gem-Dinitroalkyl Benzenes: A Novel Class of IOP-Lowering Agents for the Treatment of Ocular Hypertension

Gem-dinitroalkyl benzenes: a novel class of IOP-lowering agents for the treatment of ocular hypertension

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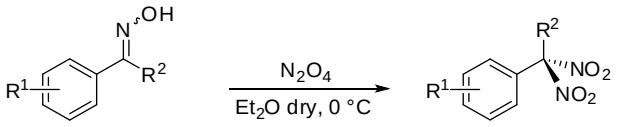
ABSTRACT: Primary open angle glaucoma is the second most common cause of blindness worldwide. Nitric oxide has recently received particular attention as a potential anti-glaucoma agent. In this work, gem-dinitroalkyl benzenes are evaluated for their capability to act as a new class of IOP lowering agents. These derivatives have been endowed with a variety of NO-release capacities and found to relax contracted rat aorta strips in a concentration-dependent manner. They have been studied for their IOP-lowering activity in a transient ocular hypertensive rabbit model at 1% dose. The most effective IOP-lowering products were compounds **9**-**11** and **13**, whose activity was similar to that of Molsidomine 120 min after administration. Compounds **9** and **13** were selected for evaluation using carbomer-induced glaucoma as the chronic model of IOP. They cause a significant reduction in IOP in the first 24 hours and their activity is maintained over five days, displaying a Molsidomine-like profile.

Primary open angle glaucoma (POAG), the most common form of glaucoma, is the second most common cause of blindness worldwide and shows higher incidence in developing than in developed countries. Its prevention and treatment have been major objectives for the World Health Organization (WHO) VISION 2020 program.[1] Current pharmacological treatments for the disease aim to lower intraocular pressure (IOP), the principal risk factor for the disease, which is caused by an imbalance in the rate of aqueous humor (AH) production by the ciliary process and/or by reduced AH drainage through either the trabecular meshwork, Schlemm's canal or the uveo-scleral route.[2] Several classes of drugs are used to achieve this aim, including beta-blockers, alpha-agonists, carbonic anhydrase inhibitors, miotics and prostaglandin analogues.[3] The use of nitric oxide as a potential anti-glaucoma agent has recently become the focus of particular attention .[4] NO is an endogenous messenger that is almost ubiquitous in the human body where it exerts a variety of effects. It is produced under the action of NO synthase (NOS), an enzyme which exists in three isoforms; the constitutively expressed endothelial NOS (e-NOS, NOS-III), neuronal NOS (n-NOS, NOS-1) isoforms and the inducible NOS isoform (i-NOS, NOS-II).[5,6] All three NOS isoforms are present in the eye, where NO mediates a multitude of ocular effects including IOP maintenance and the control of basal ocular vascular tone through the activation of the soluble guanylate cyclase (sGC) signaling pathway.[4,7-8] Moreover, a number of products are able to produce NO in physiological conditions (NO-donors) and can exert a variety of effects in the eye, including ocular blood flow modulation. In fact, some have been studied as potential anti-glaucoma drugs.[4,9] A number of hybrid products that derive from the coupling of anti-glaucoma drugs with nitrooxy (-ONO_2), NO-donor moieties, have therefore been developed.[10] Two prostaglandin F2alpha (PGF2α) hybrids are in clinical trials,[11-13] and one has been approved by FDA.[14]

A new class of IOP lowering agents, 1,1-dinitroethyl/dinitromethyl benzenes, are herein described as a continuation of our studies into potential anti-glaucoma agents [9,15]. The preparation of these products, their physical-chemical characterization and an investigation into their ability to spontaneously release nitrite at physiological pH and via reaction with cysteine are reported. The ability of this class of compound to reduce IOP in transient ocular hypertensive rabbit model (tOHT) and to relax rat aorta strips, which had been pre-contracted with L-phenylephrine, is discussed herein.

