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A diazen-1-ium-1,2-diolate analog of 7-azabenzobicyclo[2.2.1]heptane: Synthesis, nitric oxide and nitroxyl release, in vitro hemodynamic, and anti-hypertensive studies

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ABSTRACT

1-(7-Azabenzobicyclo[2.2.1]heptane)diazen-1-ium-1,2-diolate (**16**) was designed with the expectation that it would act as a dual nitric oxide (NO) and nitroxyl (HNO) donor that is not carcinogenic or genotoxic. Compound **16**, with a suitable half-life (17.8 min) in PBS at pH 7, released NO (19%) and HNO (22%) during a 2 h incubation in PBS at pH 7. In addition, compound **16** exhibited a significant in vitro positive inotropic effect, increased the rates of contraction and relaxation, and increased coronary flow rate, but did not induce a chronotropic effect. Furthermore, compound **16** (13.7 mg kg^{-1}, po dose) provided a significant reduction in the blood pressure of mice up to 3 h post-drug administration. All these data suggest that compound **16** constitutes an attractive 'lead-compound' that could have potential applications to treat cardiovascular disease(s) such as congestive heart failure.

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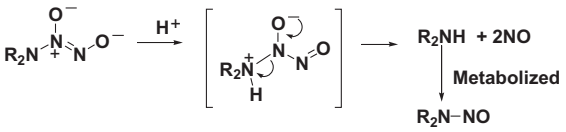
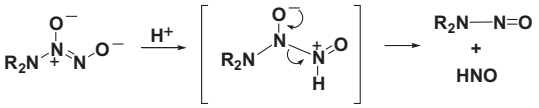
The potent carcinogenicity, toxicity, and potential genotoxicity of nitrosamines [$R^1(R^2)N$–NO; $R^1(R^2)$ = dialkyl, cycloalkyl] has presented medicinal chemistry challenges in the design of biologically active amines. *N*-Nitrosodialkylamines such as *N*-nitrosodimethylamine [$(CH_3)_2N$–NO] are activated via a sequence of metabolic transformations that involve (i) cytochrome P450 mediated α-hydroxylation to give $CH_3(CH_2OH)N$–NO that (ii) subsequently fragments to furnish an active methyldiazonium ion (H_3C–$N^+\equiv N$) prior to (iii) methylation of DNA to produce a DNA adduct.[1] One aspect of our medicinal chemistry research program has been focused on the design of hybrid ester prodrugs wherein diazen-1-ium-1,2-diolated derivatives of amines are conjugated to a cyclooxygenase-1 (COX-1) and/or cyclooxygenase-2 (COX-2) inhibitor. These prodrugs fragment under physiological conditions to release nitric oxide (NO), and/or nitroxyl (HNO), and the parent amine from the *N*-amino-diazen-1-ium-1,2-diolate moiety, together with the parent COX inhibitor. The decomposition pathway of the *N*-amino-diazen-1-ium-1,2-diolate moiety is dependent upon the site of protonation. The site of protonation is influenced by the nature (primary or secondary) of the N^3-amino group, the relative basicity of the N^3-amino and diazen-1-ium-1,2-diolate N^2-nitrogen atoms, the pH of the reaction media and energy (stability) of tautomeric species.[2–4] In this regard, protonation at the amine N^3-nitrogen and then decomposition would ultimately furnish 2 molecules of NO and the amine (R_2NH).[5] Subsequent metabolism of R_2NH may result in formation of a toxic nitrosamine (R_2N–NO) as illustrated in Eq. 1:



(1)

On the other hand, protonation of the diazen-1-ium-1,2-diolate N^2-nitrogen and then decomposition would furnish the nitrosamine (R_2N–NO) directly along with a HNO species as shown in Eq. 2 below:[5]



(2)

Thus, *N*-aminodiazeniumdiolates derived from secondary amines undergo protonation at the amine nitrogen to release 2 molecules of NO (Eq. 1) whereas those derived from primary amines furnish varying ratio's of NO and HNO (Eq. 2).[2–5]

The toxicity of *N*-nitrosodimethylamine [$(CH_3)_2N$–NO] indicated above and *N*-nitrosopyrrolidine (**1**), in conjunction with numerous reports showing that *N*-nitroso-L-proline (**2**) is

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non-tumorigenic in animals,[6–11] prompted the synthesis of the O^2-acetoxymethyl diazen-1-ium-1,2-diolate derivative of L-proline (**3**) that fragments under physiological conditions in the presence of an esterase to release the natural amino acid L-proline (**4**) and 2 molecules of NO.[12] Coupling **3** to the selective ulcerogenic COX-1 inhibitors aspirin and indomethacin furnished the respective hybrid ester NO donor prodrugs **5** and **6** that are transformed to non-ulcerogenic selective COX-2 inhibitors which retain the anti-inflammatory activity of the parent drugs aspirin and indomethacin (see structures **1**–**6** in Fig. 1).[12]

