; Rauckman. E. J.; Drayer, D. P. *Invest. Radiol.* **1984**, *19*, 553. (b) Akaike, T.; Maeda, H. *Methods Enzymol.* **1996**, *268*, 211.

(6) (a) Tomalia, D. A.; Naylor, M. A.; Goddard, W. A., III *Angew. Chem., Int. Ed. Engl.* **1990**, *29*, 138. (b) Wörner, C.; Mülhaupt, R. *Angew. Chem., Int. Ed. Engl.* **1993**, *32*, 1306. (c) Jansen, J. F. G. A.; de Brabander-van den Berg, E. M. M.; Meijer, E. W. *Science* **1994**, *266*, 1226. (d) Scherrenberg, R.; Coussens, B.; van Vliet, P.; Edouard, G.; Brackman, J.; de Bradander, E.; Mortensen, K. *Macromolecules* **1998**, *31*, 456. (e) Rietveld, I. B.; Smit, J. A. M. *Macromolecules* **1999**, *32*, 4608.

(7) (a) Matyjaszewski, K.; Shigemoto, T.; Fréchet, J. M.; Leduc, M. *Macromolecules* **1996**, *29*, 4167. (b) Bosman, A. W.; Janssen, R. A. J.; Meijer, E. W. *Macromolecules* **1997**, *30*, 3606.

(8) Doyle; M. P.; Hoekstra, J. W. *J. Inorg. Biochem.* **1981**, *14*, 351.

(9) Kuthan, H.; Ullrich, V.; Estabrook, R. *Biochem. J.* **1982**, *203*, 551.

(10) (a) A. Abragam. *The Principles of Nuclear Magnetism*; Clarendon Press: Oxford, 1961; p 300. (b) Wyard, S. J. *Proc. Phys. Soc.* **1965**, *86*, 587. (c) Frank, N. L.; Clerac, R.; Sutter, J.-P.; Daro, N.; Kahn, O.; Coulon, C.; Green, M. T.; Golhen, S.; Ouahab, L. *J. Am. Chem. Soc.* **2000**, *122*, 2053.

(11) (a) Fontecave, M.; Pierre, J.-P. *Bull. Soc. Chim. Fr.* **1994**, *131*, 620. (b) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1995**, *117*, 12078.

(12) (a) Sawada, Y.; Yamazaki, I. *Biochim. Biophys. Acta* **1973**, *327*, 257. (b) Asada, K.; Takahashi, M.-A.; Nagate, M. *Agric. Biol. Chem.* **1974**, *38*, 471. (c) Finkelstein, E.; Rosen, G. M.; Rauckman, E. J. *J. Am. Chem. Soc.* **1980**, *102*, 4994. (d) Pou, S.; Tsai, P.; Porasuphatana, S.; Halpern, H. J.; Chandramouli, G. V. R.; Barth, E. D.; Rosen, G. M. *Biochim. Biophys. Acta* **1999**, *1427*, 216.

(13) (a) Pou, S.; Pou, W. S.; Bredt, D. S.; Snyder, S. H.; Rosen, G. M. *J. Biol. Chem.* **1992**, *267*, 24173. (b) Wever, R. M. F.; van Dam, T.; van Rijn, H. J. M.; de Groot, F.; Rabelink, T. J. *Biochem. Biophys. Res. Commun.* **1997**, *237*, 340. (c) Xia, Y.; Roman, L. J.; Masters, B. S. S.; Zweier, J. L. *J. Biol. Chem.* **1998**, *273*, 22635. (d) Vásquez-Vivar, J.; Kalyanaraman, B.; Martásek, P.; Hogg, N.; Masters, B. S. S.; Karoui, H.; Tordo, P.; Pritchard, K. A., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9220. (e) Xia, Y.; Tsai, A.-L.; Berka, V.; Zweier, J. L. *J. Biol. Chem.* **1998**, *273*, 25804. (f) Stroes, E.; Hijmering, M.; van Zandvoort, M.; Wever, R.; Rabelink, T. J.; van Faasen, E. E. *FEBS Lett.* **1998**, *438*, 161. (g) Pou, S.; Keaton, L.; Surichamorn, W.; Rosen, G. M. *J. Biol. Chem.* **1999**, *274*, 9573. (h) Vásquez-Vivar, J.; Hogg, N.; Martásek, P.; Karoui, H.; Pritchard, K. A., Jr.; Kalyanaraman, B. *J. Biol. Chem.* **1999**, *274*, 26736. (i) Yoneyama, H.; Yamamoto, A.; Kosaka, H. *Biochem. J.* **2001**, *360*, 247. (j) Rosen, G. M.; Tsai, P.; Weaver, J.; Porasuphatana, S.; Roman, L. J.; Starkov, A. A.; Fiskum, G.; Pou, S. *J. Biol. Chem.* **2002**, *277*, 40275.

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