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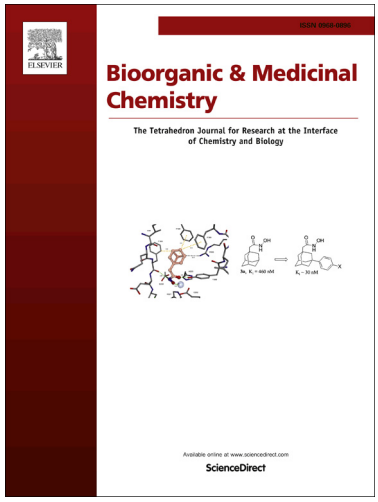

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New Acyloxy Nitroso Compounds with Improved Water Solubility and Nitroxyl (HNO) Release Kinetics and Inhibitors of Platelet Aggregation

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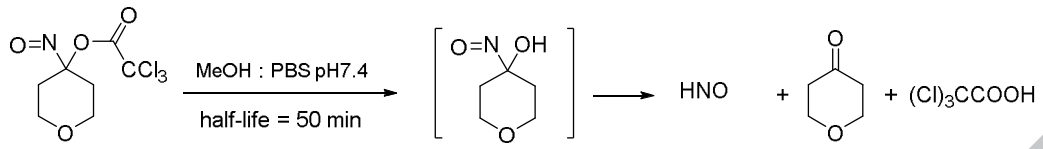
Abstract

New acyloxy nitroso compounds, 4-nitrosotetrahydro-2*H*-pyran-4-yl 2,2,2-trichloroacetate and 4-nitrosotetrahydro-2*H*-pyran-4-yl 2,2-dichloropropanoate were prepared. These compounds release HNO under neutral conditions with half-lives between 50 and 120 minutes, identifying these HNO donors as kinetically intermediate to the much slower acetate derivative and the faster trifluoroacetic acid derivative. These compounds or HNO-derived from these compounds react with thiols, including glutathione, thiol-containing enzymes and heme-containing proteins in a similar fashion to other acyloxy nitroso compounds. HNO released from these acyloxy nitroso compounds inhibits activated platelet aggregation. These acyloxy nitroso compounds augment the range of release for this group of HNO donors and should be valuable tools in the further study of HNO biology.

Graphical abstract



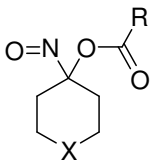
Keywords: Acyloxy nitroso compounds, nitroxyl, thiols, heme proteins, platelet aggregration

1. Introduction

Nitroxyl (HNO) is a nitrogen-containing compound chemically related to the well-known signaling agent nitric oxide (NO).[1] Nitroxyl demonstrates a distinct chemical and biological profile compared to other redox-related nitrogen oxides, such as hydroxylamine (NH_2OH), nitric oxide (NO), nitrite (NO_2^-), and nitrate (NO_3^-).[1,2] HNO acts as a potent vasodilator in a mechanism different from NO.[3,4] Other biological activities of HNO include its ability to inhibit platelet aggregation,[5] to block tumor growth through inhibition of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and to act as an anti-oxidant.[6,7] Nitroxyl enhances myocardial contractility and relaxation, properties that have led to clinical trials of HNO-donating drugs for congestive heart failure.[8,9,10] Nitroxyl reacts with thiols to give an *N*-hydroxysulfenamide intermediate that can further react with excess thiol to give the corresponding disulfide and hydroxylamine or rearranges to a sulfinamide.[11,12] For example, HNO directly influences myofilament function through modification of key cysteine residues in actin, myosin and tropomyosin and oxidizes cysteine residues present in the transmembrane domain of phospholamban (PLN) that ultimately regulates Ca^{+2} re-uptake into the sarcoplasmic reticulum.[13,14] HNO also reacts and /or forms coordination complexes with metalloproteins, especially iron heme proteins.[15,16] HNO dimerizes to give hyponitrous acid ($H_2N_2O_2$), which decomposes to nitrous oxide (N_2O) and water.[17,18] This reaction prevents the storage of HNO, requiring studies to be performed

using HNO donors.[19,20] Angeli's salt ($Na_2N_2O_3$, AS) is one of the most widely used HNO donors,[21,22] and under neutral pH releases one molecule of HNO and one molecule of nitrite.[22] Piloty's acid (an *N*-hydroxysulfonamide), another HNO donor, decomposes under basic conditions to release HNO and benzenesulfinate.[23] Cyanamide also releases HNO but has limited use due to the release of cyanide.[24]

Acyloxy nitroso compounds are another class of HNO donors that demonstrates varying HNO release rates based on different acyl group hydrolysis rates that depend on acyl group structure.[25,26] Acyloxy nitroso compounds also elicit numerous biological actions including vasodilation, enhanced cardiac contractility and inhibition of platelet aggregation.[27,28,29,13] These compounds do not release nitrite or cyanide and their characteristic blue color facilitates their kinetic study.[30] Previous work shows that 1-nitrosocyclohexyl acetate (**1**, Figure 1) is a slow HNO donor ($t_{1/2}$ = 800 min in PBS buffer, pH = 7.6), while 1-nitrosocyclohexyl trifluoroacetate (**2**, Figure 1) is an extremely fast HNO donor ($t_{1/2}$ = 121 milliseconds (ms) under the same conditions).[30] While capable of releasing HNO, these compounds demonstrate limited water solubility and **1** directly reacts with thiols before HNO release occurs.[31,32] Other acyloxy nitroso compounds (**4-5**) that contain a pyran core have been prepared and demonstrate improved water solubility that allows porcine liver esterase (PLE) catalyzed decomposition that greatly increases the rate of HNO release.[31,33] Despite this advance, the use of PLE may not be compatible in all circumstances and the present work describes the synthesis and evaluation of new acyloxy nitroso compounds (**6-7**, Figure 1) as HNO donors. These compounds possess electron deficient chlorinated acyl groups to enhance the HNO release rate (compared to the acetate) and should demonstrate enhanced water solubility based on the addition of the more hydrophilic pyran framework.



1, X= CH_2, R = CH_3
2, X= CH_2, R = CF_3
3, X= CH_2, R = $C(CH_3)_3$
4, X= O, R = CH_3
5, X= O, R = $C(CH_3)_3$
6 X= O, R = CCl_3
7, X= O, R = $C(Cl_2)(CH_3)$

Figure 1. Structure of compounds 1-7

2. Material and methods

2.1. Chemistry

Analytical thin layer chromatography (TLC) was performed on silica gel plates with C-4 Spectroline 254 indicator. Visualization was accomplished with UV light and 20% phosphomolybdic acid solution in EtOH. Solvents for extraction and purification were technical grade and used as received. Melting points were performed on a Mel-Temp apparatus. ^{1}H, ^{31}P and ^{13}C NMR spectra were recorded using a Bruker Advance 300 MHz NMR spectrometer. Chemical shifts (δ) are given in ppm; multiplicities are indicated by s (singlet), d (doublet), dd (doublet of doublet), ddd (doublet of doublet of doublet), t (triplet), q (quartet), p (pentet) and m (multiplet).

2.1.1. General procedure for the synthesis of acyloxy nitroso compounds (4-7)

A solution of dihydro-2*H*-pyran-4(3*H*)-one oxime (**8**, 5.22 mmol) in CH_2Cl_2 (10 mL) was added dropwise with stirring to a solution of lead (IV) acetate (5.22 mmol) and acid (52.2 mmol) in CH_2Cl_2 (25 mL) at 0 ^{o}C. A blue color appeared with the addition of the oxime. The mixture was stirred at 0 ^{o}C for 1 hr and stirring was continued at rt for 3 hrs. Water (10 mL) was added, the layers separated, and the CH_2Cl_2 layer was washed with

saturated sodium bicarbonate (3 x 10 mL), dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude residue was purified by column chromatography (10% EtOAc in hexane).

2.1.1.1. 4-Nitrosotetrahydro-2*H*-pyran-4-yl acetate (4)

Blue oil (0.41 g, 51%), UV-vis (MeOH): λmax = 667 nm, ε = 19.23 M^{-1} cm^{-1}; ^{1}H NMR δ (300 MHz, benzene-*d*$_6$) 1.99–2.05 (m, 2H, CH_2), 2.17–2.24 (m, 2H, CH_2), 1.86 (s, 3H, CH_3) 3.22–3.24 (m, 2H, CH_2), 3.68–3.76 (m, 2H, CH_2); ^{13}C NMR (75 MHz, benzene-*d6*) 167.86 (C=O), 120.07 (O-C-N), 63.17 ($2CH_2$), 34.24 ($2CH_2$), 20.32 (CH_3).

