Thiol-Induced Nitric Oxide Release from 3-Halogeno-3,4-dihydrodiazete 1,2-Dioxides

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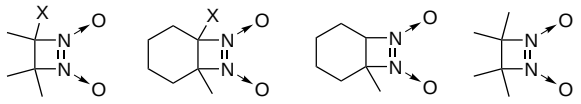
In this work we studied the mechanism of nitric oxide (NO) release underlying the vasorelaxant and antiaggregant effect of 3,4-dihydrodiazete 1,2-dioxides (DD). Six derivatives were included in the investigations, namely, 3-bromo- and 3-chloro-3,4,4-trimethyl-DD (**1a**,**b**), 3-bromo- and 3-chloro-4-methyl-3,4-hexamethylene-DD (**2a**,**b**), 3,3,4,4-tetramethyl-DD (**3**), and 3-methyl-3,4-hexamethylene-DD (**4**), and their reactivity toward thiols was analyzed. The 3-bromo- and 3-chloro-DD derivatives were found to react with thiols; this reaction can lead to NO formation, DD **2a** being the most reactive compound. 2-(Hydroxyamino)-2-methylbutan-3-one oxime (**5a**) and 2-hydroxy-2-methylbutan-3-one oxime (**6**) were the main products isolated from the reaction of **1a** with cysteine. Reaction rates of DD with thiols were dependent upon pH and concentration of the reagents. Maximum rates of NO release corresponded to thiol concentrations in the range of 1 mM. Consistent with reaction kinetics data and products isolated, a reaction mechanism was proposed. Addition of **2a** to bovine aortic endothelial cells led to strong NO release indicating a reaction with endogenous thiols. In rat mesenterial arteries, the vasorelaxant action of **2a** was only slightly influenced by addition of thiol to the incubation medium. For the most reactive DD derivatives, cytotoxic effects were observed at concentrations roughly 2 orders of magnitude higher than those inducing vasorelaxation.

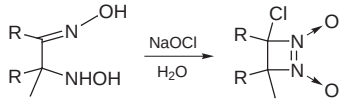
3,4-Dihydrodiazete 1,2-dioxides (diazetine dioxides, DD) were described for the first time about 20 years ago;[1] however, their biological activity was studied only recently.[2] These compounds were found to exert strong vasorelaxant and antiaggregant effects.[2] Spontaneous decomposition of DD was considered to be a source of nitric oxide (NO),[1b,2] and the rate constants of this reaction were measured for a series of DD in aqueous solutions.[2b] However, the rate constants were in the range of 10^{-7} s^{-1}, too low to account for the strong vasorelaxation induced by DD.[2a,d] Typical concentrations of diazetine dioxides causing significant vasodilation were determined to be in the range of 10^{-6} M.[2] It has been shown that the activity of various NO-liberating compounds is considerably influenced by thiols.[3] Diazetine dioxides are known to react with various nucleophilic and reducing agents. These reactions can proceed via diazetine ring opening and may be accompanied by a loss of one of the nitrogen atoms of the heterocycle.[4] The ability of DD to react with thiols was questioned[2a] and is not explicitly investigated. This study was accomplished in order to elucidate the contradiction between the strong vasorelaxant effect of DD and its low rate of spontaneous decomposition. It is demonstrated that 3-halogen derivatives of diazetine dioxides readily react with thiols and that the reaction can lead to NO formation.

Chart 1



X = Br(**a**), Cl(**b**)

Scheme 1



R = CH_3 (**a**), R + R = $(CH_2)_4$ (**b**)

Results

The compounds used for this investigation are shown in Chart 1. Synthesis was carried out according to the literature (cf. Experimental Section) except for chloro derivatives **1b** and **2b** which were obtained by treatment of the corresponding 1,2-hydroxyamino oximes **5a**,**b** with hypochlorite solution according to Scheme 1.