The synthesis of 1,1-dinitroethyl/dinitromethyl benzene derivatives **8**-**14**, bearing different electron-withdrawing and electron-donating substituents on the aromatic ring, was easily accomplished by means of a well-known procedure firstly reported by Ponzio,[16] and further reinvestigated by H. Suzuki in 1988.[17] The treatment of stereoisomeric (*E*)/(*Z*) mixtures,[18] of acetophenone oxime derivatives **1**-**5** with a solution of dinitrogen tetroxide in dry diethyl ether (Et_2O) at low temperature afforded the corresponding 1,1-dinitroethyl benzene derivatives **8**-**12** in moderate yields. The reaction also proceeded with benzaldoxime derivatives **6** and **7** to afford the corresponding 1,1-dinitromethyl benzenes **13** and **14** in slightly lower yields (Scheme 1). Partition coefficient (logP) values for the *gem*-dinitro derivatives **8**-**12**, distribution coefficient values at physiological pH ($logD^{7.4}$) for compounds **13**-**14**, obtained using the shake-flask technique at room temperature, and ionization constants determined by potentiometric titration for derivatives **13** and **14**, are reported in Table 1.

Scheme 1. Synthesis of *gem*-dinitro derivatives 8-14.



1-5 R^2 = Me
6-7 R^2 = H

8-12 R^2 = Me
13-14 R^2 = H

Oxime	Product	R^1	R^2	Yield (%)[a)]
1	**8**	H	Me	46
2	**9**	4-Me	Me	50
3	**10**	4-CF_3	Me	53
4	**11**	4-OMe	Me	52
5	**12**	4-OTEG[b)]	Me	53
6	**13**	H	H	41
7	**14**	4-OMe	H	39

a) Isolated yields, b) OTEG = 2-(2-(2-methoxyethoxy)ethoxy)ethoxy.

An analysis of the data shows that the partition coefficients for compounds **8**-**12** are well-distributed over one logarithmic unit interval (1.63 to 2.92). Compound **8**, without any substitution on the aromatic ring, shows a logP value of 2.34 which falls in the optimal lipophilicity for corneal permeation.[19 and ref. therein reported]

The introduction of a substituent in the *para*-position of the aromatic ring modulates the lipophilicity, according to the series **10** > **9** > **8** > **11** > **12**. These data are in good accordance with those calculated using the Hansch π constants of the related substituents (π_{CF3} = 0.88, π_{CH3} = 0.56, π_{OCH3} = -0.02). Compound **12** is the least hydrophobic of the series due to the presence of the polar oxa-alkyl substituent, whose Hansch π constant value is unknown. Our experimental results indicate that it should be close to -0.70 ($logP_{(12)}$-$logP_{(8)}$). Lipophilicity differs markedly for products **13** ($logD^{7.4}$= -1.14) and **14** ($logD^{7.4}$= -1.21). The presence of the acidic benzylic carbon atoms, which bear the two nitro groups, means that derivatives **13** and **14** can exist in neutral or ionized forms, depending on their ionization constants (pK_as) and the pH of the medium. Since this feature has an important influence on the lipophilic-hydrophilic balance of the products, their pK_as were measured via potentiometric titration, giving values of 3.90 and 3.77 respectively. This means that the products exist prevalently in the ionized form at physiological pH (Table 1).

The capacity of the *gem*-dinitro derivatives to release nitrite was evaluated over 24 h of incubation in phosphate buffer (PBS, pH 7.4) both in the absence and in the presence of an excess of L-cysteine (1:50), by Griess reaction. The results reported in Table 1 showed the extent of nitrite release by the compounds at 1 h and 6 h incubation, expressed as percent mol/mol of NO_2^-. No nitrite formation occurs for 1,1-dinitroethyl derivatives **8**-**10** in the absence of the thiol cofactor in these experiments, while the amount of nitrite released over 24 h of incubation increases significantly, and in a linear time-dependent manner, in the presence of a large excess of L-cysteine (see Figure 1A for kinetic plots). The efficiency of nitrite release occurs according to the series **10** > **8** ≥ **9**. The exact mechanism of the reaction will require additional dedicated investigation, although the behavior could be explained by the conventional nucleophilic displacement of a nitro group under the action of the thiol cofactor, or perhaps more probably, an electron transfer nucleophilic displacement (electron transfer chain process)[20] Derivative **11**, which bears the relatively efficient, electron donating methoxy group (σ_p= - 0.27) in the *para*-position, is able to release nitrite spontaneously over 24 h incubation in PBS (Figure 1B). The thiol-independent mechanism is proposed to explain this finding (Scheme 2), since *p*-OCH_3 acetophenone was detected as the predominant degradation product by HPLC. Similar behavior is shown by compound **12** which bears a polar glycolic alkoxy substituent in the para-position of the aromatic ring (Figure 1B).