In contrast to the L-proline-diazen-1-ium-1,2-diolate prodrugs **5** and **6**, which undergo protonation of the L-proline nitrogen atom prior to release of NO (Eq. 1), analogs of celecoxib having a tetrahydropyridyl (**7**) or piperidyl (**9**) diazen-1-ium-1,2-diolate moiety attached to the central five-membered pyrazole ring are highly unstable unisolable compounds. Compounds **7** and **9** that undergo protonation at the diazen-1-ium-1,2-diolate N^2-position (Eq. 2) and spontaneously release HNO and the respective *N*-nitrosamine product **8**[13] or **10**[14] as illustrated in Figure 2. Similarly, the unstable and unisolabile (**11**),[15] and the stable and isolabile (**13**),[16] *N*-methylanilino diazen-1-ium-1,2-diolates both undergo protonation at the N^2-diazen-1-ium-1,2-diolate position and release HNO to furnish the respective nitrosamines **12** and **14** (Eq. 2). Compound **13**, having a half-life of 11 min at pH 7.4 and 37 °C, released the nitrosamine **14** (100%) and HNO (100%).

Compounds containing a five-membered L-proline ring (**3**, **5**, **6**) released NO[12] while those having a six-membered tetrahydropyridyl (**7**),[13] piperidyl (**9**),[14] or anilino (**11**[15] and **13**[16] ring released HNO. These data indicate that the release of NO or HNO is dependent upon (i) ring size and the electronic effect of the substituent (pyrazole 4,5-olefinic bond in compound **11** and the 4-cyano substituent in compound **13**) at the *para*-position of an aniline ring. Ohwada et al. recently reported[1] that embedding the nitrosamine functionality within a 7-azabenzobicyclo[2.2.1]heptane structure, such as in compound **15**, blocks the mutagenicity of these nitrosamines. This lack of mutagenicity is attributed to the fact that these nitrosamines are inert to α-hydroxylation which is the first trigger event of mutagenicity. The larger calculated α-H bond dissociation energies for these bicyclic nitrosamines by magnitudes up to 20–30 kcal/mol compared to those for monocyclic nitrosamines and *N*-nitrosodimethylamine is a logical explanation for inhibition of the α-hydroxylation reaction. This important discovery by Ohwada et al. prompted us to investigate the diazen-1-ium-1,2-diolate derivative of 7-azabenzobicyclo[2.2.1]heptane (**16**, see structure in Fig. 3). It was envisaged that **16** has the potential to act as a dual NO/HNO donor. This belief is based on some structural similarities (i) between compounds **16**, IPA/NO (**17**; HNO donor at pH >5 and a HNO and NO donor in the pH 5–8 range),[4] the tetrahydropyridyl compound **7** and the piperidyl compound **9** that each have either

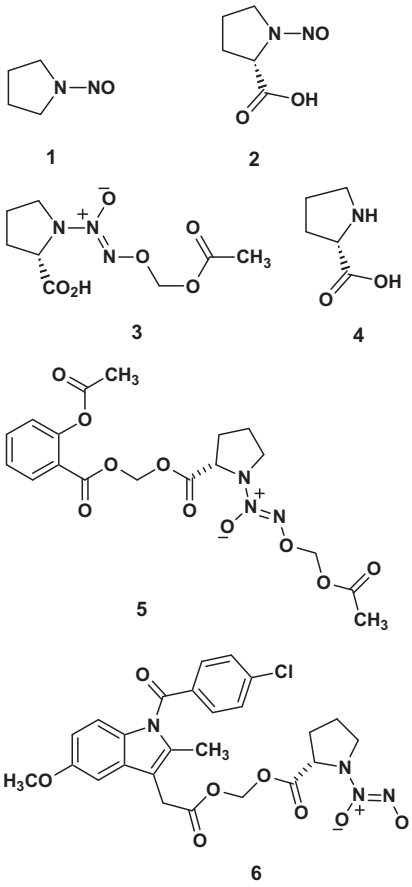


Figure 1. Structures of the putative mutagen *N*-nitrosopyrrolidine (**1**), non-tumorigenic *N*-nitroso-L-proline (**2**), the O^2-acetoxymethyl derivative of the L-proline-diazen-1-ium-1,2-diolate (**3**), L-proline (**4**), and the hybrid NO donor ester prodrugs **5** and **6**.