To check the reactivity of DD toward compounds containing SH groups, various thiols (e.g., cysteine, glutathione, *N*-acetylcysteamine) were added to solutions of the compounds **1**−**4** in phosphate buffer. The reaction mixtures were analyzed at different times using HPLC. 3-Halogen-substituted 3,4-dihydrodiazete 1,2-dioxides **1a**,**b** and **2a,b** were found to react readily with thiols at 25 °C, while their halogen-free analogues **3** and **4** did not. The reactivity of the different thiols with

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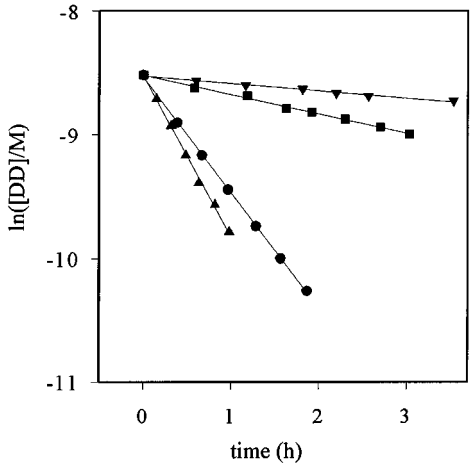


Figure 1. Decrease of DD **1a** (●), **1b** (▼), **2a** (▲), and **2b** (■) concentrations during the reaction with 5 mM cysteine in 0.1 M potassium phosphate buffer (pH 7.5) at 30 °C. Reaction rate constants are 0.052, 0.0033, 0.072, and 0.0086 M^{-1} s^{-1}, respectively.

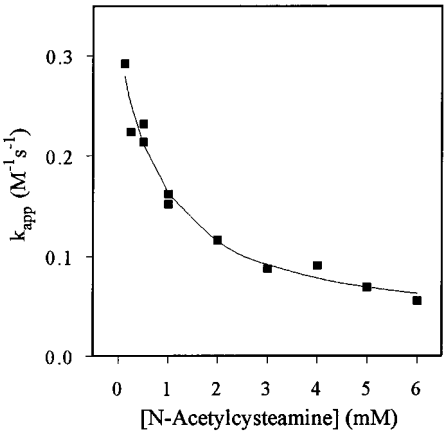


Figure 2. Dependence of apparent rate constants of DD **2a** (initial concentration, 0.025 mM) reaction with *N*-acetylcysteamine in 0.1 M potassium phosphate buffer at 30 °C upon initial thiol concentration. The curve corresponds to the function $k_{app} = a + 1/(b + c[RSH])$, where $a = -k_1$, $b = (k_{-1} + k_3)/k_{-1}k_1$, and $c = k_2/k_{-1}k_1$ (cf. Scheme 3). Optimum curve fitting was obtained using $a = 0.01$, $b = 3.39$, and $c = 1.61$.

respect to DD did not differ strongly. At a thiol concentration of 1 mM, pH 7.5, and 30 °C, the apparent rate constants (k_{app}, the rate of **2a** decomposition divided by initial concentrations of **2a** and thiol, $k_{app} = -d[DD]/dt \times ([DD] \times [RSH])^{-1}$) were 0.2, 0.17, and 0.1 M^{-1} s^{-1} for cysteine, *N*-acetylcysteine, and glutathione, respectively. In Figure 1, the logarithm of diazetine dioxide concentrations is plotted vs time in the presence of 5 mM cysteine. Linearity of these plots implies pseudo-first-order kinetics for this reaction, **2a** being the most reactive compound. Figure 2 demonstrates that k_{app} depended on the initial thiol concentration, pointing out that the reaction proceeds via a complex mechanism.

With respect to the thiol, the reaction was found to have pseudo-first-order kinetics with a rate constant of $k_t = d[RSH]/dt \times ([DD_0] \times [RSH])^{-1} = 0.69$ M^{-1} s^{-1}. This constant was not dependent on the reagent concentrations (data not shown in detail).

pH profiles of k_{app} measured from the inital rate of **2a** decomposition in the presence of glutathione and cysteine are shown in Figure 3. These pH profiles were

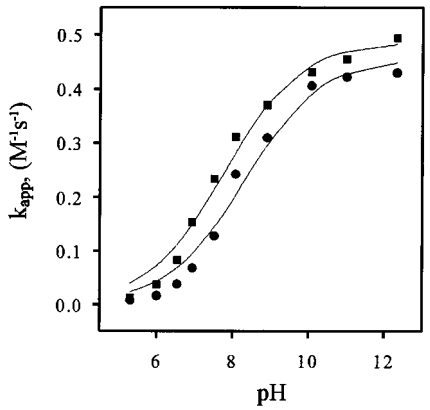
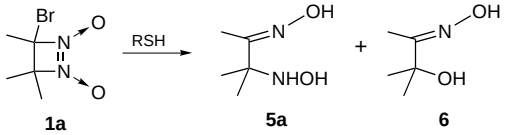


Figure 3. Dependence of the reaction rate of **2a** with cysteine (■) and glutathione (●) upon pH of the reaction mixture at 30 °C. Starting concentrations in 0.1 M potassium phosphate buffer: **2a**, 50 μM; thiol, 1 mM.