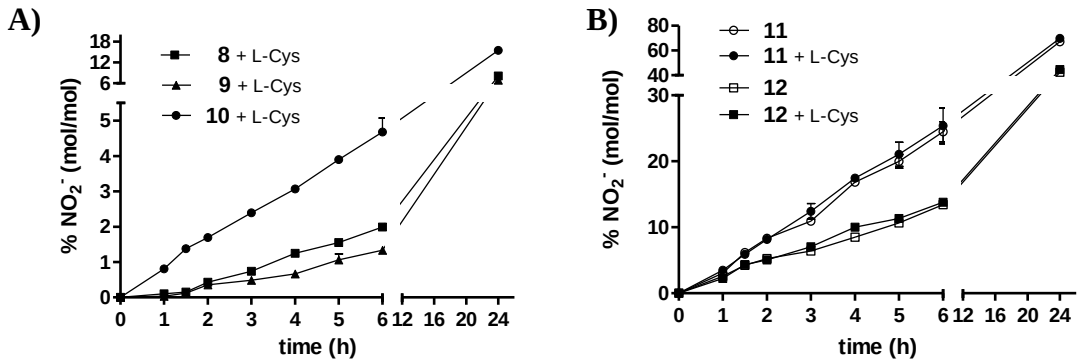


Figure 1. (A). Nitrite (NO_2^-) release kinetics for compounds **8**, **9**, **10** in a phosphate buffer at pH 7.4 in the presence of L-cysteine (L-Cys, 50x) over time (24 h); nitrite release is not observed in the absence of L-Cys. **(B)** Nitrite (NO_2^-) release kinetics for compounds **11** and **12** in phosphate buffer at pH 7.4 in the absence (○ **11**, □ **12**) and in the presence of L-Cys (50x, ● **11**, ■ **12**) over time (24 h). Results are expressed as percentage (% mol/mol) of nitrite released. The symbols represent data from three or more replicates and error bars represent standard deviation from the mean.

As mentioned above, 1,1-dinitromethyl derivatives **13** and **14** are acidic compounds. Compound **13** is able to release nitrite only under the action of cysteine (Table 1), while the *para*-methoxy substituted compound **14** only releases nitrite spontaneously at acidic pH. HPLC analyses of an acidic solution of compound **14** after 24 h of incubation highlight the presence of anisic acid as final product, which can be rationalized as being a Nef-like reaction mechanism, as proposed in Scheme 3. On the other hand, the spontaneous nitrite donor **11**, which bears the same electron-donating substituent, behaves as a non pH-dependent spontaneous nitrite donor (Figure 2).

Table 1. Physico-chemical properties (lipophilicity descriptors log*P* and log$D^{7.4}$, ionization constant), and extent of nitrite release in the presence and absence of L-cysteine at physiological pH.

	Physico-chemical properties			**% NO_2^- ± SE [c]**			
				- L-cys		**+ L-cys**	
Compound	**logP ±SE [a]**	**log$D^{7.4}$ ±SE [a]**	**pK_a [b]**	**1 h**	**6 h**	**1 h**	**6 h**
8	2.34 ±0.11	-	-	-	-	< 0.5	2.0 ±0.3
9	2.69 ±0.11	-	-	-	-	< 0.5	1.3 ±0.3
10	2.92 ±0.05	-	-	-	-	0.8 ±0.1	4.6 ±0.2
11	2.20 ±0.12	-	-	3.0 ±0.1	24.9 ±1.2	3.5 ±0.1	25.4 ±1.2
12	1.63 ±0.09	-	-	2.6 ±0.2	12.8 ±1.2	2.7 ±0.3	13.8 ±0.5
13	-	-1.14 ±0.04	3.77 ±0.01	-	-	3.0 ±0.07	30.0 ±0.8
14	-	-1.21 ±0.04	3.90 ±0.01	1.3 ±0.1[d]	4.0 ±0.2 [d]	-	-

