Published in final edited form as:
Nitric Oxide. 2014 November 15; 0: 70–78. doi:10.1016/j.niox.2014.08.013.

Analysis of the HNO and NO donating properties of alicyclic amine diazeniumdiolates

Gaurav Bharadwaj[a,1], **Patricia G.Z. Benini**[a,1], **Debashree Basudhar**[a], **Cyf N. Ramos-Colon**[a], **Gail M. Johnson**[a], **Marti M. Larriva**[a], **Larry K. Keefer**[b], **Daniela Andrei**[b], and **Katrina M. Miranda**[a,*]

[a]Department of Chemistry and Biochemistry, University of Arizona, Tucson, Arizona 85721, USA

[b]Chemical Biology Laboratory, National Cancer Institute at Frederick, Frederick, Maryland 21702, USA

Abstract

Nitroxyl (HNO) donors have been shown to elicit a variety of pharmacological responses, ranging from tumoricidal effects to treatment of heart failure. Isopropylamine-based diazeniumdiolates have been shown to produce HNO on decomposition under physiological conditions. Herein, we report the synthesis and HNO release profiles of primary alicyclic amine-based diazeniumdiolates. These compounds extend the range of known diazeniumdiolate-based HNO donors. Acetoxymethyl ester-protected diazeniumdiolates were also synthesized to improve purification and cellular uptake. The acetoxymethyl derivative of cyclopentylamine diazeniumdiolate not only showed higher cytotoxicity toward cancer cells as compared to the parent anion but was also effective in combination with tamoxifen for targeting estrogen receptor α-negative breast cancer cells.

Keywords

Nitroxyl; Nitric oxide; Diazeniumdiolate; IPA/NO; Angeli's salt; Tamoxifen

1. Introduction

Nitric oxide (NO) is a well-studied physiological signaling molecule with functions ranging from regulation of blood pressure, cellular defense against invading pathogens and neurotransmission to prevention of platelet aggregation [1–5]. Nitroxyl (HNO), the product of one-electron reduction of NO, has recently emerged as a promising pharmacological agent. Donors of HNO have been shown to enhance myocardial contractility [6,7], to induce vasodilation [8], and to provide protection against ischemia-reperfusion injury [9], through mechanisms distinct from those of NO [10]. HNO has also been used clinically in treatment of alcoholism [11]; the alcohol deterrent agent cyanamide undergoes metabolic activation to

*Correspondence author. University of Arizona, Department of Chemistry and Biochemistry, Tucson, Arizona 85721, USA,. kmiranda@email.arizona.edu (K. M. Miranda).
[1]These authors contributed equally.

HNO, which then inhibits aldehyde dehydrogenase [12]. Recently, HNO has also been reported to inhibit tumor growth and angiogenesis [13].

Due to irreversible dimerization [14,15], HNO cannot be stored, necessitating use of donor compounds for production of HNO [16–18]. Angeli's salt [19] ($Na_2N_2O_3$; Scheme 1) has been used extensively to study the chemical biology of HNO. Angeli's salt spontaneously releases HNO in aqueous solution [20,21], with a pH-independent rate between pH 4–8 [20]. Despite the utility of Angeli's salt as an HNO donor, the short half-life of ~2 min [22] under physiological conditions inhibits analysis of the effects of chronic exposure to HNO. Derivatization of Angeli's salt to form a more stable HNO donor has been unsuccessful to date. Moreover, decomposition of Angeli's salt produces nitrite, which also has biological activity [23]. Continuous progress in the pharmacology of HNO requires development of a versatile platform for systematically generating reliable, tunable, controlled fluxes of HNO in physiological media.

A variety of organic donors have been produced (Scheme 1). For example Piloty's acid ($C_6H_5SO_2NHOH$) [24] and derivatives have long been known to function as base-sensitive HNO donors [25]. Although the rate of HNO release is tunable, these compounds often tend to generate NO under aerated conditions [26]. Acyl nitroso compounds generate HNO on reaction with nucleophiles [27], but are unstable intermediates [28]. The most general route of synthesizing such compounds is via oxidation of N-acyl hydroxylamine derivatives [29]. Acyloxy nitroso compounds, which are synthesized by oxidation of oximes [30], have more recently been found to function as HNO donors [31,32]. HNO release can be mediated either by enzymatic or spontaneous hydrolysis of the ester bond [33].

Diazeniumdiolates, also known as NONOates, are adducts of an NO dimer with nitrogen, carbon, oxygen or sulfur-based nucleophiles [34,35]. Angeli's salt is formally in this class. Secondary amine-based diazeniumdiolates generate NO upon spontaneous decomposition, with reliable half-lives of decomposition ranging from 2 s to 20 h, depending on the amine backbone [36–38]. Importantly, diazeniumdiolates can be readily O^2-derivatized [39–42], which facilitates purification and increases stability and decomposition half-life, among other effects. Moreover, derivatization allows conversion of diazeniumdiolates to prodrugs that can be bioactivated, thus providing a route to rational design for targeted delivery [43]. For instance, acetoxymethylation produces compounds that are sensitive to esterase-mediated hydrolysis [44] to release the parent diazeniumdiolate along with acetic acid and formaldehyde (Scheme 2).

Unlike the large number of secondary amine based diazeniumdiolates, isopropylamine (IPA/NO) and cyclohexylamine (CHA/NO) diazeniumdiolates are currently the only examples of stable primary amine analogues in the literature [45]. Very recently, the unstable methylamine diazeniumdiolate was characterized and was trapped as the more stable O^2-benzyl derivative [42]. Under physiological conditions, IPA/NO has a short half-life of 6.7 min [46] and generates HNO via a tautomerization pathway (Scheme 3) [46–48]. At lower pH, protonation of the nitroso oxygen, followed by tautomerization and N-N bond cleavage, leads to NO production. This mechanism of NO production is applicable to secondary amine diazeniumdiolates as well [49].

O^2-derivatization such as acetoxymethylation of IPA/NO increases HNO production over the parent diazeniumdiolate due to access to a unique decomposition pathway (Scheme 4) [50,51]. After deprotonation of the amine proton, 1–4 acyl migration occurs via a cyclic intermediate and expulsion of formaldehyde. Subsequent fragmentation by N-N bond cleavage produces an acylnitroso derivative that on hydrolysis produces HNO. Esterase-mediated decomposition also occurs as shown in Scheme 2. A β-galactosidase-sensitive derivative has also recently been described [52].

Currently, whether other primary amine diazeniumdiolates release NO or HNO on decomposition is not known. To establish primary amine diazeniumdiolates as an alternative to currently existing HNO donors and to verify NO/HNO production, herein, we report the synthesis and characterization of a series of structurally related primary alicyclic amine-based diazeniumdiolates.

2. Materials and methods

Unless otherwise noted, chemicals were purchased from Sigma-Aldrich and used without further purification. NO was purchased from Matheson or Air Liquide. Concentrations of ionic diazeniumdiolate stock solutions (>10 mM; prepared in 10 mM NaOH and stored at −20 °C) [22] were determined directly prior to use from the measured molar extinction coefficient at 250 nm. Concentrations of O^2-derivitized diazeniumdiolates were similarly ascertained, but in ethanol. Typically, the assay buffer consisted of the metal chelator diethylenetriaminepentaacetic acid (DTPA; 50 μM) in calcium- and magnesium-free Dulbecco's phosphate-buffered saline (PBS; pH 7.4). By sequestering contaminating metals, addition of DTPA quenched the oxidation of HNO to NO [53]. Stock solutions of glutathione (GSH; > 10 mM) were made in assay buffer and stored at 4 °C. All reactions were performed at 37 °C except those involving measurement with an NO-specific electrode (room temperature). Thin layer chromatography was carried out using Analtech silica gel GF (250 μm) glass-backed plates. Flash chromatography was performed using the indicated solvent system on silica gel 60 (230–450 mesh size; Alfa Aesar).

2.1. Instrumentation

UV-visible spectroscopy was performed with an Agilent Hewlett-Packard 8453 diode-array spectrophotometer equipped with an Agilent 89090A thermostat. BioTek Synergy 2 microplate readers were also utilized for absorbance and fluorescence measurements. Electrochemical detection was accomplished with a World Precision Instruments Apollo 4000 system equipped with NO, O_2 and H_2O_2 sensitive electrodes (Sarasota, FL). Solution pH was determined by use of a ThermoElectron Orion 420A+ pH meter. ^1H and ^{13}C NMR was carried out on a Bruker DRX-500 instrument. Low resolution mass spectra were recorded on a JEOL HX1 10A instrument. CHN analysis was performed at Midwest Microlab (Indianapolis, IN).

2.2. Synthesis of alicyclic amine diazeniumdiolates

Sodium 1-(N-cyclohexylamino)diazen-1-ium-1,2-diolate (CHA/ NO; **2**) was synthesized according to a previously published procedure [45]. For synthesis of other

diazeniumdiolates, in general, a solution of the appropriate alicyclic amine in diethyl ether was placed in a 250 mL Parr bottle. The solution was deaerated with argon, cooled in dry ice, charged with 40 psi of NO, and allowed to stir for 24–48 h. The resulting solid precipitate was collected by filtration and washed with diethyl ether. The ammonium salt was converted to the sodium salt by dissolution in methanol and addition of one equivalent of 25% methanolic sodium methoxide. After several minutes of stirring, the sodium salt was precipitated by addition of diethylether and was collected as a white solid upon vacuum filtration. Note that as hygroscopic sodium salts, ionic diazeniumdiolates are not amenable to characterization by high resolution mass spectrometry or elemental analysis. **Caution:** Preparations of primary amine diazeniumdiolates can be unstable in the solid state, sometimes decomposing suddenly without warning. Minimize the storage amount (typically <250 mg) and store in containers with large head space. Additionally, using exactly 1 eq of base is a crucial step to avoid generation of highly reactive species. See reference 46 for additional details.

2.2.1. Sodium 1-(N-cyclopentylamino)diazen-1-ium-1,2-diolate (CPA/NO; 1)—A solution of cyclopentylamine (15 mL, 0.15 mol) in 25 mL of diethylether gave 3.7 g (14% yield) of the desired sodium salt. ^{1}H NMR (DMSO): δ 1.48–1.56 [m, 4H], 1.59–1.66 [m, 4H], 3.86–3.88 [m, 1H], 6.00–6.01 [b, 1H]; ^{13}C NMR (DMSO): δ 24.67, 31.27, 58.19; UV (10 mM NaOH): λ_{max} 250 nm (ε = 8,200 $M^{-1}cm^{-1}$).

2.2.2. Sodium 1-(N-cycloheptylamino)diazen-1-ium-1,2-diolate (CHPA/NO; 3)—A solution of cycloheptylamine (30 mL, 0.22 mol) in 60 mL of diethylether gave 4.2 g (19% yield) of the desired sodium salt. ^{1}H NMR (DMSO): δ 1.23–1.52 [m, 8H], 1.58–1.67 [m, 4H], 3.40–3.43 [m, 1H], 5.89–5.90 [b, 1H]; ^{13}C NMR (DMSO): δ 23.79, 28.00,31.63, 56.00; UV (10 mM NaOH): λ_{max} 250 nm (ε = 8,700 M^{-1} cm^{-1}).