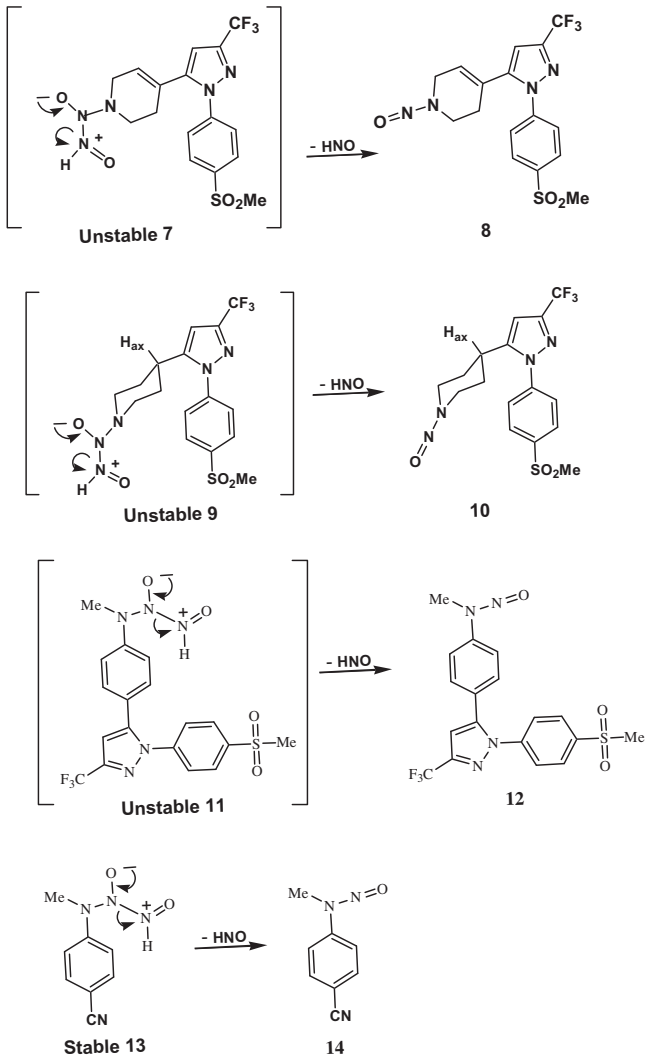


Figure 2. Structures of diazen-1-ium-1,2-diolate derivatives of unstable tetrahydropyridyl (**7**), unstable piperidyl (**9**), and unstable *N*-methylanilino (**11**) analogs of celecoxib, and the stable derivative of *N*-methyl-4-cyanoaniline (**13**).

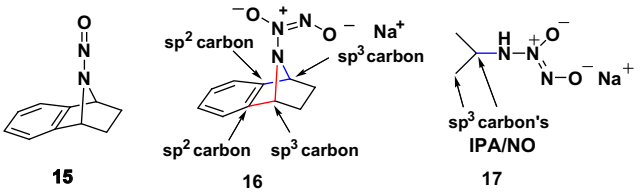


Figure 3. Structures of the non-mutagenic nitrosamine **15**, diazen-1-ium-1,2-diolate derivative of 7-azabenzobicyclo[2.2.1]heptane (**16**) and IPA/NO (**17**).

one or two amino sp^3 α-carbons, and (ii) between **16** which has two amino sp^2 β-carbons in the phenyl ring and the anilino compounds **11** and **13** which each have sp^2 α- and β-carbons in the phenyl ring (see structures **16** and **17** in Fig. 3). We now report the synthesis, biological half-life, NO and HNO release data, some in vitro hemodynamic properties, and anti-hypertensive activity of the target compound **16**.

7-Azabenzobicyclo[2.2.1]heptane (**18**, an alternative chemical name is 1,2,3,4-tetrahydronaphthalen-1,4-imine), required for the synthesis of the target compound **16**, was prepared using synthetic methodologies previously reported.[1] Subsequent reaction of compound **18** with NO gas (40–50 psi) in diethyl ether containing a solution of NaOMe in MeOH (25% w/v) at 23 °C for 24 h afforded the target product sodium 1-(7-azabenzobicyclo[2.2.1]heptane)diazen-1-ium-1,2-diolate (**16**) in 52% isolated yield (see Scheme 1). Ether is used as a solvent such that the product **16** precipitates from solution and is readily isolated by filtration and subsequent washing with dry ether. Compound **16**, after drying under vacuum, is a highly stable product that has currently been stored at 25 °C for a period of 2 months. In contrast, IPA/NO is an unstable and highly explosive compound. It has been reported that IPA/NO preparations can be unstable in the solid state, sometimes decomposing suddenly and without warning or apparent provocation long after isolating the material as a solid.[4]

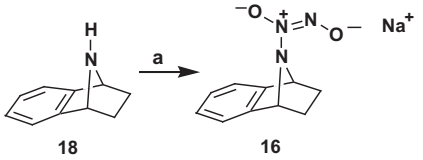
The half-life of **16** was measured using a previously reported spectrophotometric method[17] via monitoring the decrease in the absorbance of the diazeniumdiolate chromophore at about 259 nm in pH 7.4 phosphate buffer at 37 °C. Spectral changes occurring with respect to incubation times are shown in Figure S1 in Supplementary data. A linear regression of the $\ln(A_t - A_\infty)$ versus time plot showed good linearity ($R^2 = 0.993$). The observed half-life ($t_{1/2}$) for **16** in pH 7.4 phosphate buffer solution containing 5% DMSO at 37 °C was 17.8 min, which is about 7.7-fold longer than that of IPA/NO ($t_{1/2} = 2.3$ min).[18] This longer half-life for **16** for may provide a longer in vivo duration of action.