a) measured using the shake-flask method at room temperature; b) determined by potentiometric titration with the GlpK_a apparatus ($n \geq 3$); c) percentage NO_2^- (mol/mol) ± SE, released with respect to the quantity of compound incubated at 37 °C in phosphate buffer (pH=7.4) in the absence and in the presence of an excess of L-Cysteine (1:50), $n \geq 3$.; d) determined after incubation at 37 °C in 30 mM HCl (pH 1.5) in the absence of L-Cysteine.

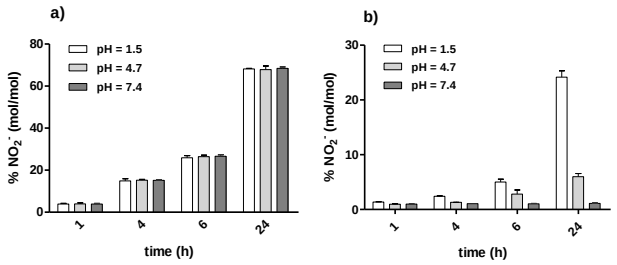
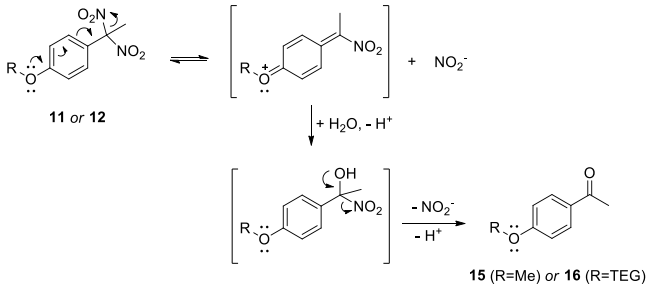


Figure 2. pH-dependence of nitrite (NO_2^-) release for compounds **11** (**a**) and **14** (**b**). The results are expressed as a percentage (% mol/mol) of nitrite released with respect to the quantity of compound incubated. The bars represent data from three or more replicates and error bars represent standard deviation from the mean.

Scheme 2. Proposed mechanism for spontaneous nitrite release from compounds 11 and 12.



The vasodilating activity of all the final products was assessed on denuded rat aorta strips that had been pre-contracted with L-phenylephrine under normoxic conditions. All 1,1-dinitroethyl derivatives **8-12** were able to relax the contracted strips in a concentration-dependent manner. Their EC_{50} values are reported in Table 2. When the experiments were repeated in the presence of 1 μM ODQ (1*H*-[1,2,4]oxadiazolo-[4,3-*a*]quinoxalin-1-one), a well-known inhibitor of sGC, their potencies were partly reduced according to the involvement of NO in the vasodilation (Table 2). This behavior could be due to the ability of the compounds to release nitrite at physiological pH under the action of the thiol cofactor (compounds **8**-**10** and **13**) or spontaneously (compounds **11**, **12** and **14**).

Scheme 3. Proposed Nef-like reaction mechanism for the spontaneous nitrite release from 14.

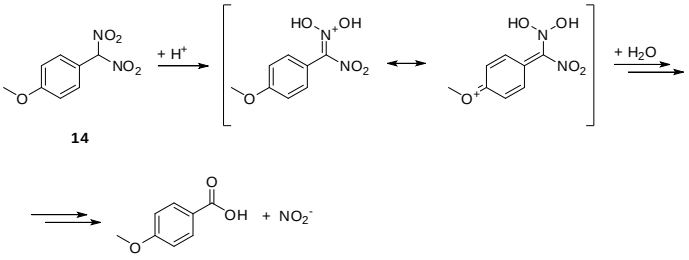


Table 2. Vasodilating activity of compounds 8-14.