2.2.3. Sodium 1-(N-cyclooctylamino)diazen-1-ium-1,2-diolate (COA/NO;4)—A solution of cyclooctylamine (15 mL, 0.11 mol) in 25 mL of diethylether gave 2.9 g (17% yield) of the desired sodium salt. ^{1}H NMR (DMSO): δ 1.46–1.65 [14H, m], 3.44–3.46 [1H, m], 5.86–5.87 [b, 1H]; ^{13}C NMR (DMSO): δ 23.57, 25.32, 26.82, 29.43, 55.14; UV (10 mM NaOH): λ_{max} 250 nm (ε = 8,300 M^{-1} cm^{-1}).

2.2.4. Attempted synthesis of sodium 1-(N-cyclobutylamino)diazen-1-ium-1,2-diolate and sodium 1-(N-cyclopropylamino) diazen-1-ium-1,2-diolate—Similar methodology as above was used in order to synthesize the corresponding diazeniumdiolate salts. However, the final products could not be isolated due to instability.

2.3. General synthesis of acetoxymethylated diazeniumdiolates

A solution of bromomethyl acetate in THF was added to a slurry of the relevant sodium salt in dry DMSO at room temperature. The reaction mixture was stirred overnight, whereupon 15 mL of water was added, and stirring was continued for another 10 min. The residue was extracted with dichloromethane, washed with $NaHCO_3$ (5%), dried over anhydrous sodium sulfate, filtered, and concentrated under vacuum. Column chromatography was performed using hexane: acetone (4:1) to give the desired product.

2.3.1. O^2-(Acetoxymethyl) 1-(cyclopentylamino)diazen-1-ium-1,2-diolate (AcOM-CPA/NO;5)—

A solution of bromomethyl acetate (183 mg, 1.19 mmol) in 3 mL of THF was added to a slurry of **1** (200 mg, 1.19 mmol in 10 mL of DMSO) according to the above general procedure to give the desired product as a colorless oil (187 mg, 72%). ^{1}H NMR ($CDCl_3$) δ 1.47–1.53 (m, 2H), 1.61–1.72 (m, 4H), 1.94–2.00 (m, 2H), 2.12 (s, 3H), 4.15–4.21 (m, 1H), 5.75 (s, 2H), 6.22 (d,J = 7.5 Hz, 1H); ^{13}C NMR ($CDCl_3$) δ 20.83, 23.91, 31.13, 58.71, 87.08, 169.31; UV (ethanol) λ_{max} (ε) 239 nm (10,500 M^{-1} cm^{-1}); MS (LCQ, ESI ionization method): 240.1 (MNa^+peak). Anal. Calcd for $C_8H_{15}N_3O_4$: C, 44.23; H, 6.96; N, 19.34. Found: C, 44.44; H, 6.95; N, 19.40.

2.4. Decomposition profile

The rate constants of decomposition for ionic diazeniumdiolates at 37 °C were measured spectrophotometrically by monitoring the decrease in absorbance near 250 nm in assay buffer of the desired pH. The rate of decomposition of **5** in the presence or absence of the esterase in 2% guinea pig serum (by volume) was similarly determined at 238 nm. Kinetic analysis was performed by fitting the data to an exponential decay ($A = \Delta A e^{-kt} + A_\infty$).

2.5. Detection of nitrogen oxides

Trapping of HNO or NO by metmyoglobin (metMb) [17,54–56] or 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-2DA) [51,57] assays, electrochemical detection [17,58], and quantitation of HNO [59] were performed as previously described. Details are presented in the Supporting Information. Figures are representative data sets, each from $n \geq 3$ individual experiments.

2.6. Cell culture

Estrogen receptor α-negative (ER-; MDA-MB-231) or positive (ER+; MCF-7) human breast cancer cells (American Type Culture Collection, Manassas, VA) were grown as monolayers in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS, Hyclone), penicillin (50 units/mL), and streptomycin (50 units/mL; Life Technologies, Inc., Grand Island, NY) at 37 °C in 5% CO_2 and 80% relative humidity. Single cell suspensions were obtained by trypsinization (0.05% trypsin/EDTA, Life Technologies), and cells were counted using a Beckman cell counter or bright line hemocytometer (Sigma-Aldrich).

2.7. Clonogenic cell viability assay

Cells were plated at 400,000 cells per 60-mm dish and grown for 48 h. Cells were treated in growth media containing different concentrations (0.5–20 mM) of diazeniumdiolates for 24 h. After treatment, the cells were washed twice with PBS, trypsinized, counted and plated at a density of 100, 1,000 or 10,000 per 60-mm plate. For each condition, cells were plated in triplicate, and each experiment was repeated at least twice. After 10–12 d, the colonies were stained with crystal violet (0.5% w/v) and counted using a Stemi microscope.

2.8. MTT viability assay

Cytotoxicity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Cells were plated at 8,000–10,000 cells per well in a 96-well plate and

grown overnight. Cells were treated with different concentrations (10–100 μM) of **5** or control (ethanol, < 0.1% by volume) for 48 h. Thereafter, 10 μL of 5 mg/ mL MTT was added to each well, and the plate was incubated for 1 h at 37 °C. After removal of the media, 100 μL of DMSO was added to each well, and the absorbance was recorded at 550 nm. Growth inhibition is reported as the percentage of the corresponding control. Figures are representative data sets, each from $n \geq 3$ individual experiments. For analysis of combined effects of **5** with tamoxifen, cells were treated with 10 μM tamoxifen, 75 μM of **5** or a combination for 48 h.

3. Results and discussion

The utility of NO-donating diazeniumdiolates is extensive due to ease of use as well as controllable release time. Here, we strove to expand the available number of HNO-releasing diazeniumdiolates. Since CHA/NO has been previously synthesized [45], we focused on a small series of alicyclic primary amine analogues. Alicyclic amine diazeniumdiolates were synthesized by exposing solutions of amine in diethyl ether to high pressures of NO (Scheme 5). Evidence of diazeniumdiolate formation was verified by the presence of a 250 nm peak, characteristic of the [N(O)NO]-moiety [22]. The molar extinction coefficients for these compounds (7,900–8,700 M^{-1} cm^{-1}) were in good agreement with those of other diazeniumdiolates (6,000–10,000 M^{-1} cm^{-1}) [22,34,37,40,46]. Cyclobutylamine- and cyclopropylamine-based diazeniumdiolates could not be isolated as the sodium salts due to low stability.

3.1. Decomposition rate

Diazeniumdiolates are typically stable as solids and in highly basic solution and can be stored at −20 °C for long periods [22]. Decomposition, which follows first order kinetics, is accelerated with decreasing pH. The newly prepared alicyclic amine diazeniumdiolates decayed as expected (Fig. 1, with **1** as the representative example). The rate constants of decomposition and half-lives at pH 7.4 and 37 °C are summarized in Table 1. Similar decay rates indicate a lack of a significant ring effect on the stability of these diazeniumdiolates. The deceleration of decomposition with increasing pH (Fig. 2; 100-fold variance in rate constant) is also consistent with other known ionic diazeniumdiolates [46,60].

3.2. NO/HNO release profile

Numerous methods exist for detecting NO. Conversely, direct detection of HNO is complicated by irreversible dimerization [14,15], and assessment of HNO release is often indirect and qualitative. Our group has developed a protocol for HNO detection from donor compounds [47]. HNO production can be rapidly assessed with an NO-specific electrode in the presence of ferricyanide, which oxidizes HNO to NO [58]. The differences in the signal intensities for **1–4** in assay buffer alone (Fig. 3, blue bars) parallel the trend in decay rates (Table 1). The current intensity was elevated (2–8 fold) during decomposition of the ionic diazeniumdiolates in the presence of ferricyanide (Fig. 3, red bars). This elevation indicates significant production of HNO from these new primary amine-based diazeniumdiolates at physiological pH. The similar signal maxima also suggest similar product distribution for **1–4**.

As previously demonstrated with IPA/NO (5 μM) [47], the alicyclic amine diazeniumdiolates also exhibit a pH-dependent nitrogen oxide release profile (Fig. 4, with **1** as the representative example). At physiological pH and above, these alicyclic amine diazeniumdiolates primarily release HNO, as indicated by the increase of the signal intensity in the presence versus absence of ferricyanide. At lower pH, the independence of the signal intensity to ferricyanide denotes that NO is the major decomposition product. Thus, the release of HNO near physiological pH is established to be a general property of primary amine-based diazeniumdiolates. As these results are purely qualitative, the extent of HNO/NO production requires analysis by other techniques.

3.3. Quantification of HNO release

We have recently developed a high throughput assay for quantitatively detecting HNO by trapping with GSH and labeling with a specific fluorogenic reagent [59]. At pH 7.4, the HNO yield was approximately 50% (Table 2). The small deviations between **1–4** are consistent with the trend in signal maximum as shown in Fig. 3 (red bars), and again suggesting little influence of ring size on the HNO donating capabilities. A decrease in pH to 7.0 reduced the yield of HNO by half. These HNO yields for **1–4** can be compared to 59 ± 2 and 36 ± 1% at pH 7.4 and 7.0, respectively, for IPA/NO [59]. Given the high sensitivity of HNO yield near neutral pH, use of primary amine diazeniumdiolates as HNO donors requires careful control of pH.

3.4. Cell viability

HNO donors have the potential to be applied in treatment of heart failure,[7] as preconditioning agents against ischemia reperfusion injury [9] and as alcohol deterrents [11]. To be able to use primary amine-based diazeniumdiolates as pharmacological precursors of HNO, low cytotoxicity is a requirement. Typically, simple ionic diazeniumdiolates become cytotoxic at millimolar concentrations [13,58]. Similar cytotoxicity values were observed for **1–4** as evaluated by clonogenic assay in MCF-7 breast cancer cells (Fig. 5). The decomposed diazeniumdiolate products required higher initial concentrations of **1–4** (≥20 mM; data not shown), supporting a role of the released nitrogen oxides on cell viability.

HNO has also been shown to inhibit breast cancer [13] and neuroblastoma [61] proliferation in mouse xenografts as well as in culture, through increased apoptosis. Due in part to short half-life and spontaneous decomposition outside cells, ionic diazeniumdiolates may have limited potential from a chemotherapeutic point of view. The sensitivity of cells to released HNO will depend on the extent of HNO diffusion and consumption, for example by dimerization. Conversion to neutral species that can be activated (e.g., enzymatic, hydrolytic or photolytic) has been used as a strategy to overcome the limitations of the ionic precursors, including improvement of cellular uptake and delivery of HNO [43,50].

3.5. Synthesis of acetoxymethyl-protected diazeniumdiolates

The acetoxymethyl ester derivative of **1** was prepared (Scheme 6) by reacting with bromomethylacetate under anhydrous conditions. Acetoxymethyl derivatives of the other alicyclic diazeniumdiolates were also prepared similarly (data not shown), but AcOM-

CPA/NO (**5**) in general was chosen for further detailed characterization due to the fact that the corresponding anion (**1**) had the lowest cytotoxicity among the four potential precursors (Fig. 5).