2.1.1.2. 4-Nitrosotetrahydro-2*H*-pyran-4-yl pivalate (5)

Blue oil (0.67 g, 44%), UV-vis (MeOH): λmax = 665 nm, ε = 19.8 M^{-1} cm^{-1}; ^{1}H NMR δ (300 MHz, benzene-*d*$_6$) 1.23 (s, 9H, $3CH_3$), 1.57–1.62 (m, 2H, CH_2), 1.97–2.07 (m, 2H, CH_2), 3.21–3.57 (m, 2H, CH_2), 3.75–3.77 (m, 2H, CH_2); ^{13}C NMR (75 MHz, benzene-*d*$_6$): 185.47 (C=O), 119.44 (O-C-N), 63.52 ($2CH_2$), 38.78 ($C(CH_3)$), 29.93 ($2CH_2$), 26.71 ($3CH_3$).

2.1.1.3. 4-Nitrosotetrahydro-2*H*-pyran-4-yl 2,2,2-trichloroacetate (6)

Blue oil (0.76 g, 53%), UV-vis (MeOH): λmax = 660 nm, ε = 16.06 M^{-1} cm^{-1}; ^{1}H NMR δ (300 MHz, benzene-*d*$_6$) 1.42–1.50 (m, 2H, CH_2), 2.02 (ddd, *J* = 13.8, 11.2, 5.1 Hz, 2H, CH_2), 3.36–3.50 (m, 2H, CH_2), 3.67 (ddd, *J* = 11.9, 5.0, 2.8 Hz, 2H, CH_2); ^{13}C NMR (75 MHz, benzene-*d*$_6$) 159.37 (C=O), 124.17 (O-C-N), 90.09 ($C(Cl)_3$), 63.1 ($2CH_2$), 29.23($2CH_2$); Anal. Calcd. For $C_7H_8Cl_3NO_4$: C, 30.41; H, 2.92; N, 5.07; Found: C, 30.38; H, 2.87; N, 4.44.

2.1.1.4. 4-Nitrosotetrahydro-2*H*-pyran-4-yl 2,2-dichloropropanoate (7)

Blue oil (0.65 g, 58%), UV-vis (MeOH): λmax = 663 nm, ε = 18.5 M^{-1} cm^{-1}; ^{1}H NMR δ (300 MHz, benzene-d_6) 1.33−1.54 (m, 2H, CH_2), 1.85−1.98 (m, 2H, CH_2), 2.00 (s, 3H, CH_3) 3.33−3.51 (m, 2H, CH_2), 3.57−3.64 (m, 2H, CH_2); ^{13}C NMR (75 MHz, benzene-d_6) 163.9 (C=O), 122.76 (O-C-N), 80.18 ($C(Cl)_2CH_3$), 63.21 ($2CH_2$), 33.91 (CH_3) 29.41 ($2CH_2$); Anal. Calcd. For $C_8H_{11}Cl_2NO_4$: C, 37.52; H, 4.33; N, 5.47; Found: C, 37.43; H, 4.41; N, 4.64.

2.2. Acyloxy nitroso compounds decomposition kinetics and products

UV-vis spectrometry was performed on a Cary 100 Bio UV-vis spectrophotometer or Cary 50 Bio UV-vis spectrophotometer (Varian, Walnut Creek, CA). Gas chromatographic detection of nitrous oxide was performed on 7890A Agilent Technologies gas chromatograph.

2.2.1. Gas chromatographic detection of nitrous oxide

A solution of **4-7** (40 µmol) in 0.8 mL [1:1 MeOH: phosphate buffered saline (PBS, 100 mM, pH 7.4) or 1:1 MeOH: 0.1 M NaOH] was placed in a 10 mL round bottom flask, sealed with a rubber septa and flushed with argon. The samples were incubated at rt and at desired time points headspace aliquots (25 µL) were injected via a gas-tight syringe onto a 7890A Agilent Technologies gas chromatograph equipped with a micro-electron capture detector and a 30 m x 0.32 m (25 µm) HP-MOLSIV capillary column. The oven was operated at 200 °C for the duration of the run (4.5 minutes). The inlet was held at 250 °C and run in split mode (split ratio 1:1) with a total flow (N_2 as carrier gas) of 4 mL/min and a pressure of 37.9 psi. The µECD was held at 325 °C with a makeup flow (N_2) of 5 mL/min. The retention time of nitrous oxide was 3.4 min, and yields were calculated based on a standard curve for nitrous oxide (Matheson Tri-Gas). Similar experiments were done with an added aliquot (13 µL) of a stock solution [5 mg PLE dissolved in PBS (100mM, pH 7.4, 1 mL) that gives a total of 85U in 1 mL] of porcine liver esterase (9U/µmol) of substrate.

2.2.2. Kinetic UV-vis Decomposition Assay

Phosphate buffered saline (PBS, 100 mM, pH 7.4, 1 mL) was added to a solution of **4** and **5** (60 μmol) in MeOH (1 mL) at rt in a sealed cuvette. UV-vis measurements were taken every 15 min for 10 hrs at 667 nm. In other experiments, a solution of NaOH (0.1 M, 1 mL) was used and similar measurements made. Similar measurements were performed with the addition of PLE (200 μL, 9 U/μmol substrate). Further experiments were performed in the presence of thiols. A solution of GSH (70.2 μmol) in PBS (100 mM, pH = 7.4, 1 mL) was added to a solution of **4-7** (35.1 μmol) in MeOH (1 mL) at rt in a sealed cuvette. UV-vis measurements were taken every 1 min for 90 min at 665 nm. Similar experiments were performed using another concentration of GSH (70.2 μmol) in NaOH (0.1 M, 1 mL).

2.3. Reaction of acyloxy nitroso compounds with GAPDH

The UV-vis assay of GAPDH inhibition and reversibility measurement of **4**, **6**, and **7** were performed as described.[32] High-resolution mass spectra were obtained using a Thermo Scientific LTQ Orbitrap mass spectrometer equipped with a heated electrospray ionization source operated in positive ion mode.

2.3.1. Assay of GAPDH modification by 6 and 7 using HPLC-HRMS spectrometry

A solution of lyophilized GAPDH [1000 U/mL in PBS (100 mM, pH 7.4)] was prepared and the concentration was assessed using UV-vis spectrometry (ε_{280}= 14600 M^{-1} cm^{-1}).[34] An aliquot of protein was added to a solution of **6** or **7** (5 μL; 10 mM in MeOH) and Millipore water to give a final concentration of the protein (100 μM) and of HNO donor (1mM) in 50 μL total volume, which was incubated for 1 hr at rt. Trypsin (20 μg/mL in 1 mM HCl) was added at a ratio of 20:1 and samples were incubated at 37 ^{o}C for 17 hrs. Samples (50 μL) were diluted with HPLC grade MeOH (200 μL) and 2% formic acid in

HPLC grade water (200 µL) for a final peptide concentration of 10 µM. The samples were analyzed by HPLC-HRMS on a Thermo Scientific LTQ Orbitrap XL. Separations were achieved using a Thermo Scientific Hypersil GOLD C18 column (150 x 2.1 mm, 1.9 µm). During LC, solvent A (0.1 % formic acid in H_2O) and B (0.1 % formic acid in MeOH) were held at a flow rate of 400 µL/min. Conditioning the column involved transitioning from acetonitrile to 95 % A and 5 % B over 15 min, and held for 5 min. LC analysis consisted of injections (10 µL) using a gradient elution of 5 to 95 % B over 10 min, followed by lowering to 5 % B over 10 sec, and held for 1 min. After each analysis two blanks were run. ESI parameters consisted of a spray voltage of 4 kV, capillary voltage of 40 V, and a capillary temperature of 325 °C. Positive mode high resolution mass spectra were collected with 30,000 Hz resolution from 200-2000m/z. Extracted masses with an error of +/- 5 ppm were found to be 853.434 (in the I144-K160 peptide), 750.398 (in the V233-R246 peptide), 852.4256 [M(disulfide(RSSR) in the I144-K160 peptide], 765.898 [M(sulfinamide(RSONH2) in the V233-R246 peptide], and 766.391 [M(sulfinic acid(RSOOH) in the V233-R246 peptide] . The same experiment was done in the absence of any acyloxy nitroso compound as a control.