Compound	EC_{50} (μM) ± SE	EC_{50} (μM) ± SE + ODQ 1 μM
Molsidomine	0.10 ± 0.02	18 ± 3
8	50 ± 2	- [a]
9	24 ± 4	99 ± 5
10	4.0 ± 0.7	55 ± 3
11	20 ± 2	64 ± 3
12	54 ± 6	- [b]
13	- [c]	-
14	4.4 ± 0.7	64 ± 4

a) % relax at 100 μM: 19 ± 2; b) % relax at 100 μM: 40 ± 5; c) % relax at 100 μM: 24 ±1

Nitrite, the principal metabolite of the aerobic oxidation of NO, is a circulating store in blood of NO. Its transformation from NO can either occur non-enzymatically via chemical disproportionation or via reduction under the acidic and highly reducing conditions which occur in some disease states, as well as via a redox reaction with a number of metal-containing proteins.[21,22] Nitrite can also react with thiols to give nitrosothiols which are involved in vascular smooth muscle relaxation.[23,24] It is able to relax aortic vessels under normoxic conditions at high concentrations (100 to 1000 µM), via a NO-dependent pathway,[23,25] but its vasodilator potency is higher in hypoxic vessels (10 µM), via both NO-dependent and independent pathways.[25-27] The vasodilator potency of tested compounds follows the series **10** ≥ **14** > **11** ≥ **9** > **8** ≥ **12** > **13**. They are less potent than molsidomine but much more potent than sodium nitrite. This behavior can be reasonably attributed to the extremely slow passive diffusion rate of the nitrite anion (HNO_2 pK_a = 3.14), across the highly lipophilic membrane of smooth muscle cells. This should not be the case for the tested compounds which can more easily cross the membrane and release nitrite inside the cell. The situation is different for 1,1-dinitromethyl derivatives **13** and **14**. Both products should arrive inside the cell in low amounts since they are quite strong acids (pK_a= 3.77 and 3.90 respectively), which exist in large prevalence as hydrophilic anions at physiological pH (7.4), and are thus endowed with reduced membrane crossing capacity. The feeble vasodilating capacity of **13** could be justified by its non-optimal hydrophilic lipophilic balance ($logD^{7.4}$ = - 1.14). By contrast, the good vasodilating capacity of **14** may be caused by the spontaneous release of nitrite, which is a sGC-dependent vasodilator.

Compounds **8-14** were studied in comparison with Molsidomine, a well-known NO-donor, for their IOP-lowering effects in a transient ocular hypertensive rabbit (tOHT) model following a 1% topical dose. The results are shown in Table 3. IOP response 120 min after administration follows the series; **9** ≥ **10** ≥ **13** ≥ **11** >> **8** ≈ **14** > **12**. The effect practically vanished after 240 min. The most active compounds, **9-11** and **13**, showed potencies and profiles that were similar to Molsidomine, which was selected as reference long-lasting NO-releaser.[9,12]

Table 3. IOP-lowering effects of tested compounds in a transient ocular hypertensive rabbit (tOHT) model.

Compound (1%)	IOP lowering efficacy (mmHg)		
	60 min	120 min	240 min
Molsidomine	-6.9 ± 0.7	-10.4 ± 1.8	-0.34 ± 0.02
8	-4.3 ± 0.2	-7.3 ± 0.1	-0.11 ± 0.01
9	-5.3 ± 0.7	-12.7 ± 0.6	+0.11 ± 0.02
10	-6.5 ± 0.3	-11.7 ± 1.4	-2.3 ± 0.1
11	-5.7 ± 0.2	-10.7 ± 1.8	-1.1 ± 0.1
12	-3.7 ± 0.2	-4.6 ± 0.6	+0.12 ± 0.01
13	-5.6 ± 0.1	-10.5 ± 0.2	+0.3 ± 0.1
14	-4.9 ± 0.2	-6.7 ± 0.7	-0.04 ± 0.01

Ocular hypotensive efficacy is expressed in mmHg, as the average difference in IOP (intraocular pressure) between compound-treated eyes, vehicle-treated eyes and their respective pre-treatment value, as shown in the following formula: Efficacy = $(IOP_{drug} - IOP_{pre\text{-}dose\ drug})$ - $(IOP_{veh} - IOP_{pre\text{-}dose\ veh})$.