3.6. Analysis of the decomposition of 5

In the presence of serum esterases, the half-life of decomposition of **5** at pH 7.4 and 37 °C (5.8 min) was similar to that of the parent diazeniumdiolate (**1**; 3.6 min). The initial peak at 238 nm shifted to 250 nm (Fig. 6), indicating cleavage of the ester bond and production of the free diazeniumdiolate (Scheme 2).

To further verify the esterase-mediated decomposition profile of **5**, electrochemical assessment was performed in the presence of 2% guinea pig serum. The decomposition profile for **5** in the presence and absence of 1 mM ferricyanide (Fig. 7) at varied pH was also similar to that of the parent diazeniumdiolates (Figs 3 and Fig 4). This further reinforces the production of free **1** via Scheme 2.

In the absence of serum, the half-life of **5** increases to 21 min at pH 7.4 and 37 °C, and decomposition accelerates with increasing pH (0.3,1.3 and 5.0×10^{-3} s^{-1} at pH 7, 8 and 9, respectively). The lack of an apparent peak at 250 nm (Fig. 8) indicates that free diazeniumdiolate is not produced during decomposition. All of these data are consistent with the previously determined mechanism for acetoxymethyl protected IPA/NO (Scheme 4) [50].

Acetoxymethyl protected IPA/NO has been previously suggested to have enhanced HNO production over the parent compound due to a unique decomposition mechanism (Scheme 4) [50]. Here, the yield of HNO in the absence of serum was assessed by trapping with GSH [59] or metMb [17,55]. Under physiological conditions, HNO production from **5** was significantly enhanced compared to **1** (Table 2; 96 vs. 54%).

MetMb (50 μM) undergoes reductive nitrosylation (Eq. 1) with all the prepared alicyclic diazeniumdiolates (Fig. S1A) [10].

$$\mathrm{metMb} + \mathrm{HNO} \rightarrow \mathrm{MbNO} + \mathrm{H}^{+} \left(8 \times 10^{5} \mathrm{M}^{-1} \mathrm{s}^{-1}\right) \quad (1)$$

As with AcOM-IPA/NO [50], a higher yield of MbNO (Fig. S1B) was observed for **5** (100 μM), suggesting increased production of HNO. Trapping of HNO was quenched in the presence of 1 mM GSH (a known scavenger of HNO) [10,62], further confirming HNO production (Fig. S1A, B). Together, these data indicate that Scheme 4 is generally applicable for acetoxymethyl-protected primary amine diazeniumdiolates. Such species therefore provide a new class of donor for studies that require longer exposure to HNO.

3.7. Intracellular NO and HNO release

To determine whether hydrolysis of **5** will occur intracellularly, DAF-FM-2DA was used as a reporter molecule in MDA-MB-231 breast cancer cells. DAF reacts with the autoxidation products of both NO [63] and HNO [57], with higher fluorescence signal in the latter case.

The diacetate DAF-FM-2DA is readily taken up by cells, where hydrolysis of the ester bonds by intracellular esterase produces the non-permeable DAF-FM. On treatment of DAF-FM-2DA loaded cells with **1–5**, an increase in fluorescence intensity was observed in a time-dependent manner compared to control (Fig. 9). A similar signal was generated for the four ionic compounds while that for **5** was ~3 fold higher, signifying enhanced HNO release within the cells.

3.8. Cell viability

Under aerobic conditions HNO can be cytotoxic [58]. This is in part due to consumption of GSH but also due to formation of reactive nitrogen oxide species that mediate DNA double strand breaks [64,65]. HNO is also known to irreversibly inhibit enzymes containing critical thiols [66–68]. Inhibition of one such enzyme, glyceraldehyde phosphate dehydrogenase (GAPDH) [67], a key player in glycolysis, has chemotherapeutic potential, given that cancer cells have a higher dependence on glycolysis than normal cells (i.e., the Warburg effect).

The cytotoxicity of **5** (Fig. 10) was substantially increased (IC_{50} of ca. 100 μM) compared to Angeli's salt, which has been previously shown to inhibit the proliferation of human breast cancer cells (highly aggressive MDA-MB-231 and less aggressive MCF-7 cells) in the millimolar range [13]. The enhanced effect of **5** can be explained in part by increased uptake thereby leading to higher intracellular concentration of HNO.

3.9. Effect of 5 on the cytotoxicity of tamoxifen

NO releasing donor compounds have been previously reported to increase the cytotoxicity of cancer drugs such as cisplatin [69], melphalan [70] and doxorubicin [71] toward cancer cells. Such analysis has not been reported for HNO donors. Here, we evaluated the potential of using **5** in combination with tamoxifen for targeting ER-breast cancer.

Tamoxifen is used clinically worldwide in treatment of early and advanced stages of ER+ breast cancer [72]. Tamoxifen is also prescribed as a chemopreventive agent for women who are at high risk of developing breast cancer. Tamoxifen exerts its anticancer properties by competing with estrogen for binding to the estrogen receptor, which is crucial for breast cancer cell proliferation [73]. Tamoxifen has also been shown to induce apoptosis in ER- cancer cells in a receptor-independent manner, albeit at quite high concentrations [74]. Successful application of tamoxifen to a broader population of breast cancer patients requires development of effective therapeutic approaches in targeting more aggressive hormone-insensitive breast cancer patients.

MB-231 cells were used for evaluating the combinatory potential of **5** with tamoxifen. Figure 11 shows the surviving fractions of MB-231 cells treated with tamoxifen (10 μM) or **5** (75 μM) with 76 ± 13 and 75 ± 6% viability, respectively. Co-administration of tamoxifen and **5** at these concentrations reduced cell viability to 47 ± 3%. It is expected that tamoxifen and HNO will have different molecular targets, thus providing multiple pathways to impact cell survival in combination. Moreover, long term, combined usage may lower occurrence of resistance compared to individual treatment. Further investigation into such antitumor

effects may lead to a better understanding of the action of HNO in combination with other drugs and the intriguing possibility of using HNO in cancer treatment.

4. Conclusions

In conclusion, we have demonstrated that diazeniumdiolates provide a general class of HNO donor. Secondary amine-based diazeniumdiolates are commercially available, and our work suggests facile synthesis and potential commercialization of primary amine-based diazeniumdiolates. These diazeniumdiolates were shown to release NO/HNO in a manner similar to that reported for IPA/NO, thus establishing the decomposition mechanism to be common to primary amine-based diazeniumdiolates. Derivatization can dramatically change the production and release time of HNO, thus facilitating exploration of chronic HNO exposure. These features also hold promise in potential application for selectively targeting cancer cells. In the future, additional compounds will be synthesized to generate a library of HNO donors with a wide range of decomposition half-lives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

This work was supported by the National Institutes of Health (R01-GM076247 to KMM) and in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

Abbreviations

AcOM-CPA/NO	O^2-(acetoxymethyl)-1-(cyclopentylamino) diazen-1-ium-1,2-diolate
Angeli's salt	sodium trioxodinitrate
CHA/NO	sodium 1-(N-cyclohexylamino)diazen-1-ium-1,2-diolate
CHPA/NO	sodium 1-(N-cyclohep-tylamino)diazen-1-ium-1,2-diolate
COA/NO	sodium 1-(N-cyclooctylamino) diazen-1-ium-1,2-diolate
CPA/NO	sodium 1-(N-cyclopentylamino)diazen-1-ium-1,2-diolate
DAF-FM-2DA	4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate
DTPA	diethylenetriaminepentaacetic acid
ER(−)	estrogen receptor α-negative
ER(+)	estrogen receptor α-positive
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GSH	glutathione
HNO	nitroxyl

IPA/NO	sodium 1-(N-isopropylamino)diazen-1-ium-1,2-diolate
metMb	ferric myoglobin
MbNO	nitrosyl myoglobin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO	nitric oxide
PBS	phosphate-buffered saline
Piloty's acid	N-hydroxylbenzenesulfonamide

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.niox.2014.08.013.

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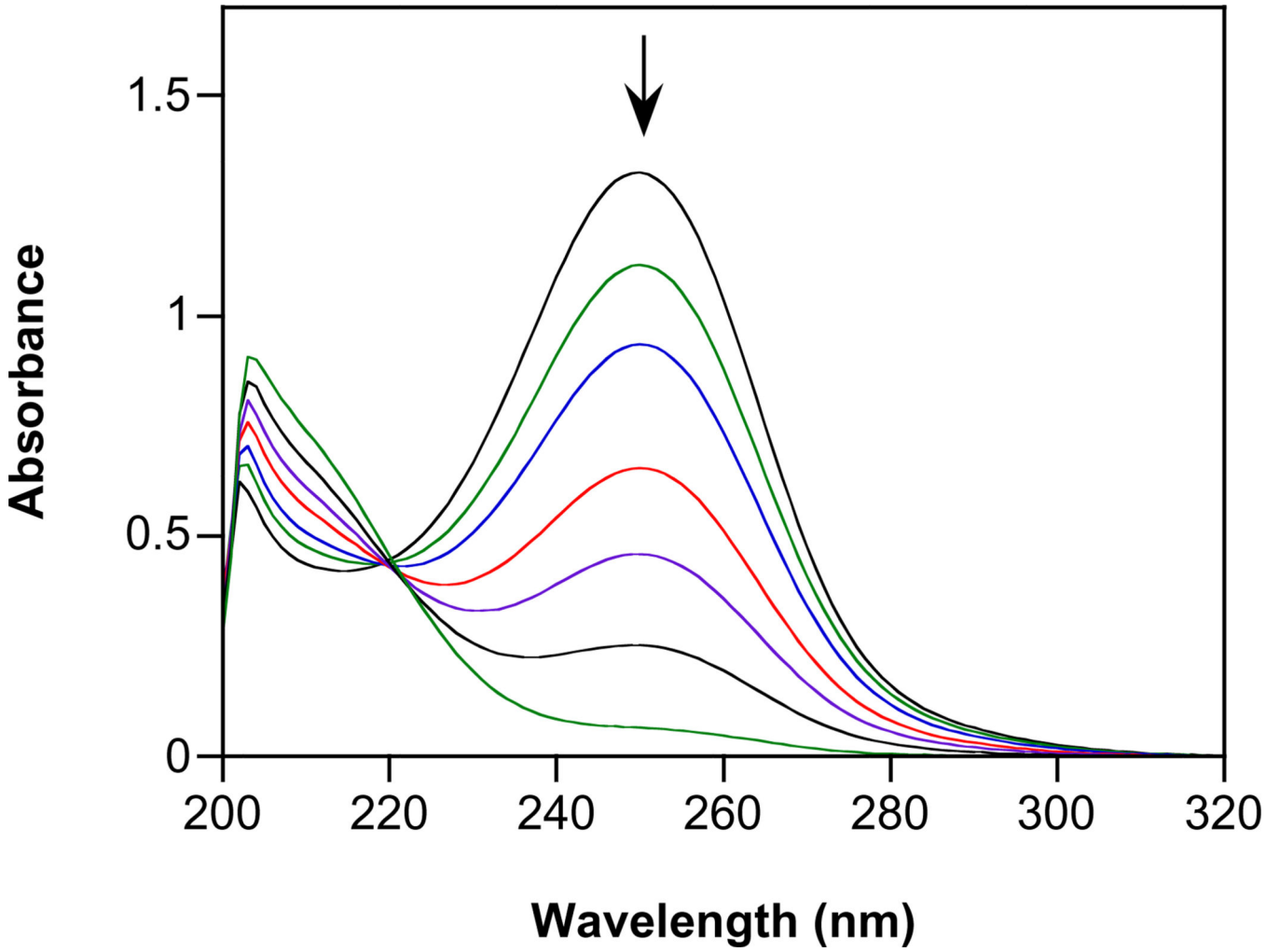


Fig. 1.