2.4. Assay of the reaction of HNO donors with heme proteins

The UV-vis assay of the reactions of **6** and **7** with both metmyoglobin and oxymyoglobin were performed as previously described.[31]

2.5. Measuring platelet aggregation and activation inhibition by 6

A BD FACS Calibur flow cytometer and Cell Quest Pro software were used for data collection and analysis used for platelet activation measurements. A 96-well plate reader (Molecular Devices, Softmax pro and Synergy H1, Gen5) and software were used for platelet aggregation measurements.[35,36]

2.5.1. Platelet aggregation inhibition

Blood was centrifuged at 100 g for 10 minutes to obtain platelet rich plasma (PRP). Platelet rich plasma was centrifuged at 4500 g for 10 minutes to obtain platelet poor plasma (PPP). Calcium chloride (2 mM, 1 µL) was added to the PRP and PPP (100 µL) that was added to each well. Compound **6** (100, 75, 50, and 10 µM) was added to the appropriate wells and allowed to incubate for 20 minutes at rt prior to addition of ADP (5 µM). Light transmission measurements were taken every 20 seconds and samples were agitated using the double orbital settings. Data was normalized so that PPP represented 100% transmission and PRP represented 0% transmission.

2.5.2. Platelet activation inhibition

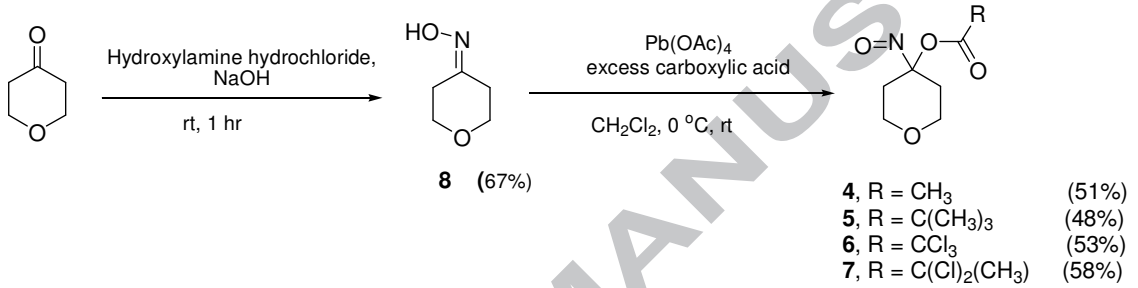
Blood and PRP were obtained as before. Platelet rich plasma (15 µL) was diluted into PBS (81.5 µL, 100 mM, pH 7.4) and **6** (1 µL; 100, 75, 50, 10, and 1 µM) was added to the mixture and incubated at rt for 20 min. A solution of ADP (5 µM; 2.5 µL) was added and the mixture was incubated for 10 minutes at rt. At this time, 10 µL of each sample was transferred into a FITC-CD62p and PE-CD41 solution of antibodies (5 µL each) and the samples were incubated for 15 minutes at rt in the dark, diluted approximately 1:40 in 1% formaldehyde and stored at 4 ºC until analysis by flow cytometry.

3. Results and Discussion

3.1. Chemistry

Acyloxy nitroso compounds **4-7** were prepared according to a reported procedure[30] (Scheme 1). Condensation of dihydro-2*H*-pyran-4(3*H*)-one with hydroxylamine hydrochloride results in dihydro-2*H*-pyran-4(3*H*)-one oxime (**8**). Oxidation of **8** with lead tetracetate (LTA) generates 4-nitrosotetrahydro-2*H*-pyran-4-yl acetate (**4**).

Similarly oxidation of **8** in the presence of excess carboxylic acid (5-10 equivalents of pivalic, trichloroacetic or, 2,2-dichloropropionic) yields 4-nitrosotetrahydro-2*H*-pyran-4-yl pivalate (**5**)**,** 4-nitrosotetrahydro-2*H*-pyran-4-yl 2,2,2-trichloroacetate (**6**) and 4-nitrosotetrahydro-2*H*-pyran-4-yl 2,2-dichloropropanoate (**7**), respectively (Scheme 1). The ^{13}C NMR spectra show the disappearance of the peak corresponding to C=N-OH (oxime) and the emergence of the carbon C(O)N=O at δ 120-124 ppm and also the presence of an additional peak corresponding to the carbonyl of the ester.

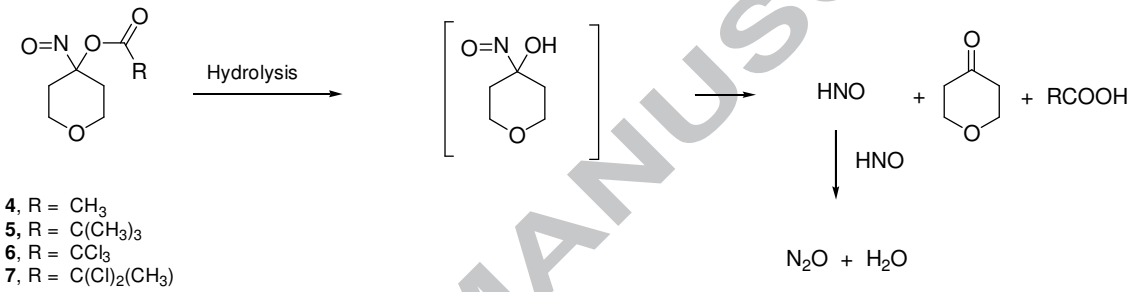


Scheme 1. Synthesis of acyloxy nitroso compounds **4-7**.

3.2. Acyloxy nitroso compounds decomposition kinetics and products

Acyloxy nitroso compounds hydrolyze to generate an unstable α-hydroxy nitroso intermediate that collapses to the ketone and HNO (Scheme 2). This mechanism predicts the rate and amount of HNO formation should directly relate to the ease of ester hydrolysis for **4-7**. Monitoring the disappearance of the absorbance at 667 nm using ultraviolet-visible (UV-vis) spectroscopy provides kinetic information on the decomposition of **4-7**. Incubation of these compounds in a 1:1 mixture of MeOH:phosphate buffer saline (PBS, 100 mM, pH = 7.4) at room temperature results in their exponential decrease over time with different rate constants and half-lives (Table 1). Compounds **4**-**5** display similar decomposition kinetics as previously reported.[31] The increased hydrolysis rate for **6** and **7** ($t_{1/2}$ = 50 and 120 min, respectively) can be attributed to the presence of the chlorine atoms, which being more

electronegative appear to accelerate the rate through inductive effects. Another kinetic study was performed by incubating these compounds in a 1:1 mixture of MeOH:0.1 N NaOH, which increases hydrolysis and results in a fast exponential decrease for **4** over time (Table 1). Compounds **6** and **7** decompose immediately upon NaOH addition as determined by the immediate visual disappearance of the blue color. Compound **5** behaves like **3** (Figure 1), which resists hydrolysis in buffer and basic conditions ($t_{1/2}$ = 2268 min under buffered conditions).[31]



Scheme 2. Hydrolysis of acyloxy nitroso compounds **4-7.**

Table 1. Rate constants and half-lives for **4-7** under different conditions.

Compound	***k*** **(min^{-1}) in 1:1 MeOH : PBS**	**$t_{1/2}$ (min) in 1:1 MeOH : PBS**	***k*** **(min^{-1}) in 1:1 MeOH : 0.1 N NaOH**	**$t_{1/2}$ (min) in 1:1 MeOH : 0.1 N NaOH**
4	8.9×10^{-4}	780	5.7×10^{-2}	12
5	3.6×10^{-4}	1900	3.7×10^{-4}	1870
6	1.4×10^{-2}	50	----	Fast
7	5.8×10^{-3}	120	----	Fast

(----) = *k* cannot be measured

Gas chromatographic (GC) head-space analysis of N_2O, the dimerization and dehydration product of HNO, provides evidence for HNO intermediacy during the

decomposition of **6** and **7** (Scheme 2). Table 2 shows the percent yield of HNO release from **6** and **7** under different conditions and time intervals. Both **6** and **7** generate increased amounts of N_2O than previously reported for **4** and **5** under similar conditions.[31] The increased amounts of N_2O produced from **6** and **7** correlated with the increased decomposition rate of these compounds as expected.

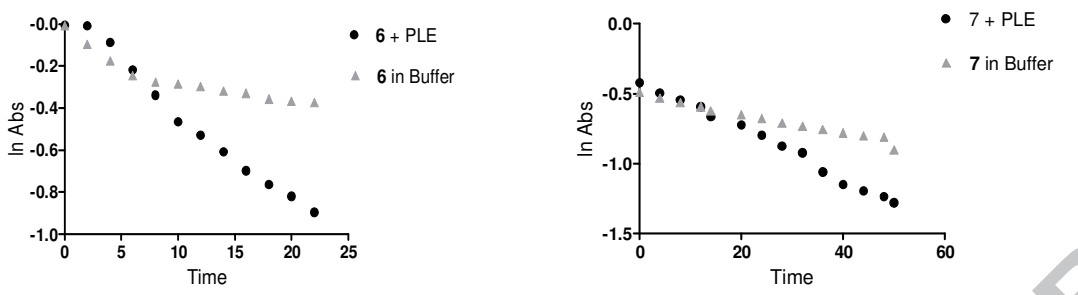
Table 2. Percent of N_2O from **6** and **7** under different conditions and times.