As mentioned above, these compounds can be considered pro-drugs of nitrite, which can be released inside the cells. It is known from the literature that nitrite displays IOP-lowering ability after topical administration in a normotensive rabbit model.[11] In a high IOP context, a significant reduction in ocular blood flow in the retinic artery,[28-29] and shear stress at the endothelial level both occur. These events are accompanied by cellular hypoxia which increases the vasodilator potency of NO releasing compounds and aqueous humor drainage.[28,30] This behavior may partly explain the discrepancy between the vasodilator potency of compound **13** and its ability to reduce IOP *in vivo*. Moreover, this compound's penetration into the eye is quite different to cellular penetration into rat aorta strips *in vitro*, which may be a plausible explanation for the observed differences. The most active compounds in each series, **9** (non-ionizable) and **13** (ionizable), were selected to be evaluated using a carbomer-induced chronic model (see experimental) as previously described by Supuran *et al.*[31]. The effects of **9** and **13** versus Molsidomine on IOP in glaucomatous albino rabbits were determined after repeated administrations over the course of five days. The results are reported in Figure 3 as IOP change (mmHg) versus time. As previously observed in the tOHT model, both compounds cause a significant reduction in IOP in the first 24 hours, while their activity is retained over five days and exhibits a Molsidomine-like profile.

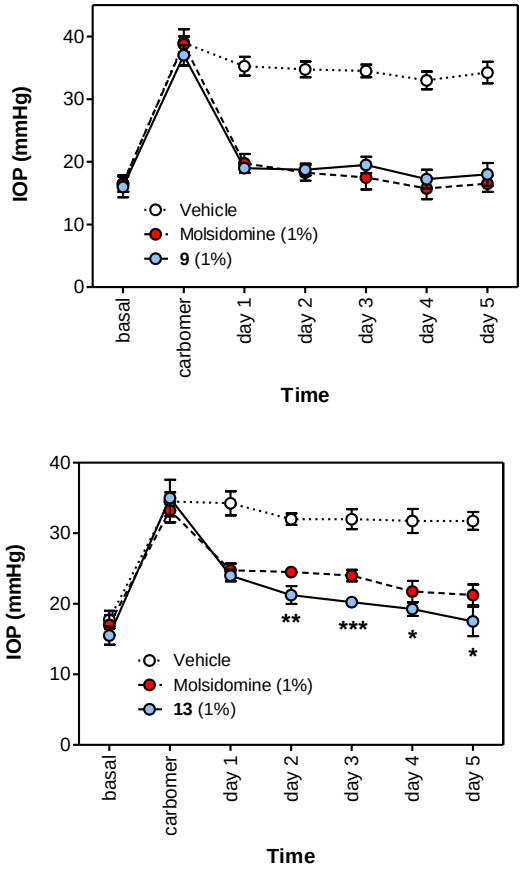


Figure 3. Effect on IOP of topical treatment with one drop (50 µL) of 1% **9** (top) or 1% **13** (bottom) versus 1% Molsidomine in carbomer-induced glaucoma in New Zealand albino rabbits. Data are presented as means ± SD (n = 4). *** p <0.001, **p < 0.01, * p < 0.05 versus Molsidomine (*t*-test)

In summary, we have developed a new class of IOP lowering agents that are endowed with varying NO-release capacity. The synthetic procedure allows a series of products to be easily obtained. These compounds were able to relax the contracted rat aorta strips in a concentration-dependent manner and show activity similar to Molsidomine in both transient ocular hypertensive rabbits and in a chronic model of IOP.

ASSOCIATED CONTENT

Supporting Information

Supporting Information is available free of charge on the ACS Publications website.
Synthetic procedures and experimental details (PDF)

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Author contributions

The manuscript was written with contributions from all authors, who all gave their approval to the final version of the manuscript.

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