Spontaneous decomposition of **1** in assay buffer at pH 7.4 and at 37 °C. Spectra are shown at 0, 1, 2, 4, 6, 10 and 18 min.

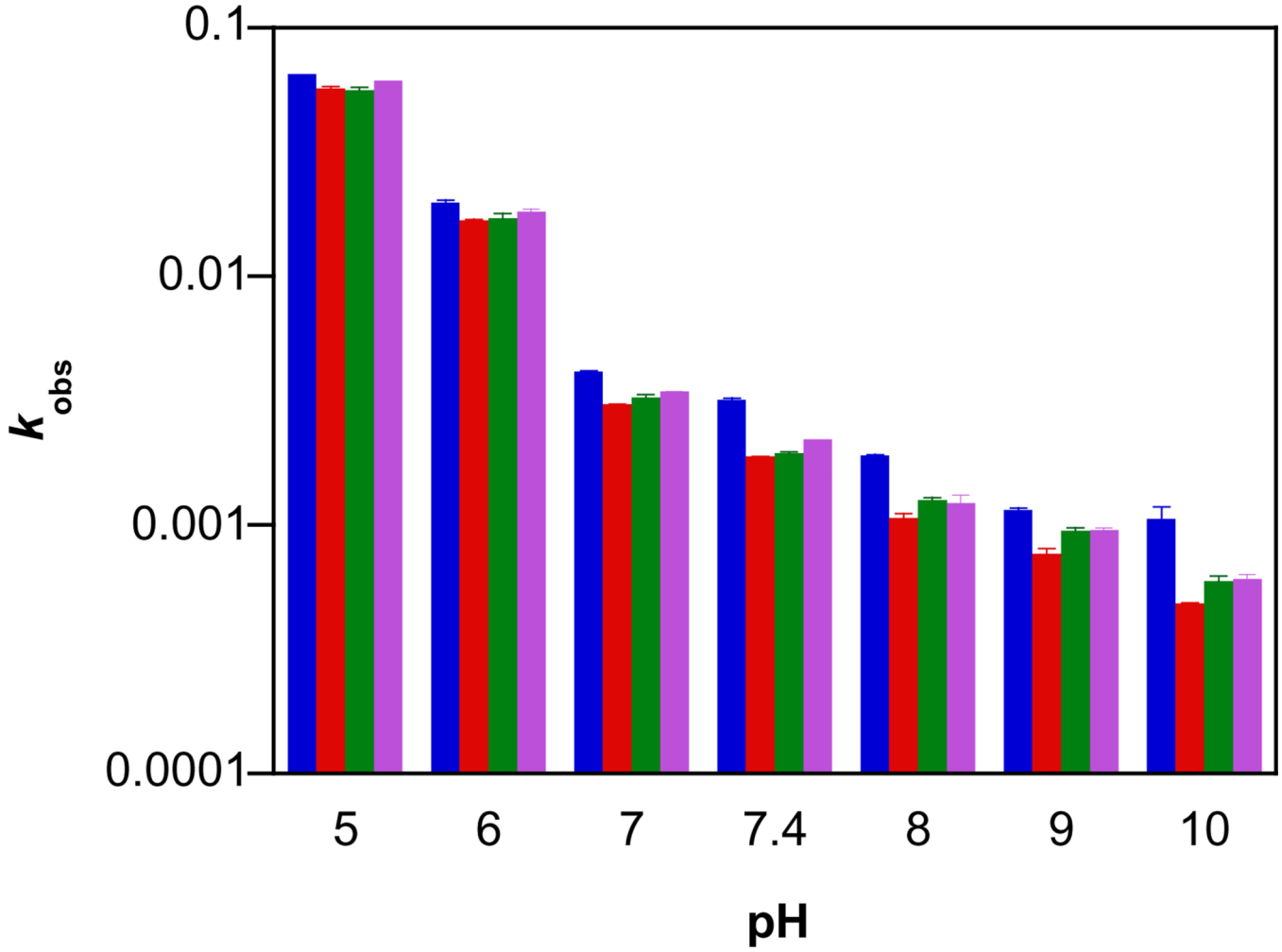


Fig. 2.

The pH-dependence of the first-order decomposition rate constants of 100 μM **1** (blue), **2** (red), **3** (green) or **4** (purple) at 37 °C in assay buffer measured at 250 nm (mean ± SD, $n \geq 3$).

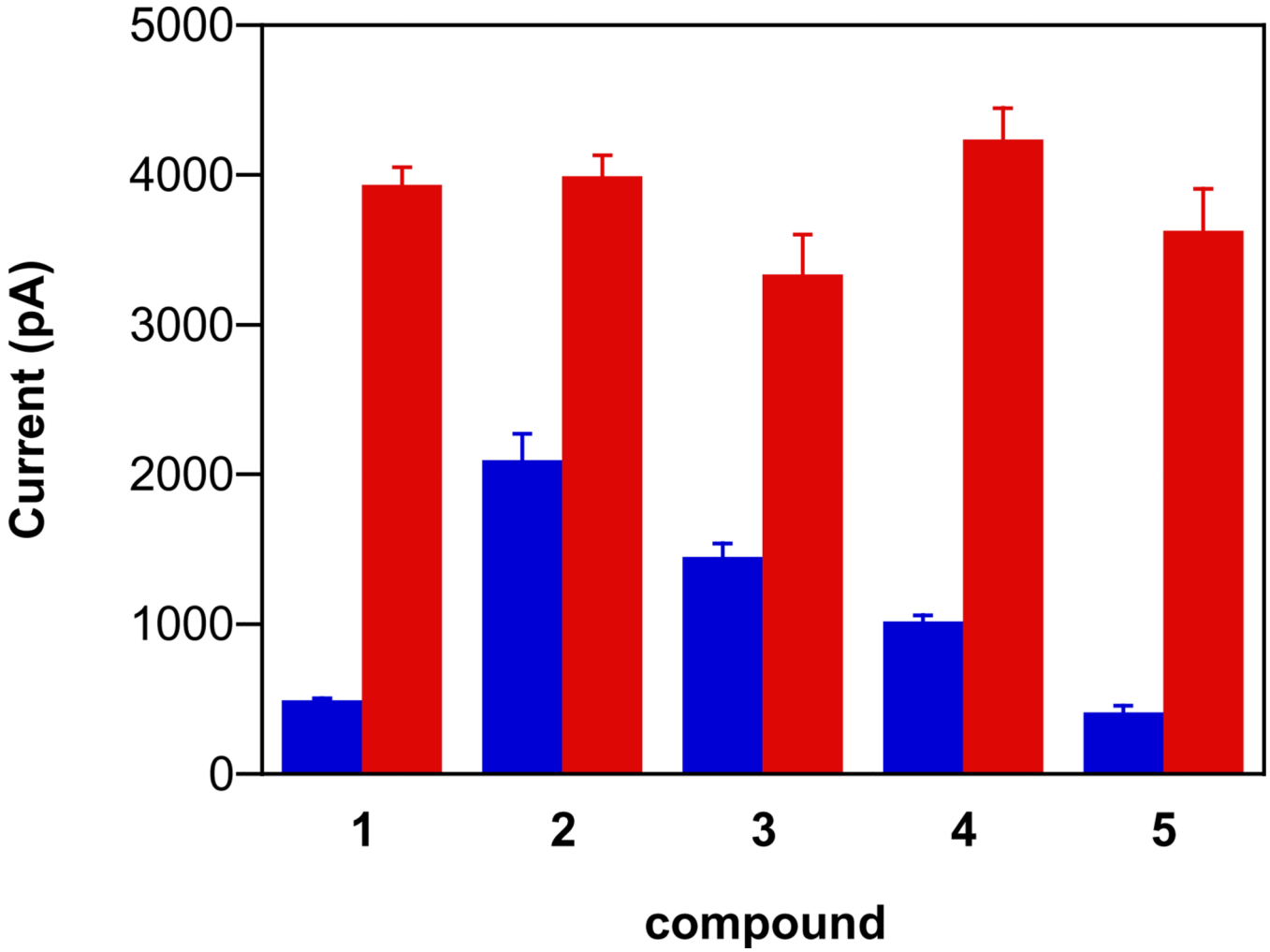


Fig. 3.

The maximum current intensity from an NO-specific electrode during decomposition of 50 μM **1–5** at pH 7.4 (blue bars) and with 1 mM ferricyanide (red bars) at room temperature (mean ± SD, $n \geq 3$). For **5**, the assay buffer contained 2% guinea pig serum.