Nitric oxide (NO) is an effective vasodilation agent, and an inhibitor of platelet aggregation and adhesion.[19] Nitroxyl (HNO), the reduced form of NO, exhibits unique biological and pharmacological properties compared to other nitrogen oxides.[20] In the cardiovascular system, HNO exerts a positive inotropic cardiac effect that is independent from β-adrenergic signaling by directly enhancing cardiac sarcoplasmic reticulum Ca^{2+} recycling.[20,21] HNO also protects heart tissue against ischemia-reperfusion injury,[22] it effectively inhibits human platelet aggregation in a rapid and concentration-dependent manner,[23] and it is resistant to superoxide radical anion.[20]

The diazen-1-ium-1,2-diolate moiety incorporated into the target compound **16** is a well established NO donor group. The half-life, and hence rate of NO release from diazen-1-ium-1,2-diolates derived from secondary dialkylamines, is generally dependent upon the nature of the alkyl substituents with smaller alkyl substituents undergoing faster NO release.[24] Therefore, it was of interest to determine the percentage nitrite, derived from the reaction of NO with oxygen in aqueous solution, that is produced upon incubation of compound **16** having a distinctive 7-azabenzobicyclo[2.2.1]heptanes ring system in phosphate-buffered saline (PBS at pH 7.4). The indirect quantification of NO as nitrite anion using the Griess reaction (see data in Table 1) was determined for incubation times of 2 and 4 h. The % nitrite arising from **16** (19.0–21.5%) was very similar to that of the reference compound Angeli's salt (20.8–21.3%) for 2 and 4 h incubations, but much lower than that from IPA/NO (62% for a 1.5 h incubation).

The percentages of HNO released from compounds **16**, IPA/NO and AS were determined by quantification of HNO-derived amide **21** produced by the selective reaction of phosphine **19** with HNO (Scheme 2) released from the test compound upon incubation in PBS at pH 7.4 using a LC–MS assay (see data in Table 1) that was developed in our research program. A detailed description of this assay has been reported,[25] and a summary of plausible reactions of NO and HNO resulting in the formation of NO_2^-, N_2O and/or HNO during the in vitro incubation of IPA/NO is described.[25] This assay uses a small amount of DMSO to solubilize the phosphine **19** which is superior to a previously reported HPLC method that requires 0.5 M NaOH to solubiize **19.**[26] Compound **16** showed a much higher HNO release (22.3%) for a 2 h incubation in PBS at pH 7.4 compared to IPA/NO (5.4%), but much lower than that for Angeli's salt (AS, 85.9%). Collectively, these nitrite and HNO release data show that **16** furnishs a balanced release of NO and HNO under physiological conditions that could be clinically relevant.

Two theoretical pathways for the fragmentation of **16** are illustrated in Figure 4. Thus, the diazen-1-ium-1,2-diolate **16** can protonate the amine nitrogen and then release 2 molecules of NO to furnish **18** which could undergo subsequent metabolism (nitrosation) to give the *N*-nitrosamine **15**. Heterolyic N–NO bond cleavage[27] of **15** may release NO to reform **18** (Path A). Protonation of the N^2-diazen-1-ium-1,2-diolate nitrogen atom and then release of HNO and the nitrosamine **15** could proceed by Path B. These two pathways illustrate plausible mechanisms for the release of



Scheme 1. Reagents and conditions: (a) NO gas (40–50 psi), Et_2O, NaOMe in MeOH (25% w/v), 23 °C, 24 h.

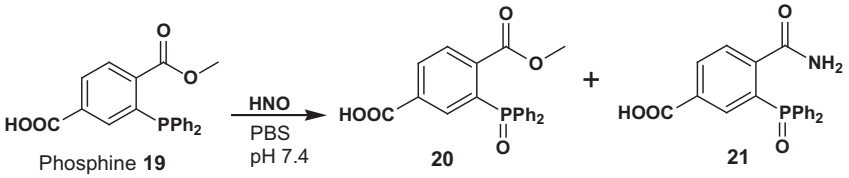
Table 1
Percent (%) nitrite (NO_2^-) and % HNO released from compound **16**, IPA/NO (**17**) and Angeli's salt (AS)

Compound	NO_2^- [a]		HNO [b]	
	2 h	4 h	2 h	48 h
16	19.0	21.5	22.3 ± 0.6	20.2 ± 0.9
IPA/NO (**17**)	62.0	—	5.4 ± 0.1	5.1 ± 1.3
AS[c]	21.3	20.8	85.9 ± 1.3	85.7 ± 3.6

[a] Percentage of nitrite produced (mean value, $n = 3$), based on a theoretical maximum release of 2 mol of NO/mol of test compound, was determined using the Griess reaction. Variation from the mean % value was ⩽0.02%. Incubations were carried out for 2 and 4 h in 2.4 mL of phosphate buffer solution (PBS, pH 7.4) at 37 °C for compounds **16** and AS, and 1.5 h for IPA/NO (**17**).