Scheme 2



found to be close to theoretical titration curves of thiol groups, and k_{app} increased with increasing pH.

To isolate the reaction products, DD **1a** was allowed to react with thiols (cysteine, dithioerythritol, or glutathione) in buffer solution (Scheme 2). At high thiol concentration (0.2 M), 1,2-hydroxyamino oxime **5a** was isolated from the reaction mixture as the only product, and 4-fold excess of thiol was necessary for the reaction to be complete under these conditions. Decrease of the thiol concentration to 40 mM did not lead to formation of other products as observed by ^{1}H NMR spectroscopy. However, when the thiol was added slowly to the reaction mixture in order to maintain its concentration in the range of 1 mM, both **5a** and hydroxy oxime **6** were formed (details described in the Experimental Section).

The effect of thiols on the rates of NO release from diazetines was measured by ozone-mediated chemiluminescence (CL) and oxyhemoglobin method. Representative results using cysteine are shown in Figure 4. At fixed thiol concentrations, the rates of thiol-mediated NO release were found to be proportional to DD concentration (data not shown); however, there was a complex dependence upon thiol concentration. Submillimolar thiol concentrations stimulated NO release from diazetines, whereas a further increase of the thiol concentration led to a decrease in the rate of NO formation; virtually no release of nitric oxide was observed when [RSH] exceeded 50 mM. Similar results were obtained by both methods of NO detection. Cysteine concentrations corresponding to maximum NO release in phosphate buffer (pH 7.4) at 25 °C for diazetine dioxides **1a** and **2a**,**b** were 0.2, 0.85, and 1.75 mM. Under these conditions, the corresponding rates of NO release of **1a** and **2b** were 0.13 and 0.08 when the value for **2a** was set to 1.0. A similar dependence of the NO release rate vs thiol concentration was also found for glutathione and dithioerythritol. The course of this dependence resembles that observed for nitroso-

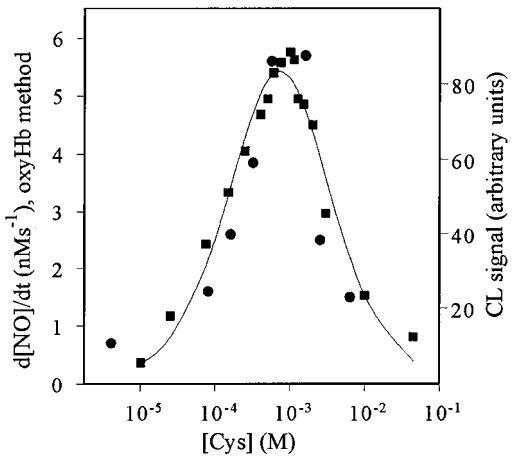


Figure 4. Dependence of the rate of NO release from 150 μM **2a** on cysteine concentration determined by chemiluminescence method (●) and oxyhemoglobin method (■) in Dulbecco buffer at 25 °C. The curve corresponds to the function d[NO]/d$t = a$[RSH]/$(1 + b$[RSH] + c[RSH]^2), where $a = k_1k_3$[**2a**]/$k_5(k_{-1} + k_3)$, $b = (k_{-1}k_4 + k_3k_4 + k_2k_5)/k_5(k_{-1} + k_3)$, and $c = k_4k_2/k_5(k_{-1} + k_3)$ (cf. Scheme 3). Optimum curve fitting was obtained using $a = 2.26 \times 10^{-5}$, $b = 500$, $c = 2 \times 10^6$.

thiol decomposition.[5] However, in contrast to these literature data, no changes in the rate of NO release were observed after addition of either neocuproine (Cu^+ chelator) or 10^{-7}−10^{-8} M Cu^{2+} and Fe^{3+} under anaerobic conditions. An absorption around 540 nm (which would indicate the presence of nitrosothiols) was not detected in the reaction mixture.

Destruction of the diazetine ring of DD was suggested to proceed via N−N bond splitting with intermediate formation of vicinal bis-nitroso compounds. The reduction of these compounds was reported to lead to formation of 1,2-nitroso oximes.[4] However, neither hydroxyamino oxime **5a** nor 3-methyl-3-nitrosobutan-2-one oxime was found to release NO in the absence or presence of thiols. Formation of **5a** was not detected after addition of 3-methyl-3-nitrosobutan-2-one oxime to cysteine solution in phosphate buffer at 25 °C.