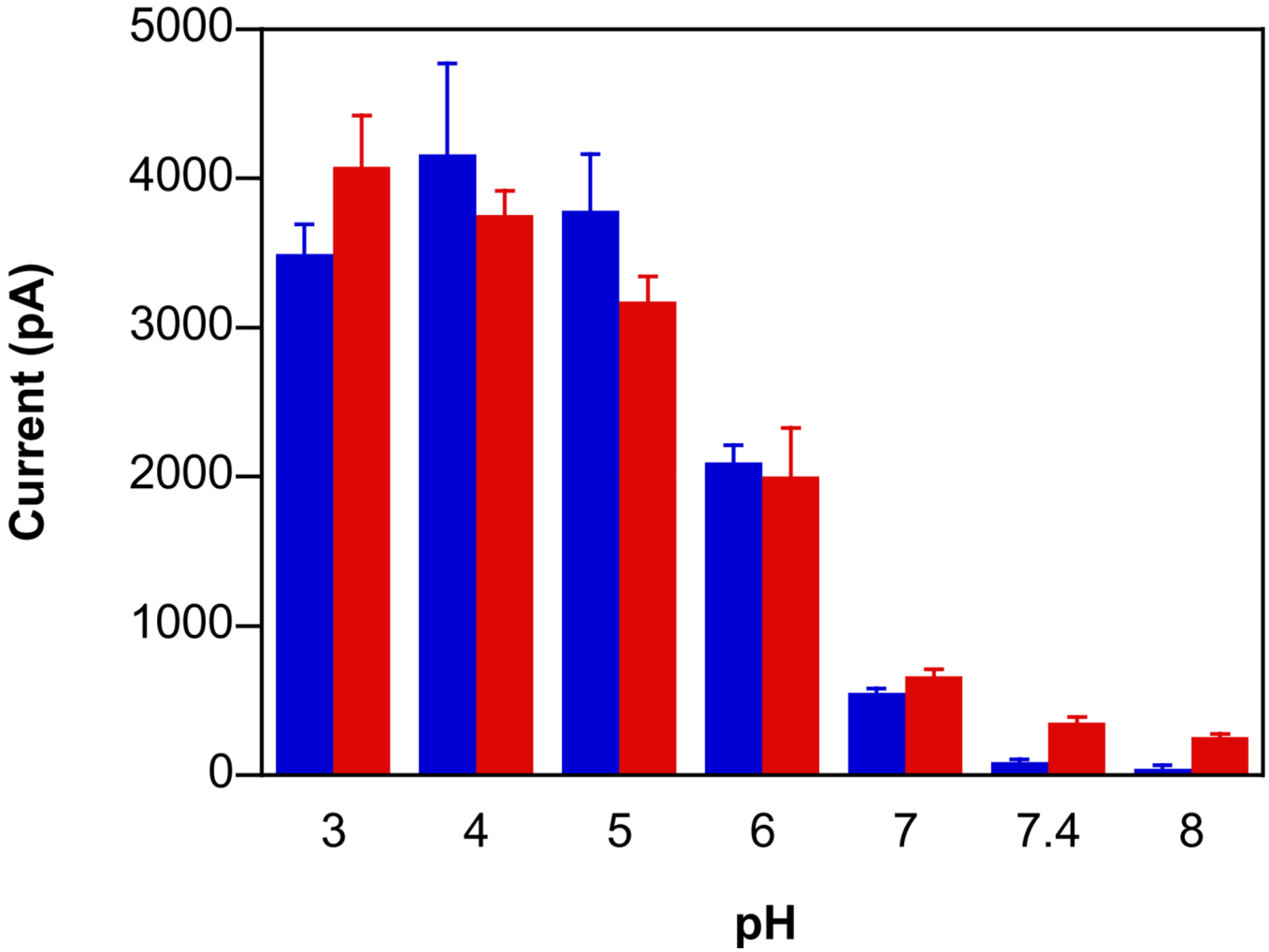


Fig. 4.

The pH-dependence of the maximum current intensity from an NO-specific electrode during decomposition of 5 μM **1** in assay buffer of pH 3–8 (blue bars) and with 1 mM ferricyanide (red bars) at room temperature (mean ± SD, $n \geq 3$).

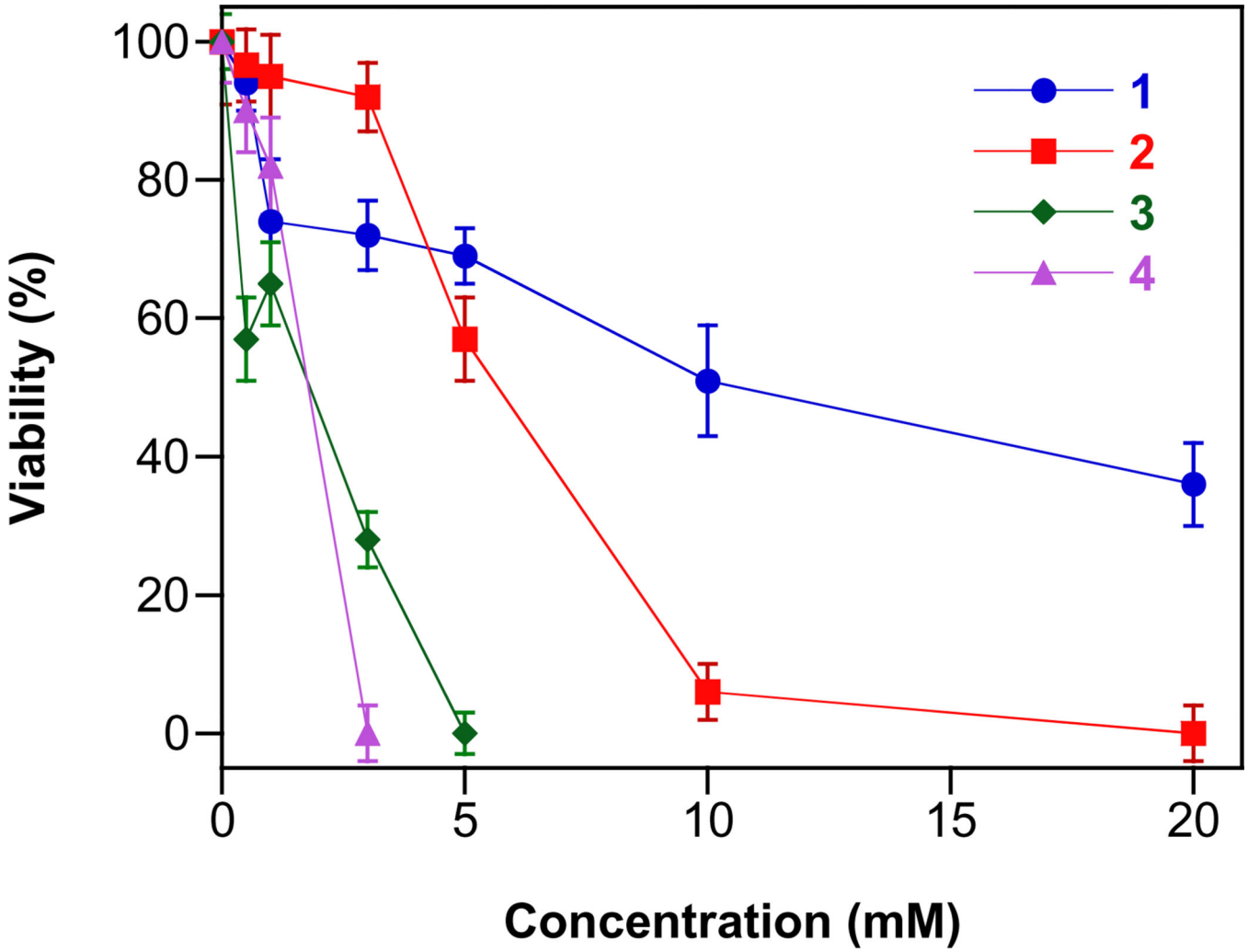


Fig. 5.
The effects of **1**–**4** (0.5–20 mM) on the viability of MCF-7 cells (mean ± SD, $n \geq 3$).

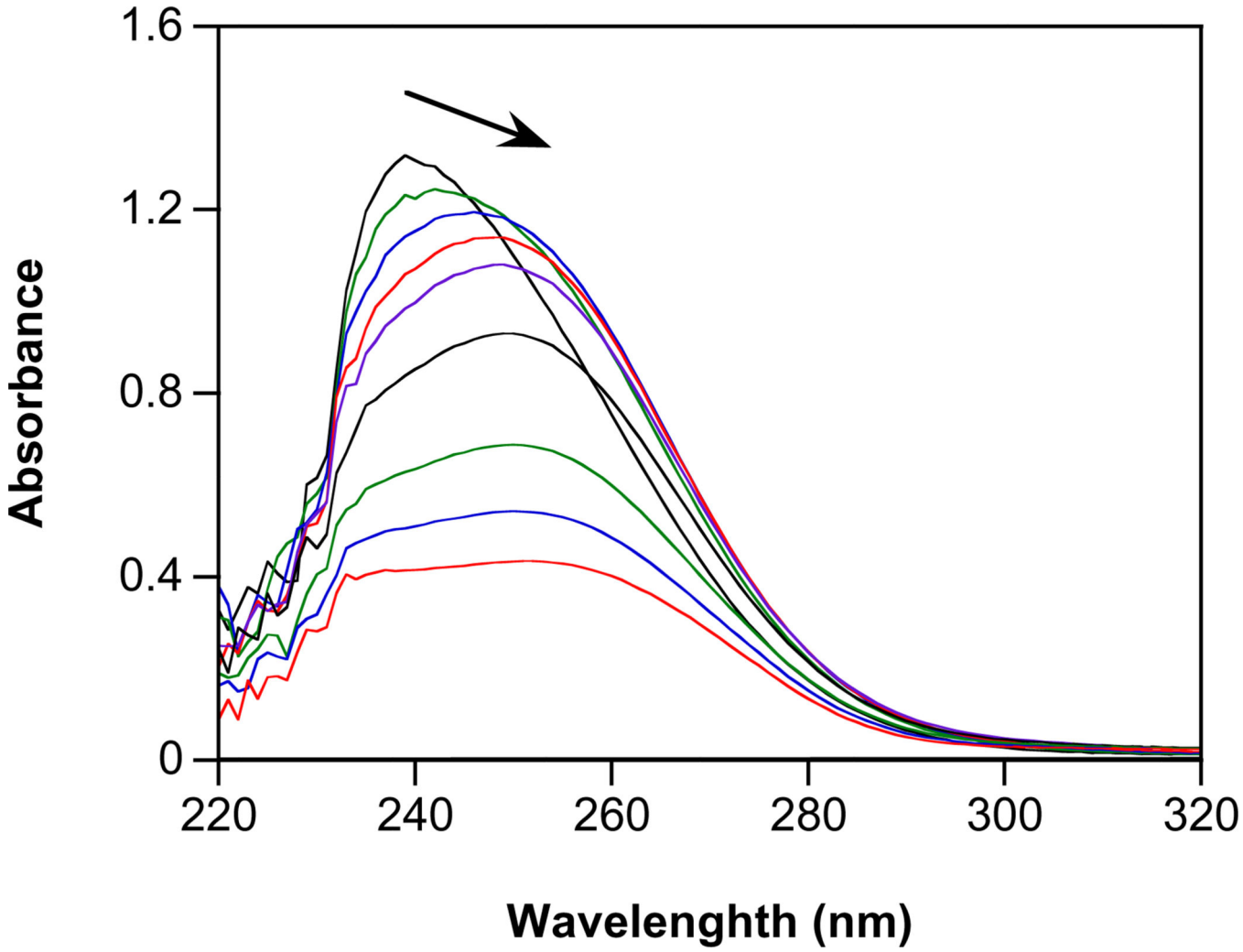


Fig. 6.

Hydrolysis of **5** in assay buffer containing 2% guinea pig serum at pH 7.4 and 37 °C. Spectra are shown at 0, 1, 2, 3, 4, 6, 10, 14 and 20 min. The noise at low wavelength is due the presence of serum proteins.