[b] Percent of HNO released is based on a theoretical maximum release of 1 mol of HNO/mol of test compound **16**, IPA/NO and AS. HNO was selectively trapped by a phosphine ligand **19** to produce an equivalent amount of a HNO-derived amide **21**. Test compounds were incubated for 2 and 4 h at 37 °C in a buffer solution contains 7.5% DMSO and 92.5% phosphate buffer (50 μM) at pH 7.4. Quantification of the HNO-derived amide **21** was performed using a LC–MS assay.[25]

[c] AS (Sodium α-oxyhyponitrite is a common and readily available dual NO/HNO donor reference compound).



Scheme 2. Reaction of phosphine ligand **19** with HNO.

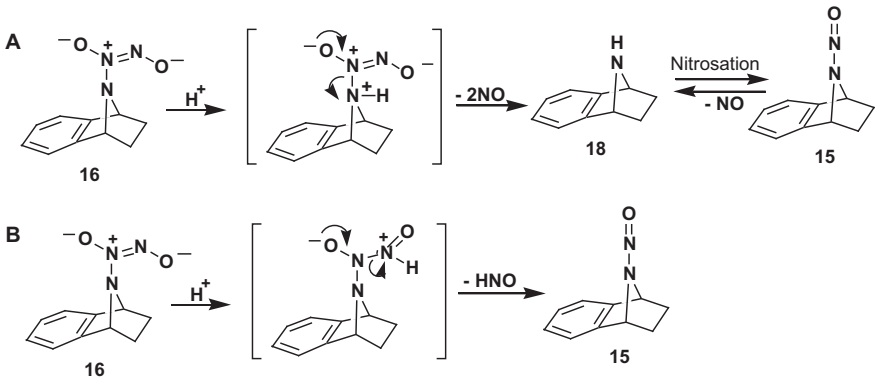


Figure 4. (A and B) Theoretical fragmentation and biotransformation of compound **16**.

both NO and HNO from compound **16**. The nitrosamine **15** is non-genotoxic[1] since it does not undergo α-hydroxylation that is a trigger event ultimately required for the O^6-alkylation of 2′-deoxyguanosine in DNA. In comparison, an isopropyl cation derived from *N*-isopropyl-*N*-(1-hydroxyethyl)nitrosamine via an intermediary isopropyl diazonium ion preferentially alkylates 2′-deoxyguanosine. This finding suggests that any isopropyl cation arising from the decomposition of IPA/NO would similarly induce a genotoxic effect.[28]

The positive inotropic effect induced by HNO is considered to be a unique biological and pharmacological action that differs from that of NO. It was therefore of interest to carry out the title studies for compound **16** which is an effective HNO donor. To determine whether compound **16** possessed positive inotropic properties, hearts from C57BL/6 mice were perfused for 20 min (Table 2). All hearts had normal baseline contractile function, measured either as left ventricular developing pressure (LVDP; inotropic effect), diastolic and systolic contraction, heart rate (HR) and coronary flow rate. Hearts perfused with compound **16** (10 μM) demonstrated a significant increase in LVDP (24%, positive inotropic effect), contraction rate (dP/dt_{max}; 30%), and coronary flow rate (53%) when compared to baseline levels. In comparison, a modest increase (9%) in the rate of relaxation (dP/dt_{min}) and a significant decrease (22%) in HR was observed (see data in Table 2). These data demonstrate compound **16** exhibits significant inotropic effects impacting cardiac function.

Systolic blood pressure (BP_{sys}, mm Hg), diastolic blood pressure (BP_{dia}, mm Hg), and heart rate (HR, beats min^{-1}) were measured at 1, 3 and 6 h time intervals following oral administration of either the vehicle alone (control group, $n = 6$), or the NO/HNO donor compound **16** (60.345 μmol kg^{-1}, or 13.7 mg kg^{-1}, po; $n = 6$) to C57 black mice. A minimum of three consecutive measurements were made for each mouse at each time interval and the mean value is the average of these three measurements. The data obtained for BP_{sys}, BP_{dias}, BP_{mean} (average of BP_{sys} and BP_{dia}) and HR are illustrated in Figure 5, Panels a–d (Table S1 listing numerical data ± standard deviation is provided as Supplementary data).

The diazen-1-ium-1,2-diolate compound **16** showed a significant reduction ($^*p < 0.05$) in BP_{sys} (mm Hg) at 1 (105.5) and 3 (106.5), but not at 6 (128.9), hours post-drug administration relative to the control group (1 h, 126.5; 3 h, 118.3; 6 h, 126.2; see data in Fig. 5a). A significant reductions in BP_{dia} was observed at 1 h (70.6), but not at 3 h and 6 h, post-drug administration (see data in Fig. 5b).The BP_{mean} profile (Fig. 5c) shows that there was a significant reduction in BP_{mean} at 1 and 3 h, but not at 6 h (1 h, 82.3; 3 h, 85.8; 6 h, 101.1) relative to control values (1 h, 106.0; 3 h, 97.5; 6 h, 105.7). These BP data indicate that **16** has a relatively short antihypertensive duration of action of about 3 h. The HR of mice was determined and the data are shown in Figure 5d (complete data is provided in Table S1 as Supplementary data). The HR at 1 h (605) and 3 h (554) and 6 h (466) was higher than that for control mice (449, 448 and 439 beats min^{-1} at 1, 3 and 6 h post-drug