Compound	**% N_2O in 1:1 MeOH : PBS 2 hrs**	**% N_2O in 1:1 MeOH : PBS 24 hrs**	**% N_2O in 1:1 MeOH : 0.1 N NaOH 2 hrs**
6	89 %	95 %	94 %
7	57 %	92 %	91 %

3.3. Decomposition kinetics and products in the presence of porcine liver esterase (PLE)

The improved water solubility of **4-7** allows the study of their hydrolysis in the presence of porcine liver esterase (PLE). Previous work shows PLE enhances the hydrolysis of **4**-**5** and experiments here yields similar kinetic profiles (Supporting Information).[31] Monitoring the disappearance of the absorption at 663 nm using UV-vis spectroscopy provides kinetic information regarding the decomposition of **6-7** in the presence of PLE.

The addition of PLE to **6-7** increases decomposition with rate constants of k = 0.087 min^{-1} and 0.03 min^{-1}, ($t_{1/2}$ = 8 and 23 min), respectively, resulting in a 6-fold and 5-fold increase from their rate in buffer (Fig. 2A and 2B). These results reveal that **6**-**7** decompose with HNO release without enzymatic aid at room temperature with half-lives in the 1-2 hour range.

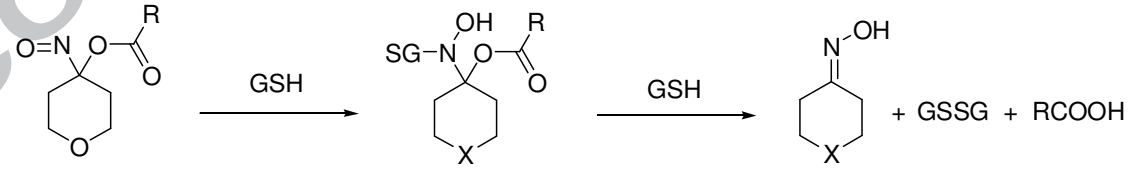


[A] **[B]**

Fig. 2. Kinetic UV-vis decomposition of [A] **6** (7 µmol) in PBS buffer (3 % DMF) -/+ PLE and [B] **7** (7 µmol) in PBS buffer (3 % DMF) -/+ PLE.

3.4. Acyloxy nitroso compounds decomposition kinetics and products in the presence of thiols

Thiols are among the most important targets for HNO as these reactions appear to elicit many of its biological effects.[11] Scheme 3 shows a possible reaction pathway for **4**-**7** and thiols that competes with hydrolysis and HNO release and has been observed for **1**.[27] In this pathway, thiol adds to the nitrogen of the nitroso group giving an intermediate that can react with another molecule of thiol to give disulfide and the corresponding oxime without HNO release.[27,37]



4, R = CH_3
5 R = $C(CH_3)_3$
6, R = CCl_3
7, R = $C(Cl)_2(CH_3)$

Scheme 3. Possible decomposition of **4-7** in the presence of GSH.

Compounds **4-7** were incubated at room temperature with glutathione (GSH, as a model thiol) in PBS buffer (pH = 7.4) and 0.1 N NaOH and the rates determined as before. Table 3 shows the decomposition rate constants and half-lives for **4-7** in the presence of 2-fold GSH in different media.

Table 3. Rate constants and half-lives for **4-7** in the presence of 2-fold GSH under different conditions.

Compound	k (min^{-1}) in PBS + 2-fold GSH	$t_{1/2}$ (min) in PBS + 2-fold GSH
4	0.011	65
5	0.007	99
6	0.173	4
7	0.0578	12

Addition of GSH significantly increases the decomposition rate of **4-7** in PBS pH = 7.4 as shown in Table 3. This increase is generally 10-fold more than the rate of decomposition in buffered conditions and the observed rates of decomposition of **4-7** in the presence of GSH are also enhanced compared to PLE-catalyzed hydrolysis suggesting that decomposition in the presence of thiol occurs via a different pathway than ester hydrolysis (Scheme 3). Presumably, under both buffered and basic conditions, some or all of the thiol exists as the thiolate (an enhanced nucleophile) that adds to the nitroso group of **4-7**, faster than hydrolysis and HNO release, to yield the corresponding oxime (Scheme 3).[31,32] GC-MS measurements of the reaction mixture of **6-7** in the presence of 2-fold GSH in PBS, support this route revealing only dihydro-2*H*-pyran-4(3*H*)-one oxime (**8**) and no dihydro-2*H*-pyran-4(3*H*)-one as the product.[27]

These studies identify **6**-**7** as kinetically distinct acyloxy nitroso compounds compared to those previously reported as they decompose with HNO release under buffered conditions with moderate half-lives ($t_{1/2}$ = 50 and 120 min, respectively). These compounds decompose slowly compared to the trifluoracetate (**2**) but much more quickly than both the acetate and pivalate derivatives (**1**, **3-5**, Figure 1), a clear function of the electron withdrawing chlorines found in the acyl group. PLE also catalyzes the hydrolysis and HNO release from **6**-**7** (faster hydrolysis rates than **4-5**) further expanding the range of available HNO delivery rates. However, **6-7** still directly with thiols, prior to complete HNO release, similar to other acyloxy nitroso compounds.[27] Compounds **6-7** appear unique in that they release HNO under buffered conditions in the absence of esterases with reasonable half-lives compared to other acyloxy nitroso compounds (**1-3**, Figure 1).

3.5. Reactions of acyloxy nitroso compounds with GAPDH

Nitroxyl inhibits GAPDH, an important thiol protein involved in glycolysis.[38,39] Recently, GAPDH has been implicated in non-metabolic functions such as transcription activation and initiation of apoptosis.[40] Glyceraldehyde-3-phosphate dehydrogenase's inhibition by HNO is responsible for HNO's observed toxicity to yeast cells.[41] GAPDH inhibition may also explain the ability of HNO to suppress the proliferation of human breast cancer cell lines by blocking glycolysis resulting in decreased HIF-1α expression and angiogenesis inhibition.[38,42] Earlier work shows that acyloxy nitroso compounds (**1**-**3**) inhibit GAPDH by modification of various protein thiols as determined by high resolution mass spectrometry (HRMS) suggesting that **4**, **6** and **7** would also inhibit GAPDH.[32]

3.5.1. Kinetic UV-vis Study of GAPDH inhibition by 4, 6, and 7

Incubation of **4, 6,** and **7** with GAPDH, results in inhibition in a concentration-dependent manner. The IC_{50}s for **4, 6,** and **7** were calculated to be 0.6, 0.78, and 0.37