EPR spectroscopy was performed in order to obtain evidence regarding the involvement of radical species in the reaction of thiols with DD. Applying the spin-trapping technique (Figure 5), we observed thiyl radical adduct formation in a reaction mixture of **2a** and glutathione using 5,5-dimethylpyrroline *N*-oxide (DMPO). Hyperfine splitting constants were determined to be a_N = 1.505 mT and a_{H^β} = 1.645 mT; these values were confirmed by spectral simulation.

Figures 6 and 7 present data on the influence of bovine aortic endothelial cells (BAEC) on the release of nitric oxide by diazetines. A very small level of NO release was observed after injection of DD **2a** solution to PBS without BAEC (Figure 6B). In the presence of BAEC, however, an increased rate of NO liberation was observed reaching a plateau after some minutes (Figure 6A). Addition of glutathione led to a strong acceleration of the NO release both in the presence and in the absence of BAEC. In a second set of experiments, it was found that superoxide dismutase strongly potentiates the CL signal arising from the reaction of thiols with **2a** or **2b** (Figure 7A,B). Figure 7C demonstrates that there was virtually no nitric oxide release from halogen-free diazetine **3** in the presence and absence of thiols.

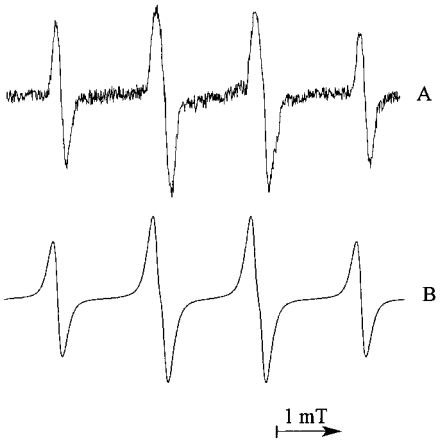


Figure 5. EPR spectrum obtained by the reaction of DD **2a** with glutathione at 25 °C in the presence of DMPO (A) and spectrum simulation using a_N = 1.515 mT, a_{H^β} = 1.640 mT, and ΔB_{pp} = 0.24 mT (B). Reagent concentrations (in Dulbecco buffer): glutathione, 2 mM; **2a**, 1 mM; DMPO, 200 mM; SOD, 50 U/mL; catalase, 1 kU/mL.

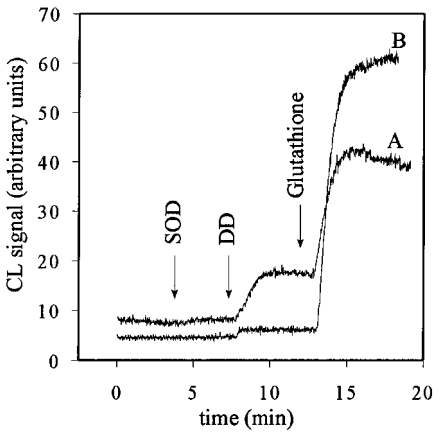


Figure 6. CL measurements of NO release from DD **2a** under aerobic conditions at 37 °C. SOD (50 U/mL), DD **2a** (30 μM), and glutathione (100 μM) were added sequentially to a cell culture flask (25 cm^2) containing 2 mL of Dulbecco buffer with (A) or without (B) BAEC.

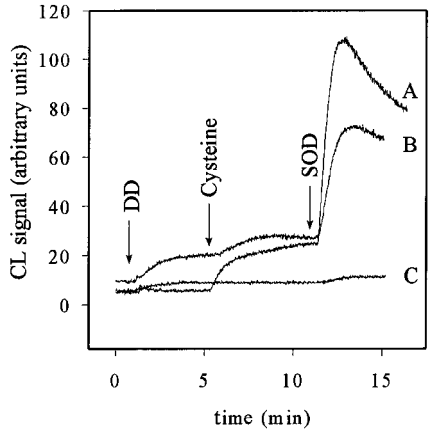


Figure 7. CL measurements of NO release from DD **2a** (A, B) and **3** (C) under aerobic conditions at 37 °C. DD (30 μM), cysteine (250 μM), and SOD (50 U/mL) were added sequentially to a cell culture flask (25 cm^2) containing 2 mL of Dulbecco buffer with (B) or without (A, C) BAEC.