Table 2
Cardiac hemodynamic parameters in isolated perfused hearts

Compound	Baseline	Compound **16** [a] (10 μM)	Compound **16** [a] (100 μM)
LVDP (cmH_2O)	169 ± 2	209 ± 8*	213 ± 3*
Rate of contraction, dP/dt_{max} (cm H_2O/ms)	4178 ± 141	5411 ± 292*	5547 ± 217*
Rate of relaxation, dP/dt_{min} (cm H_2O/ms)	−3318 ± 161	−3629 ± 138	−3945 ± 128
Heart rate, perfused (beats/min)	342 ± 11	268 ± 17*	307 ± 15*
Coronary flow rate (mL/min)	2.62 ± 0.1	4.03 ± 0.2*	3.92 ± 0.1*

[a] Values are mean ± SEM.
* $p < 0.05$ versus baseline, $n = 4$ for all groups.

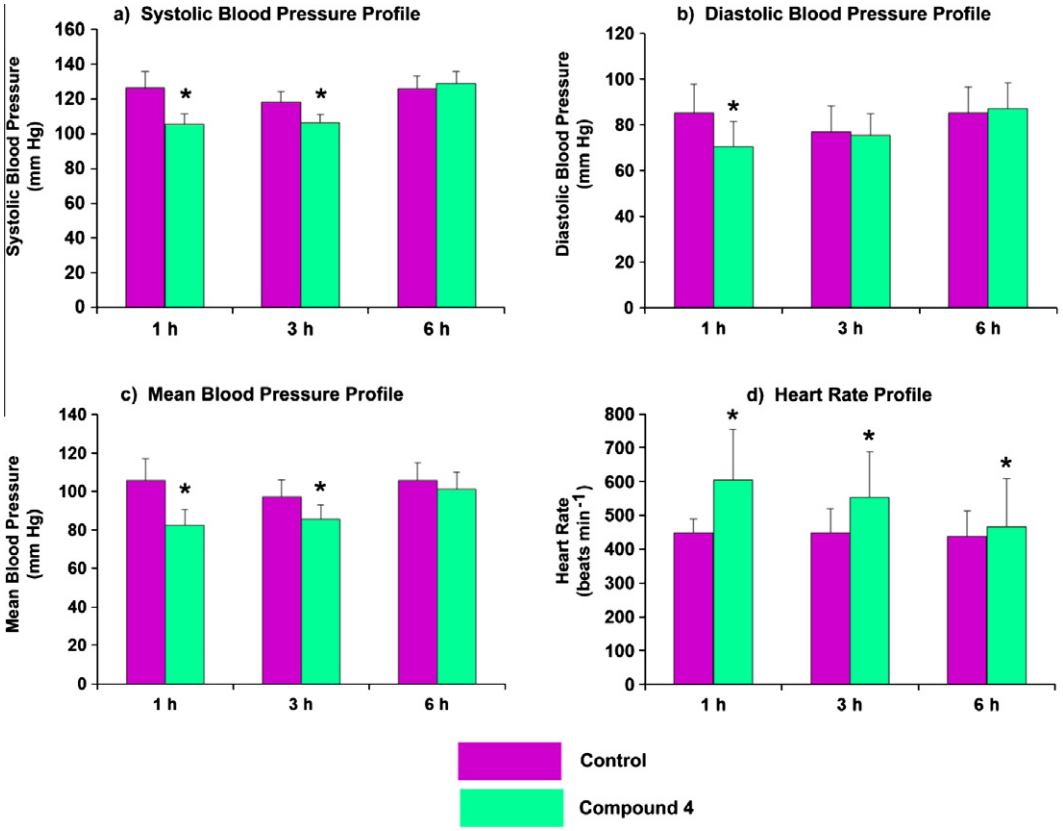


Figure 5. Systolic blood pressure (BP_{sys}), diastolic blood pressure (BP_{dia}), mean blood pressure (BP_{mean}), and heart rate (HR) in conscious mice following oral administration of either vehicle alone (control group, mean ± SD, $n = 6$), or NO/HNO donor compound **16** (60.345 mol kg^{-1} po or 13.7 mg kg^{-1} po; mean ± SD, $n = 6$). Data are presented as group mean ± SD. Statistical differences between groups are denoted as $^{*}p < 0.05$ and ns = no significant difference ($p > 0.05$).

administration, respectively). It is plausible that this in vivo increase in HR is attributed to a neuronal input that increases HR in order to compensate for the reduction in blood pressure. In comparison, a 22% reduction in HR rate was observed in the in vitro (ex vivo) perfused heart study described previously where a neuronal input is not operative.