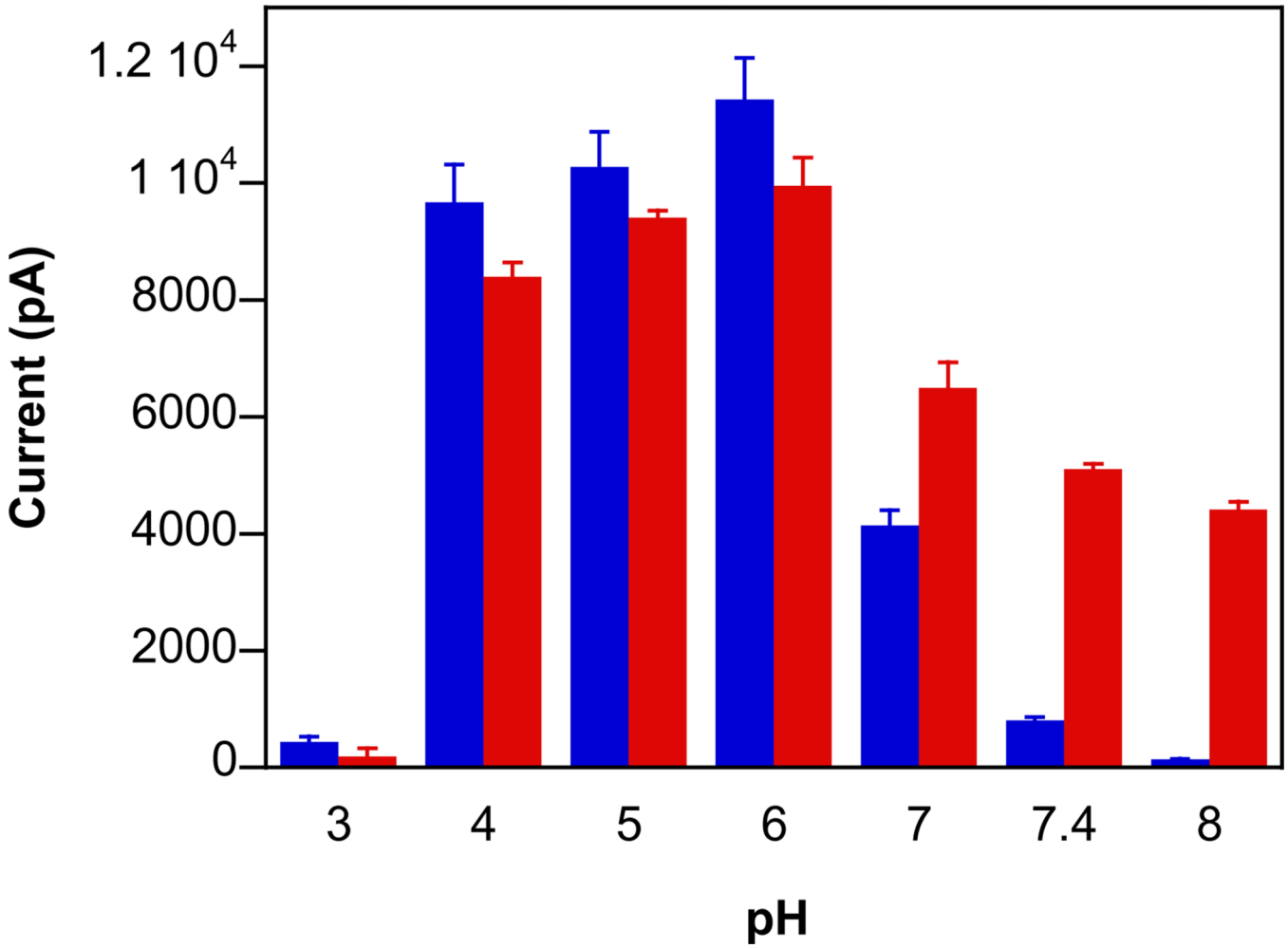


Fig. 7.

The pH-dependent maximum current intensity from an NO-specific electrode during decomposition of **5** (100 μM) in assay buffer containing 2% guinea pig serum of pH 3–10 (blue bars) and with 1 mM ferricyanide (red bar) at room temperature (mean ± SD, $n \geq 3$). The diminished signal at low pH is likely due to esterase deactivation. In order to observe signal at pH 3, all data were collected with a higher concentration than was used in Fig. 4.

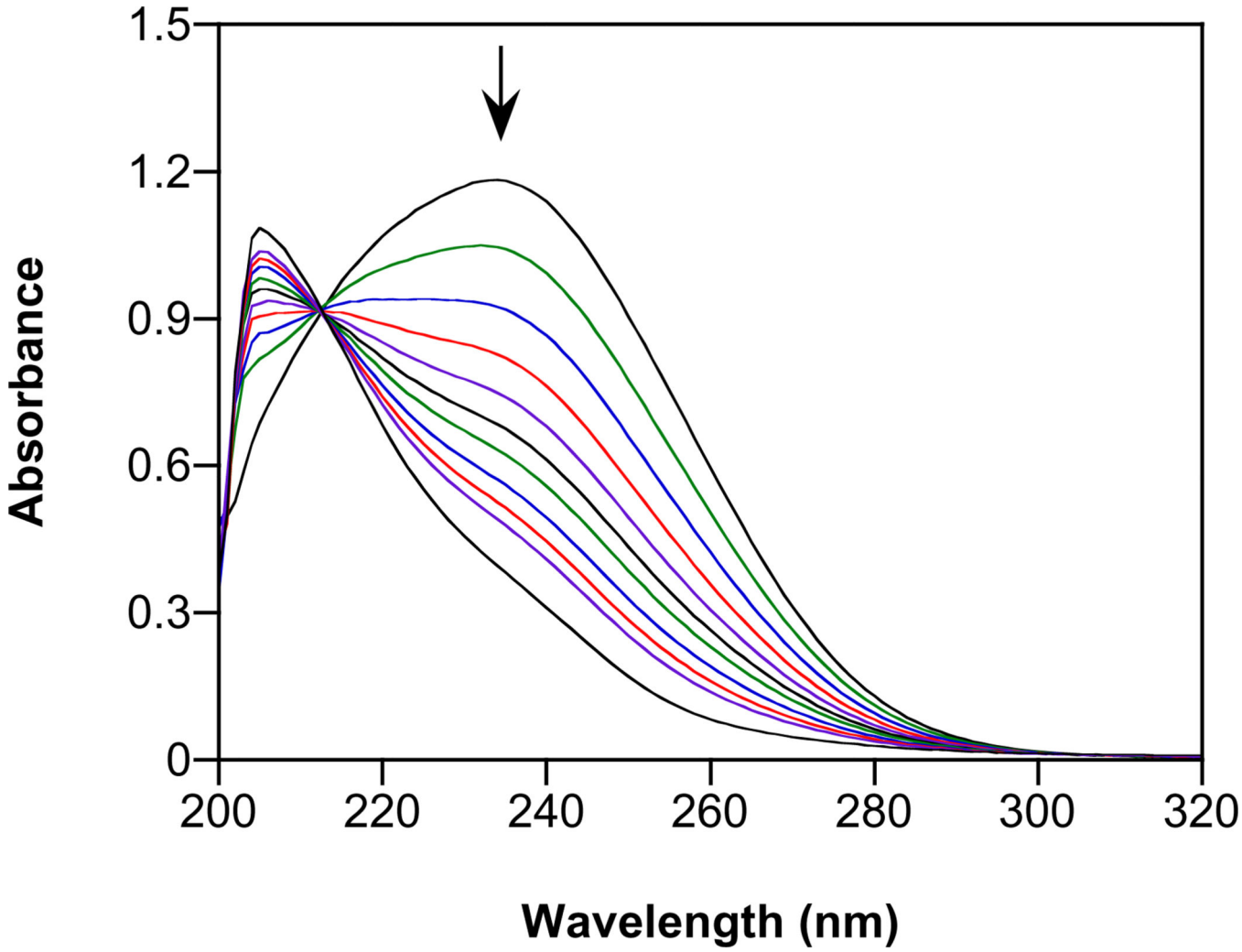


Fig. 8.

Hydrolysis of **5** in assay buffer at pH 7.4 and 37 °C ($n \geq 3$). Spectra are shown at 0, 7, 13, 20, 27, 33, 40, 50, 60, 70 and 100 min.

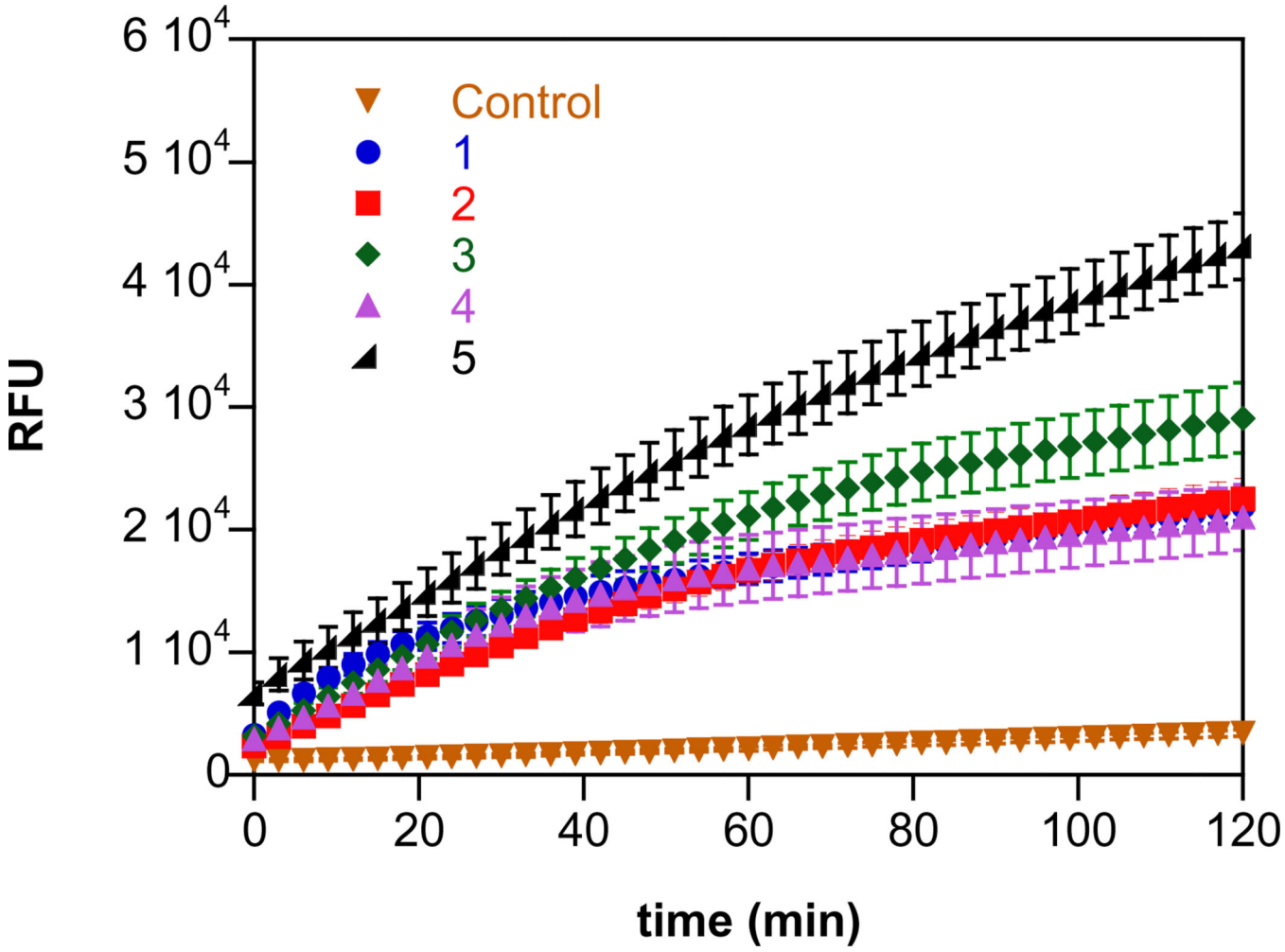


Fig. 9.