Table 1 displays cytotoxic effects exerted by DD **1a** and **2a**,**b** on BAEC. The results are quantified by an IC_{50} value representing the concentration of DD that

Table 1. Apparent Rate Constants for the Reaction of DD Derivatives with Cysteine, Cytotoxicity, and Literature Data on Inhibition of Human Thrombocyte Aggregation by DD

DD	apparent rate constants[a] (M^{-1} s^{-1})	IC_{50}[b] (mM)	IC_{50}[c] (μM)
2a	0.072	0.23 ± 0.05	1
1a	0.052	1.4 ± 0.2	10
2b	0.0033	>6	

[a] Reaction conditions according to Figure 1. [b] IC_{50} represents the concentration of DD reducing the viability of bovine aortic endothelial cell by 50% within 24 h after DD addition. [c] Literature data on half-maximum inhibition of platelet aggregation.[2e]

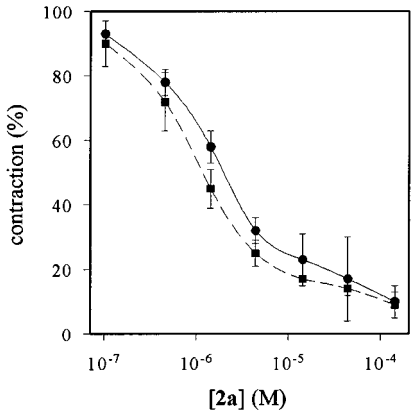


Figure 8. Concentration dependence of the vasorelaxant effect of **2a** on rat mesenteric small arteries in the absence (●) and presence (■) of 0.5 mM glutathione.

reduced the viability of BAEC by 50% within 24 h after DD addition. Obviously, higher rate constants of the reaction with thiols corresponded to more toxic compounds. Figure 8 demonstrates the vasorelaxant activity of DD **2a** that was observed at [**2a**] ≥ 10^{-7} M. Cumulative dose−response curves of rat mesenterial artery relaxation show that addition of glutathione (0.5 mM) to the incubation solution was without significant effect.

Discussion

Compounds releasing nitric oxide play a key role in NO-based pharmacotherapy. The investigation of the mechanism of NO release is an important aspect in order to prevent unwanted side effects in the application of these compounds. DDs have been shown to release nitric oxide spontaneously. However, their vasorelaxant effect cannot be explained by a spontaneous decomposition because the rate of this reaction is too low for the release of sufficient amounts of nitric oxide. The data obtained in this study clearly indicate that there is a reaction of DD with thiols which can result in NO release. This reaction involves several pathways, the main being reduction of DD to the corresponding 1,2-hydroxyamino oximes **5**. The rate constant of this reaction depends on the thiol concentration (Figure 2). This relationship can be explained assuming the first step of the reaction to be a reversible formation of an intermediate compound. The ratio of the decay rates of **2a** and *N*-acetylcysteamine (d[**2a**]/d[RSH], formal stoichiometry of the process) increases at low thiol concentration, indicating either that the intermediate is accumulated or that the partition into different pathways is thiol-dependent. Formation of a vicinal bis-nitroso derivative[4] seems to be unlikely because the plot of ln [DD] vs time was linear at various thiol concentrations (data not shown). Moreover, this dinitroso compound could hardly react with thiols in a reversible manner. From the pH dependence of k_{app} it is concluded that thiolate anions rather than protonated thiols are the reacting species. The theoretical titration curves of glutathione and cysteine are close to each other since the pK_a values of the thiol groups do not differ substantially (8.66 and 8.33, respectively).

The rates of NO release by DD increased strongly in the presence of physiologically relevant thiol concentrations. However, there was no obvious correlation between these rates and thiol concentration. Similarity between data obtained from CL and oxyhemoglobin measurements clearly demonstrates that this complex dependence of the NO release rates does not result from secondary reactions of nitric oxide. Transition-metal catalysis (as previously reported for nitrosothiol decomposition)[5] was also shown to be neglectable. Although nitrosothiol formation cannot be completely excluded in this reaction, especially in the presence of oxygen,[6] it seems to play a minor role. Thus, the observed dependence of the rate of NO release upon thiol concentration should result from the mechanism of NO formation. The lack of NO release at high thiol concentrations coincides with the observation that **5a** is the only reaction product under these conditions (Scheme 2).