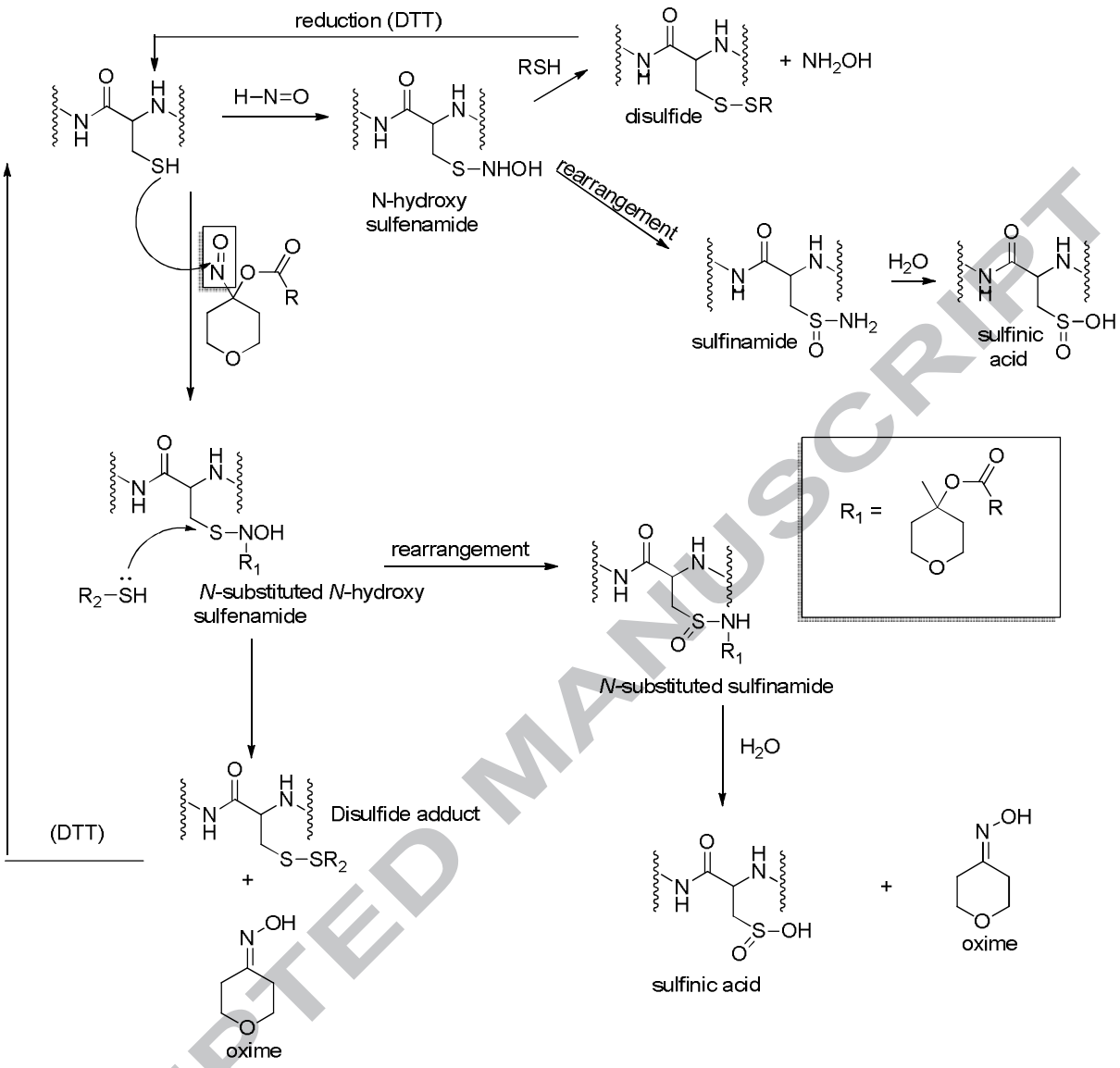
respectively (Supporting Information). Compound **4**, the slowest HNO donor, is the most potent inhibitor of GAPDH compared to **6** and **7** (faster HNO donors). This trend was previously observed in studies with AS and **1-3** with GAPDH.[[32]] The higher activity of **4** may suggest a non-HNO mediated mechanism for GAPDH inhibition that includes the direct addition of the nitroso group of the acyloxy nitroso compound to the cysteine thiol of GAPDH as previously observed.[32]

To test the reversibility of GAPDH inhibition, GAPDH was incubated with **4, 6,** and **7** for 1 hr followed by incubation with dithiothreitol (DTT, 10 mM) for 1 hr. Treatment with DTT following incubation with **4, 6,** and **7** results in partial restoration of GAPDH activity with 22, 19, and 9 % of GAPDH activity being restored by the addition of DTT to GAPDH pre-incubated with **4, 6,** and **7**, respectively (Supporting Information). The low amount of recovery suggests **6** and **7** react with GAPDH to predominantly form irreversible modifications of the protein thiol (sulfinamide and / or sulfinic acid) as the major products compared to a reversible disulfide adduct as noted in other studies of GAPDH with HNO donors. [11,32]

3.5.2. HPLC-HRMS study of GAPDH incubated with 6 and 7

Chemical modification of GAPDH with HNO can be monitored by high pressure liquid chromatography- high resolution mass spectrometry (HPLC-HRMS) with specific attention paid to the formation of a unique sulfinamide modification as a footprint for *in vivo* and *in vitro* HNO formation.[32] Acyloxy nitroso compounds **6** and **7** (1 and 10 mM in MeOH) were incubated with GAPDH [1000 U/mL in PBS (100 mM, pH 7.4)] for 1 hr at room temperature followed by overnight tryptic digestion at 37 ºC. Analysis was done on two tryptic peptides containing cysteine residues; the active site I144-K160 peptide containing two cysteine residues Cys149 and Cys 153 (active site with a mass of m/z = 1705.861 [M+1],

m/z = 853.434 [M+2] and a non-active site the V233-R246 peptide containing one isolated cysteine residue Cys 244 (non-active site with a mass of *m/z* = 1499.789 [M+1] and *m/z* = 750.3944 [M+2].[32] The active site I144-K160 peptide is the proposed site for the formation of an internal disulfide (R-SS-R) and this results in a mass change of the peptide to *m/z* = 852.4224-852.4310 [M+2] (Scheme 4).[32] The non-active site V233-R246 peptide is the proposed site for the sulfinamide ($RSONH_2$) formation and this results in a mass change of the V233-R246 peptide to *m/z* = 765.3937-765.4013 [M+2] and / or sulfinic acid (RSOOH), formed from oxidative modification of the thiol or through sulfinamide hydrolysis) to give a mass change in the V233-R246 peptide to *m/z* = 766.3890-766.3966 [M+2] (Scheme 4).[32]



Scheme 4. Proposed reaction of HNO and acyloxy nitroso compounds with GAPDH.

Treatment of GAPDH with **6** and **7** shows a mass change in the peptide I144-K160 indicating the formation of an internal disulfide between the Cys 149 and Cys 153 (Table 4). High resolution mass spectrometry of treated GAPDH with **6** and **7** shows a mass change in the peptide V233-R246 indicating the formation of sulfinamide and sulfinic acid (Table 5).

Table 4. HPLC-HRMS Analysis of the reaction of **6** and **7** with the GAPDH tryptic peptide I144-K160 compared to AS.

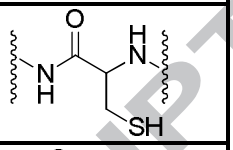
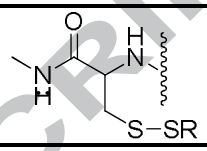
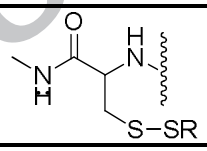
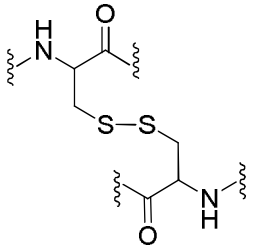
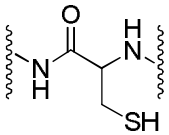
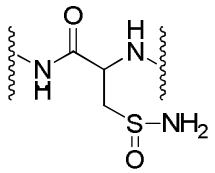
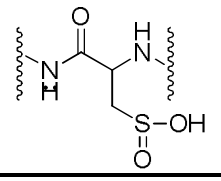
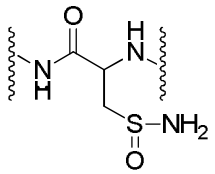
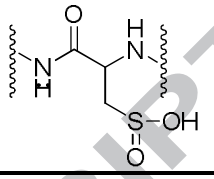
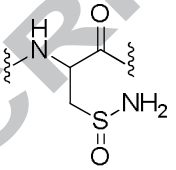
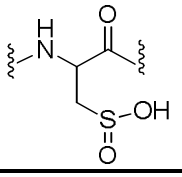
Compound	Retention time (min)	*m/z*[M+2]	Products	
Untreated GAPDH	8.9	853.4357	Unmodified cysteine, RSH	
6	7.14	852.4285	Internal disulfide; RSSR	
7	7.28	852.4239	Internal disulfide; RSSR	
AS	7.11	852.4269	Internal disulfide; RSSR	

Table 5. HPLC-HRMS Analysis of the reaction of **6** and **7** with the GAPDH tryptic peptide V233-R246 compared to AS.

Compound	Retention time (min)	*m/z*[M+2]	Products	
Untreated GAPDH	10.85	750.3984	Unmodified cysteine	
6	9.56	765.9033	Sulfinamide; $RSONH_2$	
	10.08	766.3952	Sulfinic acid; RSOOH	

7	9.69	765.9000	Sulfinamide; $RSONH_2$	
	10.16	766.3914	Sulfinic acid; RSOOH	
AS	9.58	765.9014	Sulfinamide; $RSONH_2$	
	10.04	766.3929	Sulfinic acid; RSOOH	

Compounds **6** and **7** showed a mass change in the I144-K160 peptide of *m/z* = 852.4274 and 852.4239 [M+2], respectively revealing the disulfide formation between Cys 149 and Cys 153. Compounds **6** and **7** showed a mass change in the V233-R246 peptide of *m/z* = 765.9033-756.9000 and 766.389-766.3913 [M+2], respectively revealing the formation of irreversible sulfinamide and sulfinic acid adducts, respectively with both compounds. Reaction of **6** and **7** with GAPDH gives sulfinamide and sulfinic acid in almost equal ratio and as the major products with disulfide as a minor product as shown by the ion count of the target mass.