HNO/NO release measured in MB-231 cells. The cells were exposed to 100 μL of 10 μM DAF-FM-2DA (stock diluted in PBS) for 75 min at 37 °C and washed three times with PBS to remove excess dye. Upon addition of 10 μM **5** in ethanol (<0.1%; control) or 10 μM **1–4** in 10 mM NaOH, the increase in fluorescence intensity (RFU) at 535 nm was measured as a function of time at 37 °C following excitation at 485 nm. The data are expressed as mean ± SD (*n* = 2, six replicates per plate).

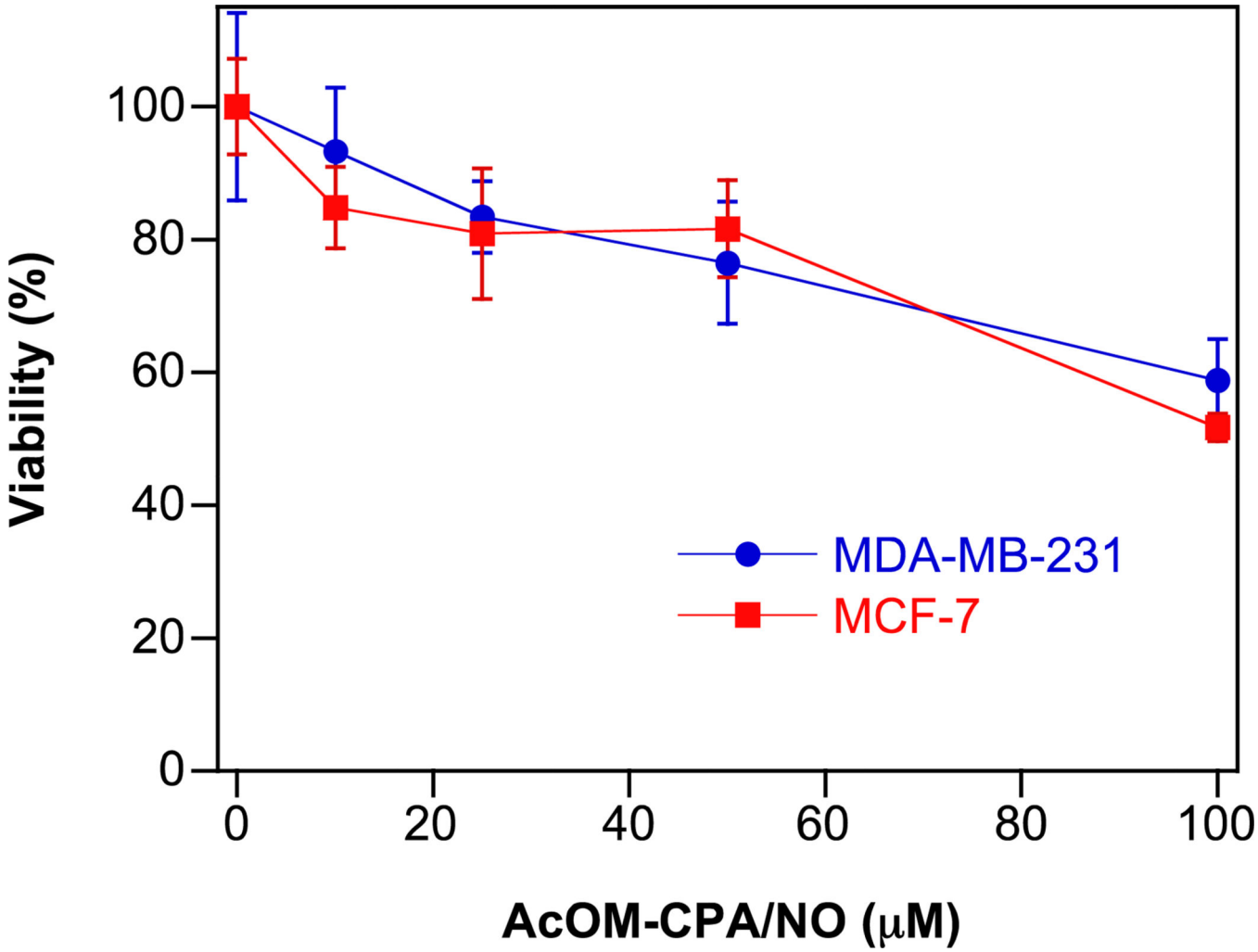


Fig. 10.

The effect of **5** (10–100 μM) on the viability of MCF-7 or MDA-MB-231 cells (mean ± SD, $n \geq 3$).

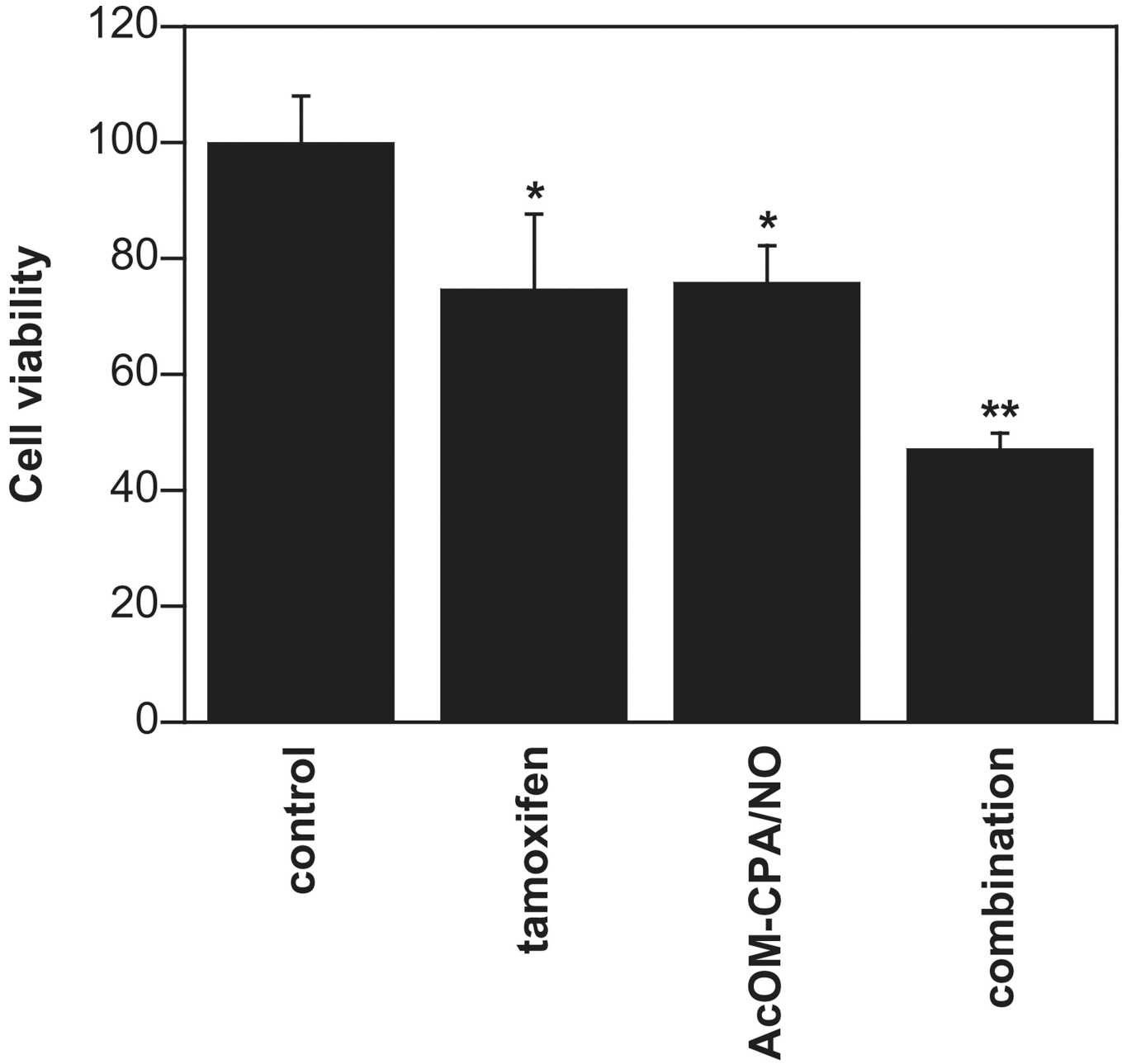
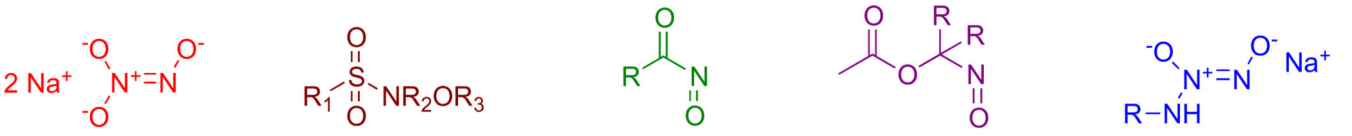


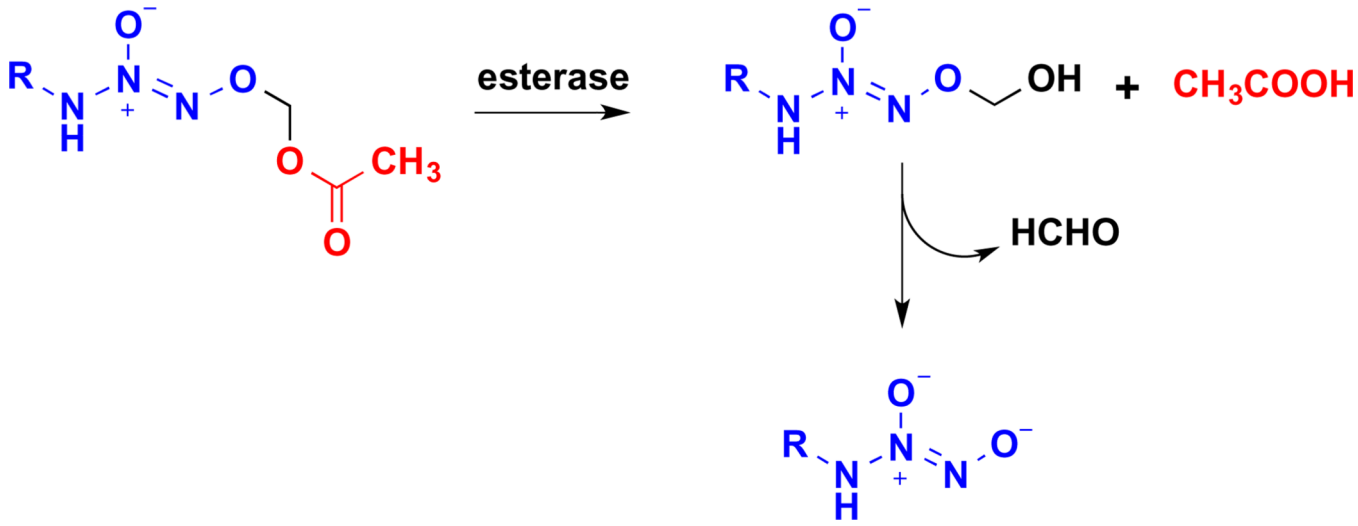
Fig. 11.