In conclusion, the hither-to-unknown 1-(7-azabenzobicyclo[2.2.1]heptane)diazen-1-ium-1,2-diolate (**16**) described in this study[29] possesses the following distinctive chemical and/or biological advantages compared to IPA/NO: (i) compound **16**, unlike the unstable and highly-explosive parent IPA/NO,[2,4] is a stable readily isolated compound, (ii) compound **16** has a longer improved half-life (17.8 min) under physiological conditions relative to IPA/NO which has a very short half-life (2.3 min),[18] (iii) in contrast to diazen-1-ium-1,2-diolate derivatives of secondary amines which release NO after protonation of the amine nitrogen atom, compound **16** also undergoes protonation at the N^2diazen-1-ium-1,2-diolate nitrogen atom to also release HNO that was quantified by selective formation of the HNO-derived amide **17** using an efficient LC–MS assay, (iv) the nitrosamine compound **15**, which is a potential metabolite of **16**, is expected to be non-carcinogenic and non-genogenic,[1] (v) compound **16** showed a significant in vitro inotropic effect, increasing the force of contraction and coronary flow rate, and (vi) compound **16** exhibited an anti-hypertensive effect since BP_{mean} was lowered significantly up to 3 h post-drug administration. These results reveal that compound **16** is a novel chemically stable compound with excellent dual NO/HNO release properties at physiological pH. Accordingly, compound **16** is a novel type of lead-compound with relevant pharmaceutical properties for the design of dual NO/HNO donors with potential applications to treat cardiovascular disease(s) including congestive heart failure.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.02.040.