Previous work with **1** (a slow HNO donor) and GAPDH, show a mass change in the V233-R246 peptide of *m/z* = 766.3929 [M+2] revealing the formation of sulfinic acid as a major product. The fast HNO donors **2** and AS show a mass change in the V233-R246 peptide of *m/z* = 765.9014 [M+2] and 766.3929 revealing the formation of sulfinamide and sulfinic acid with the sulfinamide as a major product and sulfinic acid as a minor product.[32]

Compounds **6** and **7**, with intermediate rates of HNO release ($t_{1/2}$ = 50 and 120 min, respectively) behave differently from **1** (slow HNO donor) and the fast HNO donors **2** and AS, by producing sulfinic acid and sulfinamide in an equal ratio.

These results may be explained by the proposed mechanism depicted in Scheme 4 where fast HNO donors release HNO before a direct reaction of the *C*-nitroso group with the thiol and inhibits the enzyme through HNO-mediated sulfinamide formation. Slow HNO donors directly react with the protein thiols before HNO release and inhibit the enzyme through direct addition of the *C*-nitroso group to the thiol ultimately yielding a sulfinic acid. Together, **6** and **7** appear to inhibit GAPDH by mechanisms observed for both slow and fast HNO donors, consistent with their intermediate rate of HNO production. HNO released from **6** and **7** inhibits GAPDH by disulfide and sulfinamide formation but also through direct reaction of the *C*-nitroso group with the thiol of GAPDH forming a *N*-substituted sulfinamide that easily hydrolyzes to sulfinic acid. This mechanism also accounts for the observation that the inhibition of GAPDH by **6** and **7** is partially reversed by DTT.

3.6. Reaction of acyloxy nitroso compounds with heme proteins

Five-coordinate ferric heme proteins such as metHb or metMb act as efficient traps for HNO. In the presence of AS-derived HNO, the ferric heme undergoes reductive nitrosylation, producing a ferrous-nitrosyl complex (MbNO).[43,44,45] Addition of **6** and **7** to a solution of metMb followed by UV-vis measurements show a decrease in the peaks at 503 and 630 nm representing metMb and an increase in absorbance at 540 and 580 nm corresponding to the ferrous nitrosyl complex (MbNO, Supporting Information).

The oxygenated ferrous hemes of myoglobin and hemoglobin undergo rapid oxidation to the ferric forms upon reaction with HNO generated from AS.[46] The reaction of HNO with MbO_2 is among the most rapid of the known traps for HNO. MbO_2 readily oxidizes HNO to

NO, which may have important biological implications for endogenous NO generation from HNO.[l][46][l] Subsequent reaction of the nascent NO with oxygenated heme provides additional ferric heme and NO_3^-.[47] The HNO-mediated conversion of MbO_2 to metMb can be monitored by observing the increase in absorbance at 503 and 630 nm (metMb appearance) and the decrease in absorbance at 542 and 582 nm (MbO_2 disappearance). Reaction of **6** and **7** with MbO_2 demonstrates an increase in the absorbance at 503 and 630 nm and a decrease in the absorbance at 542 and 582 nm (Supporting Information).

3.7. Platelet aggregation and activation inhibition by 6

Previous work shows the ability of HNO and acyloxy nitroso compounds to inhibit platelet aggregation suggesting that **6** or **7** may also demonstrate this important biological activity.[5,28,48] Incubation of platelet-rich plasma (PRP) with different concentrations of **6** followed by activation with adenine diphosphate (ADP) at room temperature was monitored by light absorbance. Complete inhibition of platelet aggregation occurs with **6** (100 μM) as the absorbance returns to normal as compared with the negative control (PRP no ADP added, representing 0% transmission, Figure 3). Low concentrations **6** (10 μM) had no effect as compared with the positive control (PRP + ADP) and intermediate activities were observed with amounts of **6** (50 and 75 μM) (Figure 3).

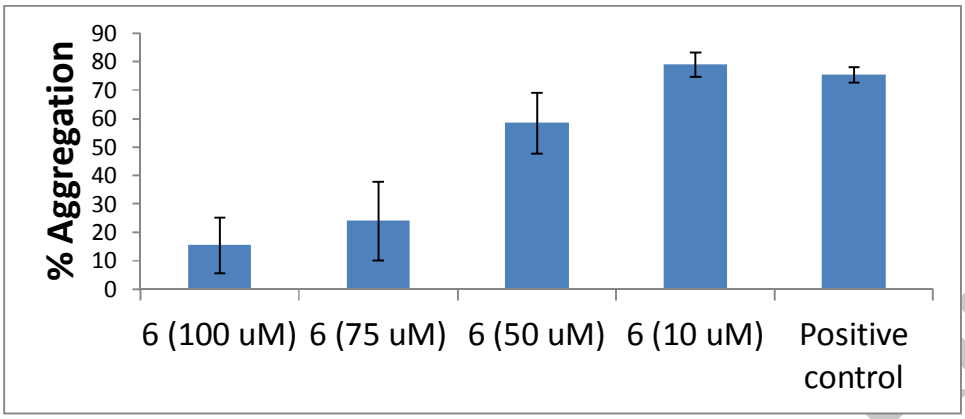


Fig. 3. Average effect of different concentrations of **6** on human platelet aggregation induced by ADP (5 µM).

Platelet-rich plasma obtained was incubated with **6** at different concentrations for 20 min at room temperature. The platelets were activated by ADP (5 µM), labeled with fluorescent antibodies, fixed with 1% formaldehyde and measured by flow cytometry. The experiment was done in triplicate and the results show a potent and similar effect on platelet activation inhibition by **6** (100, 75, 50, and 10 µM) with lower activity at (1 µM) (Fig. 4).

Compound **6** (10 µM) inhibits the expression of the activated marker (CD62P) in PRP incubated with ADP (2 µM) by 83 %, which is higher than observed with AS and SNP (Figure 4).[5] Overall, these results show that **6**, a good HNO donor, inhibits platelet aggregation, similar to previous results with acyloxy nitroso compounds,[25] and platelet activation making this class a promising group for the treatment of thrombosis. At this time, the exact mechanism of how 6 inhibits platelet aggregation remains unclear. Acyloxy nitroso compounds do activate soluble guanylate cyclase, which may explain this activity that also could be mediated through direct thiol reactions and more work will be need to differentiate these mechanisms.[49]

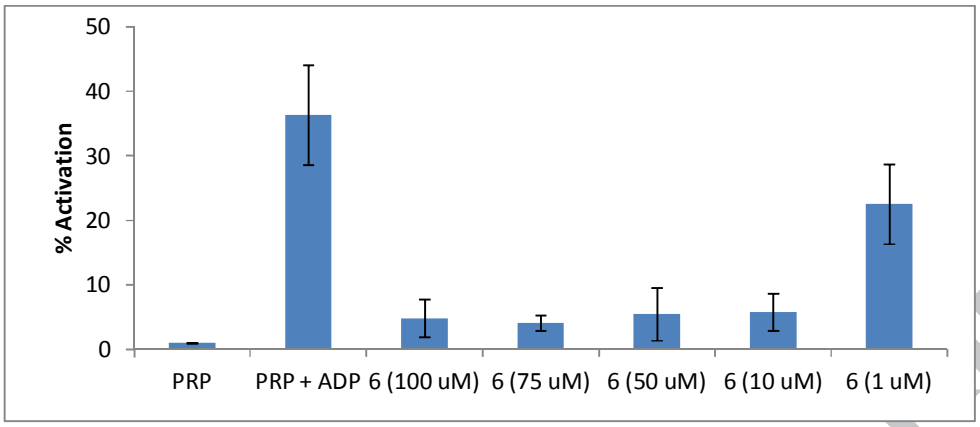


Fig. 4. Average effect of **6** on human platelet activation induced by (ADP) 5 μM.

4. Conclusion

Two new trichloro acetate-derived acyloxy nitroso compounds (**6** and **7**) were prepared and shown to release HNO under neutral conditions as determined by both gas chromatography and ferric heme protein trapping. UV kinetic analysis indicate that **6** and **7** decompose with half-lives of 50 and 120 minutes, respectively (Figure 5). These rates identify **6** and **7** as intermediate HNO donors, much slower than the trifluoroacetate (**2**) but much faster than **4** and **5** and clearly reveal the structure of the acyl group dictates the rate of hydrolysis (Figure 5). Compounds **6** and **7** should fill an important role in HNO research as donors that decompose at a slower rate that may be closer to physiological rates, which remain unknown. These compounds inhibit the thiol-containing enzyme GAPDH, similar to other HNO donors, and HPLC-HRMS experiments show that **6** and **7** form both reversible and irreversible modifications. The acyloxy nitroso compound **6** also inhibits platelet activation and aggregation. These compounds fill a kinetic need for HNO donors and may offer new approaches in the study of HNO chemistry and biology and may serve as leads for therapeutic agents that require a slower/longer HNO release profile.