The effects of tamoxifen (10 μM), **5** (75 μM) or a combination on the viability of MDA-MB-231 cells (mean ± SD, $n \geq 3$; *, $p < 0.05$ vs. control; **, $p < 0.01$ vs. control).

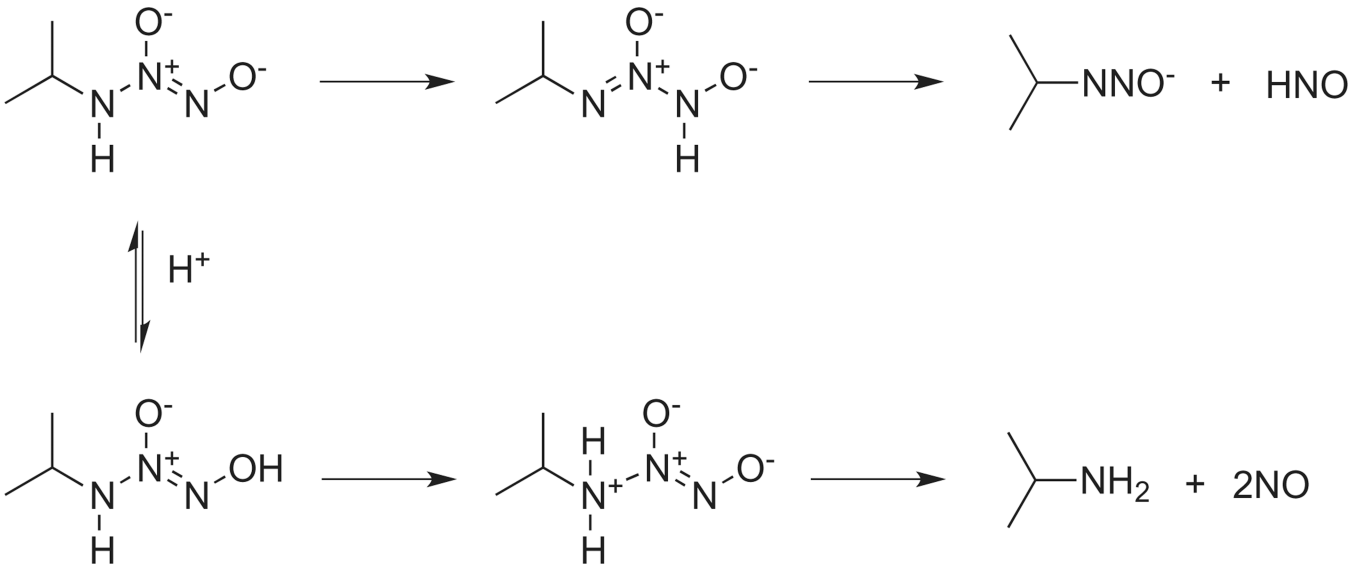


Angeli's salt | *N-hydroxysulfonamide derivatives* | *acyl nitroso compounds* | *acyloxy nitroso compounds* | *primary amine based diazeniumdiolates*

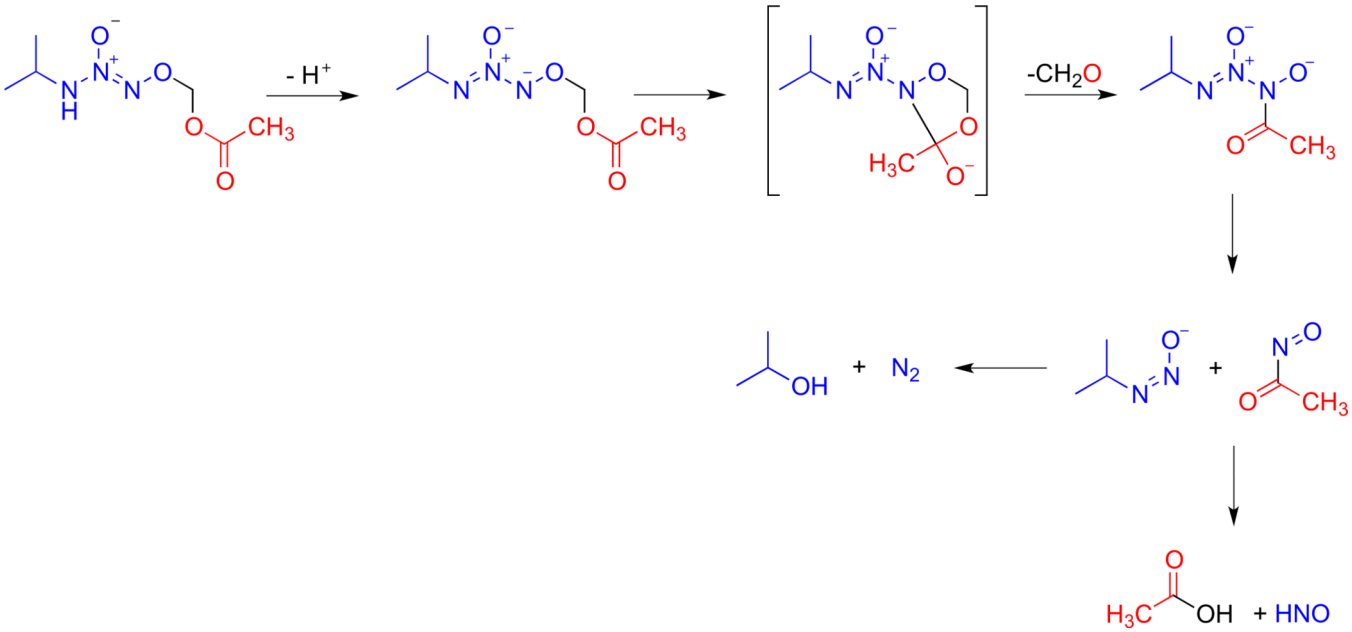
Scheme 1.
Common HNO donor structures.



Scheme 2.
Esterase-mediated decomposition of O^2-acetoxymethyl protected diazeniumdiolates.

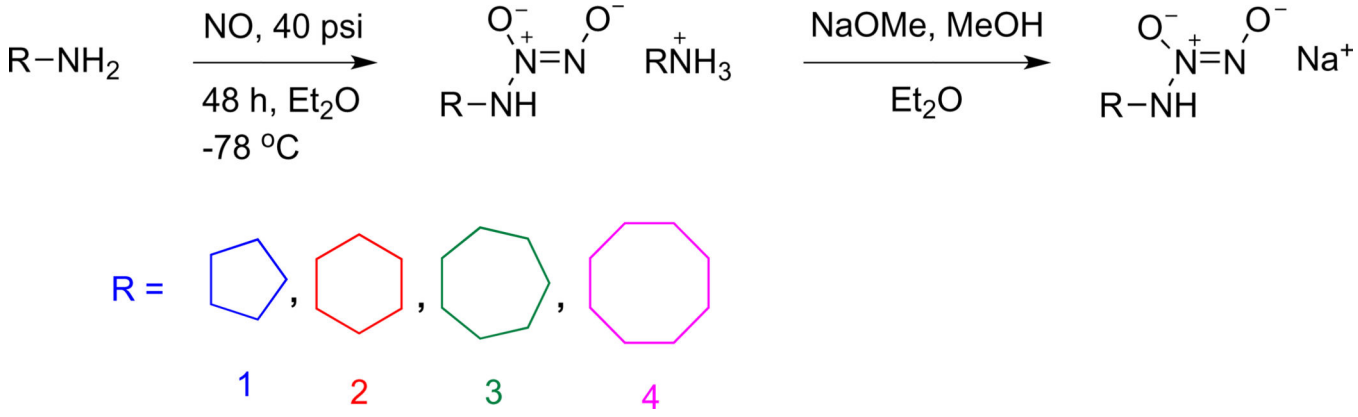


Scheme 3.
Mechanism of NO and HNO release from IPA/NO.

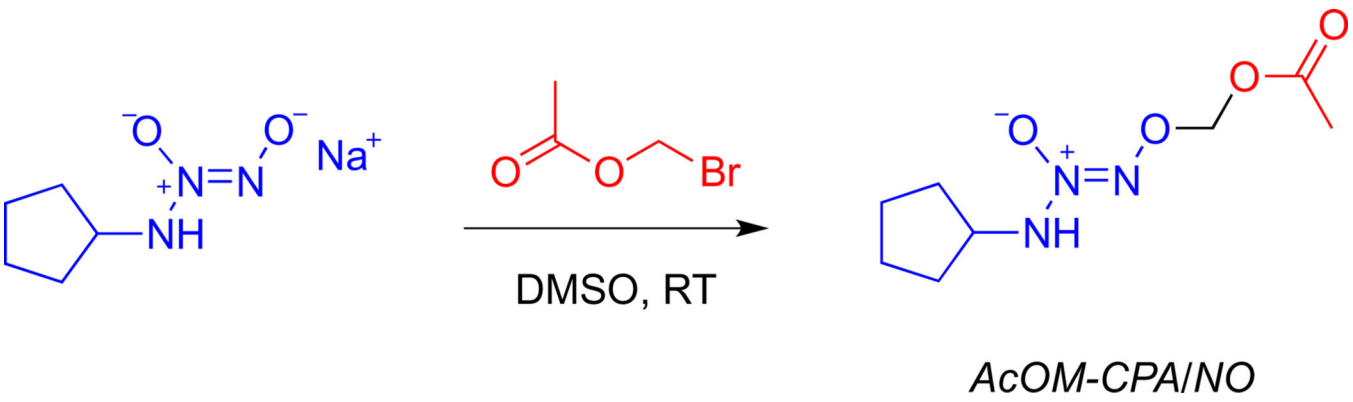


Scheme 4.

Spontaneous decomposition mechanism of acetoxymethyl-protected IPA/NO.



Scheme 5.
Synthesis of alicyclic amine diazeniumdiolates.



Scheme 6.
Synthesis of AcOM-CPA/NO (5).

Table 1

Decomposition data for alicyclic amine diazeniumdiolates at pH 7.4 and 37 °C.

Compound	$k \times 10^{-3}$ (s^{-1})	half-life (min)
1	3.2 ± 0.07	3.6 ± 0.08
2	1. 9 ± 0.06	6.1 ± 0.02
3	1. 9 ± 0.01	5.9 ± 0.04
4	2.1 ± 0.02	5.4 ± 0.07

Table 2

Percent HNO released from **1**–**5** at pH 7.0 or 7.4 using 50 μM GSH as the trapping agent.

Compound	% HNO at pH 7.0	% HNO at pH 7.4
1	28 ± 1	54 ± 4
2	25 ± 1	50 ± 4
3	24 ± 1	46 ± 4
4	31 ± 1	59 ± 2
5	ND	96 ± 3

A higher diazeniumdiolate concentration was used at pH 7.0 (60 μM) versus 7.4 (40 μM) to ensure that a detectable signal was generated. ND, not determined. For **5**, the measurement was performed in the absence of serum.