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29. *General:* Melting points were measured in capillaries using a Thomas–Hoover capillary apparatus and are uncorrected. ^{1}H and ^{13}C NMR spectra were measured on a Bruker AVANCE III 600 NMR spectrometer using D_2O as solvent. Infrared (IR) spectra were recorded as films on NaCl plates using a Nicolet 550 series II Magna FTIR spectrometer. The reaction of **18** with NO to prepare **16** was carried out using a Parr 5100 Low Pressure Reactor. The purity of compounds was assessed on the basis of elemental analysis and these microanalyses were performed for C, H, and N by the Microanalytical Service Laboratory, Department of Chemistry, University of Alberta. 7-Azabenzobicyclo[2.2.1]heptane (**18**) was prepared according to the reported method.[1] Nitric oxide gas was purchased from Praxair (Edmonton, Canada). All other reagents, purchased from the Aldrich Chemical Co. (Milwaukee, WI), were used without further purification. IPA/NO (**17**) and the phosphorous compounds **19**–**21** were prepared using the methods previously reported.[25] Animal experiments used male and female mice aged 3–5 months that were 22–33 g in weight. Mice were treated in accordance with guidelines and protocols approved by Health Science Laboratory Animal Services (HSLAS), University of Alberta. C57BL/6 mice were purchased from Charles River Laboratories (Pointe Claire, PQ).
*Sodium 1-(7-azabenzobicyclo[2.2.1]heptane)diazen-1-ium-1,2-diolate (**16**):* A solution of 7-azabenzobicyclo[2.2.1]heptane (**18**, 900 mg, 6.19 mmol) in dry diethyl ether (50 mL) was mixed with a solution of 25% NaOMe in MeOH (0.42 mL, 7.42 mmol). The resulting solution was flushed with argon, charged with 40–50 psi of NO, and stirred at 23 °C. After 24 h, the pressure was released, the product that had precipitated was collected by filtration, the precipitate was washed with ether (2 × 10 mL), and the solid was dried under vacuum to give the target product **16** (731 mg, 51.9%) as a stable non-explosive grey-white powder; mp 275–277 °C; IR (film) 2960, 1232, 1095 cm^{-1}; UV–vis, 5% DMSO in PBS pH 7.4, λ_{max} = 259 nm (ε = 2213 M^{-1} cm^{-1}, ^{1}H NMR (600 MHz, D_2O): δ 1.30–1.33 (2H, m, CH*H*′ + CH*H*′), 2.13–2.18 (2H, m, C*H*H′ + C*H*H′), 5.15–5.20 (2H, m, NC*H* + NC*H*′), 7.18–7.33 (4H, m, Ar*H*s); ^{13}C NMR (150 MHz, D_2O): 23.55 (2 × CH_2), 66.42 (2 × NCH), 121.43 (ArCH), 128.35 (ArCH), 142.07 (ArC). Anal. Calcd for $C_{10}H_{10}N_3NaO_2$: C, 52.87; H, 4.44; N, 18.50. Found: C, 51.67; H, 4.10; N, 16.75.
*Measurement of compound **16** half-life:* The half-life of compound **16** was measured spectrophotometrically by monitoring the decrease in absorbance of the diazeniumdiolate chromophore at about 259 nm wavelength using a micro-volume UV–vis spectrophotometer (Thermo Scientific NanoDrop 2000). A solution of **16** in DMSO solution (150 μL of 6 mM) was added to the buffer solution (2.85 mL of pH 7.4 50 mM PBS) in a cuvette. The final concentration of **16** was 300 μM. The mixture was stirred for incubation times from 0 to 24 h at 37 °C. Wavelength scannings within the 200–400 nm range were recorded with respect to incubation time. The absorbance at each wavelength was recorded and overlapped absorbances were stacked to form a graphic (see Fig. S1 in Supplementary data) illustrating the spectral changes accompanying the decomposition (fragmentation) of compound **16** with respect to incubation time. The maximum absorbance at each time point at 259 nm was recorded to calculate the half-life. Absorbance − time ($A - t$) data was obtained and first-order rate constants were calculated from a linear regression of the $\ln(A_t - A_\infty)$ plots, which generally showed good linearity (R^2, 0.993). The $t_{1/2}$ value was calculated using the following equations: $\ln[A]_t = -kt + \ln[A]_0$ and $t_{1/2} = \ln(2)/k$.
Nitric oxide release assay: In vitro nitric oxide release was estimated by quantification of nitrite produced by the reaction of nitric oxide with oxygen and water using the Griess reaction upon incubation of the test compound (2.4 mL of 5.0 × 10^{-2} mM) in phosphate buffer solution (PBS) at pH 7.4 at 37 °C for 2/4 h time intervals. The amount of nitric oxide released at each time interval was determined for test compounds **16**, IPA/NO (**17**) and AS using the reported procedure.[30]
LC–MS quantification of phosphine-mediated HNO trapping: A solution of phosphine **19** in DMSO (25 μL), the test compound (**16**, IPA/NO or AS) in DMSO (25 μL) and internal standard compound 4-hydroxybenzophenone in DMSO (25 μL) were added to a PBS solution pH 7.4 (925 μL), providing final concentrations of **19** (1 mM), test compound (200 μM), and 4-hydroxybenzophenone (5.05 μM). The incubation mixture was stirred at 37 °C in a sealed vial for 2 h or 48 h. Hydrogen peroxide solution (30% w/v, 5 μL) was added into the incubation mixture to quench the reaction of **19** and HNO. An aliquot (500 μL) was removed and the solution was evaporated to dryness under vacuum (using a Thermo Scientific Savant DNA 120 SpeedVac Concentrator) at about 60 °C. Methanol (500 μL) was added to dissolve the residue. After centrifugation, an aliquot of the clear methanol solution (250 μL) was analyzed by LC–MS (Water's Micromass ZQTM 4000 LC–MS, operating in the ESI negative mode, equipped with a Water's 2795 Separation Module). Separations were performed in triplicate using a Kromasil 100-5-C18 (100 μM pore size, 5 μM particle size) reverse phase column (2.1 mm diameter × 50 mm length), preceded by a Kromasil 100-5-C18 2.1× guard column. Separations were effected using a gradient going from MeCN:1% aqueous formic acid (40:60, v/v) to MeCN:1% aqueous formic acid (60:40, v/v) over a 12 min period at a flow rate of 0.25 mL/min. Operating parameters were as follows: Capillary voltage = 3.5 kV; Cone voltage = 20 V; Source temperature = 140 °C; Desolvation temperature = 250 °C; Cone nitrogen gas flow = 100 L/h; Desolvation nitrogen gas flow = 550 L/h. The identities of products **20** (retention time of 6.62 min), 2**1** (retention time of 3.63 min) and the internal standard 4-hydroxybenzophenone (retention time of 10.89 min) were confirmed by MS (LC–MS chromatograms are provided as Supplementary data). The amount of compound **21** produced upon incubation of the test compound was determined from a standard curve (that was linear over a concentration range of 0.2–10 μg/mL, R^2 >0.997) prepared using an authentic sample of compound **21**. The LC–MS HNO release data presented in Table 1 is the mean of triplicate experiments. A control experiment involving incubation of compound **19** and 4-hydroxybenzophenone under identical conditions, but not containing a test compound, and subsequent LC–MS analysis showed the absence of **21**.
*Determination of cardiac hemodynamic properties for compound **16**:* C57BL/6 mouse hearts were perfused in the Langendorff mode as described previously.[31–33] Briefly, hearts were perfused in a retrograde fashion at constant pressure (90 cm H_2O) with continuously aerated (95% O_2/5% CO_2) Krebs-Henseleit buffer at 37 °C. Hearts were first stabilized for 20 min with buffer and then perfused with compound **16** (10 or 100 μM) for 20 min. Heart rate (HR), diastolic and systolic rates, left ventricular developing pressure (LVDP) and coronary flow rate were measured.
In vivo blood pressure measurement: Non-invasive blood pressure and heart rate measurements were performed according to a protocol approved by the Health Sciences Animal Welfare Committee at the University of Alberta (Edmonton, Canada). Following oral administration of test compound **16** (60.345 μmol kg^{-1}, or 13.7 mg kg^{-1}, po dose) dissolved in water containing 1% methyl cellulose, changes in BP_{sys}, BP_{dia}, BP_{mean} and HR in C57 black mice were measured in a conscious state at 1, 3 and 6 h time intervals using our previously reported method.[34]
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