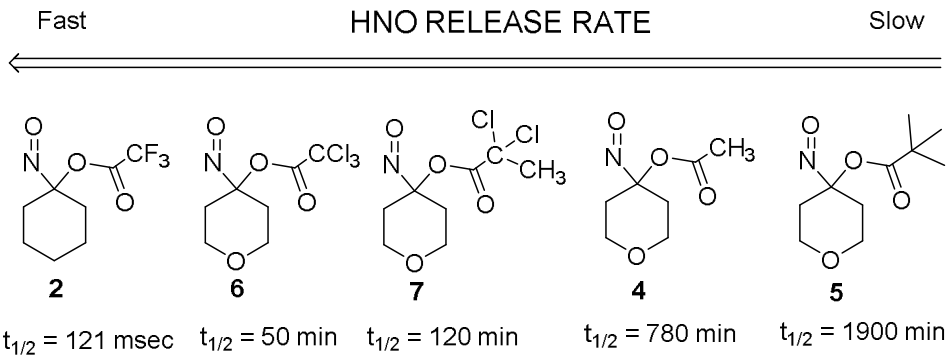


Fig. 5. Summary of acyloxy nitroso compounds and their decomposition half-lives in PBS (pH = 7.4).

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5. References

[1] J.C. Irvine, R.H. Ritchie, J.L. Favaloro, K.L. Andrews, R.E. Widdop, B.K. Kemp-Harper, The Cinderella of the Nitric Oxide Story, Trends Pharmacol. Sci. 29 (2008) 601.

[2] N. Paolocci, M.I. Jackson, B.E. Lopez, K. Miranda, C.G. Tocchetti, D.A. Wink, A.J. Hobbs, J.M. Fukuto, The Pharmacology of Nitroxyl (HNO) and Its Therapeutic Potential: Not Just the Janus Face of NO, Pharmacol. Ther. 113 (2007) 442.

[3] J.M. Fukuto, K. Chiang, R. Hszieh, P. Wong, G. Chaudhuri, The Pharmacological Activity of Nitroxyl: A Potent Vasodilator with Activity Similar to Nitric Oxide And/or Endothelium-Derived Relaxing Factor, J Pharmacol Exp Ther 263 (1992) 546.

[4] J.C. Irvine, J.L. Favaloro, R.E. Widdop, B.K. Kemp-Harper, Nitroxyl Anion Donor, Angeli's Salt, Does Not Develop Tolerance in Rat Isolated Aortae, Hypertension 49 (2007) 885.

[5] E. Bermejo, D.A. Sáenz, F. Alberto, R.E. Rosenstein, S.E. Bari, M.A. Lazzari, Effect of Nitroxyl on Human Platelets Function, Thromb. Haemost. 94 (2005) 578.

[6] D.A. Wink, M. Feelisch, J. Fukuto, D. Chistodoulou, D. Jourd'heuil, M.B. Grisham, Y. Vodovotz, J.A. Cook, M. Krishna, W.G. DeGraff, S. Kim, J. Gamson, J.B. Mitchell, The Cytotoxicity of Nitroxyl: Possible Implications for the Pathophysiological Role of NO, Arch. Biochem. Biophys. 351 (1998) 66.

[7] J.M. Fukuto, C.L. Bianco, T.A. Chavez, Nitroxyl (HNO) Signaling, Free Radic. Biol. Med. 47 (2009) 1318.

[8] N. Paolocci, W.F. Saavedra, K.M. Miranda, C. Martignani, T. Isoda, J.M. Hare, M.G. Espey, J.M. Fukuto, M. Feelisch, D.A. Wink, D.A. Kass, Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 10463.

[9] N. Paolocci, T. Katori, H.C. Champion, M.E. St John, K.M. Miranda, J.M. Fukuto, D.A. Wink, D.A. Kass, Positive inotropic and lusitropic effects of HNO/NO- in failing hearts: independence from beta-adrenergic signaling, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 5537.

[10] H.N. Sabbah, R.C. Gupta, S. Kohli, M. Wang, S. Rastogi, K. Zhang, K. Zimmermann, N. Diedrichs, B.E. Albrecht-Küpper, Chronic Therapy With a Partial Adenosine A1-

Receptor Agonist Improves Left Ventricular Function and Remodeling in Dogs With Advanced Heart Failure, Circ Heart Fail 6 (2013) 563.

[11] P.S. Wong, J. Hyun, J.M. Fukuto, F.N. Shirota, E.G. DeMaster, D.W. Shoeman, H.T. Nagasawa, Reaction between S-Nitrosothiols and Thiols: Generation of Nitroxyl (HNO) and Subsequent Chemistry, Biochemistry 37 (1998) 5362.

[12] D.W. Shoeman, F.N. Shirota, E.G. DeMaster, H.T. Nagasawa, Reaction of Nitroxyl, an Aldehyde Dehydrogenase Inhibitor, with N-Acetyl-L-Cysteine, Alcohol 20 (2000) 55.

[13] W.D. Gao, C.I. Murray, Y. Tian, X. Zhong, J.F. DuMond, X. Shen, B.A. Stanley, D.B. Foster, D.A. Wink, S.B. King, J.E. Van Eyk, N. Paolocci, Nitroxyl-Mediated Disulfide Bond Formation between Cardiac Myofilament Cysteines Enhances Contractile Function, Circ. Res. 111 (2012) 1002.

[14] V. Sivakumaran, B.A. Stanley, C.G. Tocchetti, J.D. Ballin, V. Caceres, L. Zhou, G. Keceli, P.P. Rainer, D.I. Lee, S. Huke, M.T. Ziolo, E.G. Kranias, J.P. Toscano, G.M. Wilson, B. O'Rourke, D.A. Kass, J.E. Mahaney, N. Paolocci, HNO Enhances SERCA2a Activity and Cardiomyocyte Function by Promoting Redox-Dependent Phospholamban Oligomerization, Antioxidants & Redox Signaling 19 (2013) 1185.

[15] P.J. Farmer, F. Sulc, J. Inorg, Coordination Chemistry of the HNO Ligand with Hemes and Synthetic Coordination Complexes, Biochem. 99 (2005) 166.

[16] K.M. Miranda, R.W. Nims, D.D. Thomas, M.G. Espey, D. Citrin, M.D. Bartberger, N. Paolocci, J.M. Fukuto, M. Feelisch, D.A. Wink, Comparison of the Reactivity of Nitric Oxide and Nitroxyl with Heme Proteins. A Chemical Discussion of the Differential Biological Effects of These Redox Related Products of NOS, J. Inorg. Biochem. 93 (2003) 52.

[17] P.A.S. Smith, G.E. Hein, The Alleged Role of Nitroxyl in Certain Reactions of Aldehydes and Alkyl Halides1, J. Am. Chem. Soc. 82 (1960) 5731.

[18] F.C. Kohout, F.W. Lampe, On the Role of the Nitroxyl Molecule in the Reaction of Hydrogen Atoms with Nitric Oxide, J. Am. Chem. Soc. 87 (1965) 5795.

[19] K.M. Miranda, H.T. Nagasawa, J.P. Toscano, Donors of HNO, Curr Top Med Chem 5 (2005) 649.

[20] K.M. Miranda, T. Katori, C.L. Torres de Holding, L. Thomas, L.A. Ridnour, W.J. McLendon, S.M. Cologna, A.S. Dutton, H.C. Champion, D. Mancardi, C.G. Tocchetti, J.E. Saavedra, L.K. Keefer, K.N. Houk, J.M. Fukuto, D.A. Kass, N. Paolocci, D.A. Wink, Comparison of the NO and HNO Donating Properties of Diazeniumdiolates: Primary Amine Adducts Release HNO in Vivo, J. Med. Chem. 48 (2005) 8220.

[21] K.M. Miranda, A.S. Dutton, L.A. Ridnour, C.A. Foreman, E. Ford, N. Paolocci, T. Katori, C.G. Tocchetti, D. Mancardi, D.D. Thomas, M.G. Espey, K.N. Houk, J.M. Fukuto, D.A. Wink, Mechanism of Aerobic Decomposition of Angeli's Salt (sodium Trioxodinitrate) at Physiological pH, J. Am. Chem. Soc. 127 (2005) 722.

[22] A.S. Dutton, J.M. Fukuto, K.N. Houk, Mechanisms of HNO and NO Production from Angeli's Salt: Density Functional and CBS-QB3 Theory Predictions, J. Am. Chem. Soc. 126 (2004) 3795.

[23] F.T. Bonner, Y. Ko, Kinetic, Isotopic, and Nitrogen-15 NMR Study of N-Hydroxybenzenesulfonamide Decomposition: An Nitrosyl Hydride (HNO) Source Reaction, Inorg. Chem. 31 (1992) 2514.

[24] E.G. DeMaster, B. Redfern, H.T. Nagasawa, Mechanisms of Inhibition of Aldehyde Dehydrogenase by Nitroxyl, the Active Metabolite of the Alcohol Deterrent Agent Cyanamide, Biochem. Pharmacol. 55 (1998) 2007.

[25] K. Rehse, M. Herpel, New NO-Donors with Antithrombotic and Vasodilating Activities, Part 20. Azodioxides Activated by Electron Acceptors in Geminal or Vicinal Position, Arch. Pharm. (Weinheim) 331 (1998) 104.

[26] K. Rehse, M. Herpel, New NO-Donors with Antithrombotic and Vasodilating Activities, Part 21. Pseudonitrosites and Other Azodioxides with Vicinal Electron Acceptors, Arch. Pharm. (Weinheim) 331 (1998) 111.

[27] M.E. Shoman, J.F. DuMond, T.S. Isbell, J.H. Crawford, A. Brandon, J. Honovar, D.A. Vitturi, C.R. White, R.P. Patel, S.B. King, Acyloxy Nitroso Compounds as Nitroxyl (HNO) Donors: Kinetics, Reactions with Thiols, and Vasodilation Properties, J. Med. Chem. 54 (2011) 1059.

[28] S. Donzelli, G. Fischer, B.S. King, C. Niemann, J.F. DuMond, J. Heeren, H. Wieboldt, S. Baldus, C. Gerloff, T. Eschenhagen, L. Carrier, R.H. Böger, M.G. Espey, Pharmacological Characterization of 1-Nitrosocyclohexyl Acetate, a Long-Acting Nitroxyl Donor That Shows Vasorelaxant and Antiaggregatory Effects, J. Pharmacol. Exp. Ther. 344 (2013) 339.

[29] W. Ding, Z. Li, X. Shen, J. Martin, S.B. King, V. Sivakumaran, N. Paolocci, W.D. Gao, Reversal of isoflurane-induced depression of myocardial contraction by nitroxyl via myofilament sensitization to Ca^{2+}, J. Pharmacol. Exp. Ther. 339 (2011) 825.

[30] X. Sha, T.S. Isbell, R.P. Patel, C.S. Day, S.B. King, Hydrolysis of Acyloxy Nitroso Compounds Yields Nitroxyl (HNO), J. Am. Chem. Soc. 128 (2006) 9687.

[31] J.F. DuMond, M.W. Wright, S.B. King, Water Soluble Acyloxy Nitroso Compounds: HNO Release and Reactions with Heme and Thiol Containing Proteins, J. Inorg. Biochem. 118 (2013) 140.

[32] S. Mitroka, M.E. Shoman, J.F. DuMond, L. Bellavia, O.M. Aly, M. Abdel-Aziz, D.B. Kim-Shapiro, S.B. King, Direct and Nitroxyl (HNO)-Mediated Reactions of Acyloxy Nitroso Compounds with the Thiol-Containing Proteins Glyceraldehyde 3-Phosphate Dehydrogenase and Alkyl Hydroperoxide Reductase Subunit C, J. Med. Chem. 56 (2013) 6583.

[33] L.M. Frost, S.M. Courtney, F.A. Brookfield, V.J. Kallish, Novel Nitroso Compounds as Nitroxyl Donors and Methods of Use, US2009/0281062 A1, 1-54.

[34] M.D. Bartberger, J.M. Fukuto, K.N. Houk, On the Acidity and Reactivity of HNO in Aqueous Solution and Biological Systems, Proc. Natl. Acad. Sci. U.S.A. 98 (2001) 2194.

[35] S.B. Solomon, L. Bellavia, D. Sweeney, B. Piknova, A. Perlegas, C.C. Helms, G.A. Ferreyra, S. Bruce King, N.J.H. Raat, S.J. Kern, J. Sun, L.C. McPhail, A.N. Schechter, C. Natanson, M.T. Gladwin, D.B. Kim-Shapiro, Angeli's Salt Counteracts the Vasoactive Effects of Elevated Plasma Hemoglobin, Free Radic. Biol. Med. 53 (2012) 2229.

[36] C.C. Helms, M. Marvel, W. Zhao, M. Stahle, R. Vest, G.J. Kato, J.S. Lee, G. Christ, M.T. Gladwin, R.R. Hantgan, D.B. Kim-Shapiro, Mechanisms of Hemolysis-Associated Platelet Activation, J. Thromb. Haemost. 11 (2013) 2148.

[37] H.A. Woo, H.Z. Chae, S.C. Hwang, K.-S. Yang, S.W. Kang, K. Kim, S.G. Rhee, Reversing the Inactivation of Peroxiredoxins Caused by Cysteine Sulfinic Acid Formation, Science 300 (2003) 653.

[38] B.E. Lopez, D.A. Wink, J.M. Fukuto, The Inhibition of Glyceraldehyde-3-Phosphate Dehydrogenase by Nitroxyl (HNO), Arch. Biochem. Biophys. 465 (2007) 430.

[39] B. Shen, A.M. English, Mass Spectrometric Analysis of Nitroxyl-Mediated Protein Modification: Comparison of Products Formed with Free and Protein-Based Cysteines, Biochemistry 44 (2005) 14030.

[40] A. Tarze, A. Deniaud, M. Le Bras, E. Maillier, D. Molle, N. Larochette, N. Zamzami, G. Jan, G. Kroemer, C. Brenner, GAPDH, a Novel Regulator of the pro-Apoptotic Mitochondrial Membrane Permeabilization, Oncogene 26 (2007) 2606.

[41] B.E. Lopez, C.E. Rodriguez, M. Pribadi, N.M. Cook, M. Shinyashiki, J.M. Fukuto, Inhibition of Yeast Glycolysis by Nitroxyl (HNO): Mechanism of HNO Toxicity and Implications to HNO Biology, Arch. Biochem. Biophys. 442 (2005) 140.

[42] A.J. Norris, M.R. Sartippour, M. Lu, T. Park, J.Y. Rao, M.I. Jackson, J.M. Fukuto, M.N. Brooks, Nitroxyl Inhibits Breast Tumor Growth and Angiogenesis, Int. J. Cancer 122 (2008) 1905.

[43] M. Katrina M., The Chemistry of Nitroxyl (HNO) and Implications in Biology Coordination, Chemistry Reviews 249 (2005) 433.

[44] D.A. Bazylinski, T.C. Hollocher, Evidence from the Reaction between Trioxodinitrate (II) and 15NO That Trioxodinitrate (II) Decomposes into Nitrosyl Hydride and Nitrite in Neutral Aqueous Solution, Inorg. Chem. 24 (1985) 4285–4288.

[45] M.A. Sharpe, C.E. Cooper, Reactions of Nitric Oxide with Mitochondrial Cytochrome c: A Novel Mechanism for the Formation of Nitroxyl Anion and Peroxynitrite, Biochem. J. 332 (Pt 1) (1998) 9.

[46] M.R. Kumar, J.M. Fukuto, K.M. Miranda, P.J. Farmer, Reactions of HNO with Heme Proteins: New Routes to HNO-Heme Complexes and Insight into Physiological Effects, Inorg Chem 49 (2010) 6283.

[47] M.P. Doyle, S.N. Mahapatro, R.D. Broene, J.K. Guy, Oxidation and Reduction of Hemoproteins by trioxodinitrate(II). The Role of Nitrosyl Hydride and Nitrite, J. Am. Chem. Soc. 110 (1988) 593.

[48] K. Rehse, M. Kämpfe, K.J. Schleifer, New NO-Donors with Antithrombotic and Vasodilating Activities, I: 3-Arylalkyl-N-Nitroso-5-Sydnone Imines, Arch. Pharm. (Weinheim) 326 (1993) 483.

[49] T.W. Miller, M.M. Cherney, A.J. Lee, N.E. Francoleon, P.J. Farmer, S.B. King, A.J. Hobbs, K.M. Miranda, J.N. Burstyn, J.M. Fukuto, The effects of nitroxyl (HNO) on

soluble guanylate cyclase activity: interactions at ferrous heme and cysteine thiols, J. Biol. Chem. 284 (2009) 21788.