Thiyl radical formation due to the reaction of DD with thiols was demonstrated by an EPR spin-trapping experiment; this radical can be a byproduct of the reaction or results from the superoxide-mediated oxidation of thiols.[7] However, elimination of oxygen as well as addition of superoxide dismutase had no effect on the resulting EPR spectra, suggesting that the main source of thiyl radicals is DD reaction with thiols. On the basis of the products isolated from the reaction mixtures and the kinetics data, Scheme 3 is proposed for the reaction of DD with thiols.

Reversible nucleophilic addition of a thiolate anion at the *N*-oxide oxygen atom results in formation of the unstable intermediate **7**. This sulfenic acid derivative can react with thiol leading to disulfide abstraction; further reduction produces **5**. Another pathway is the spontaneous decomposition to form intermediate **8**, which can also be reduced by thiols. Probably, further transformations include NO^- or hydroxylamine subtraction with nitrosoalkene formation. Intermediate **8** has a structure similar to *N*-nitrosohydroxylamine ethers which are able to undergo spontaneous homolytic decomposition accompanied by NO formation.[8] The resulting nitrosoalkene, being highly reactive toward nucleophilic addition,[9] can react with water or other nucleophiles present in the reaction mixture.

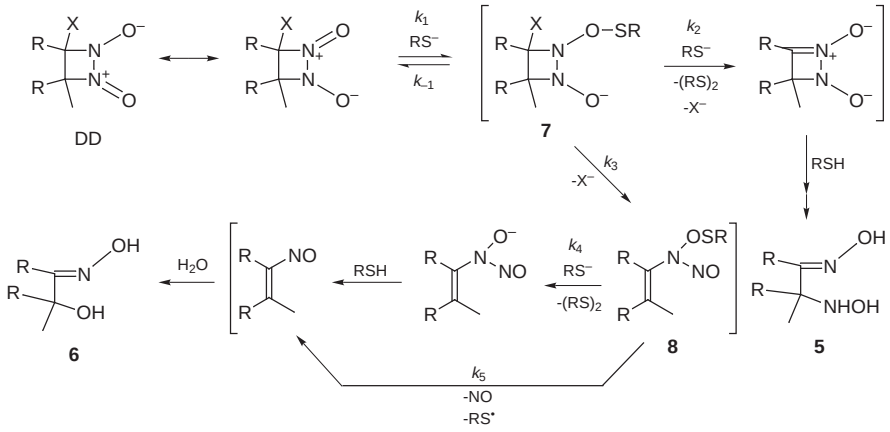
The solution of the kinetic equations for the quasi-stationary concentrations of intermediates **7** and **8** gives the following formulas for the concentration of **8** and k_{app}:

$$[\mathbf{8}] = k_1k_3[\mathrm{DD}][\mathrm{RSH}]/(k_5(k_{-1} + k_3) + (k_{-1}k_4 + k_3k_4 + k_2k_5)[\mathrm{RSH}] + k_4k_2[\mathrm{RSH}]^2)$$

$$k_{app} = -k_1 + (k_1k_{-1})/(k_{-1} + k_3 + k_2[\mathrm{RSH}])$$

Obviously, the concentration of **8** (which determines the

Scheme 3



rate of NO release) must have a maximum at a distinct thiol concentration. It is important to notice that after resolving the corresponding kinetic equations derived from Scheme 3, a good fit was found not only for the curve shown in Figure 4 but also for that shown in Figure 2. The decreased reactivity of the 3-chloro derivatives **1b** and **2b** can result from lower halogen abstraction rates leading to a decrease of k_2 and k_3.

The experiments using BAEC revealed the biological relevance of the NO release from diazetine derivatives. SOD was added in order to exclude a decrease in the extracellular NO concentration by reaction with superoxide intrinsically formed by these cells. The strong increase of the NO amount in the presence of BAEC indicates an effective interaction between cells and DD. The partition coefficient of **2a** in an octanol/water mixture was determined to be 6.4, suggesting that a penetration of this derivative into the cells and a subsequent reaction with intracellular thiols are likely to proceed. The total amount of NO detected by CL was lower in the presence of cells indicating the occurrence of NO-consuming reactions. Data of Figure 7 show that relatively high amounts of superoxide are formed in the reaction which can inactivate NO before it diffuses into the gas phase (eq 1). One of the possible sources of superoxide is the formation of thiyl radicals[7] (eqs 2 and 3):

$$NO + O_2^{\bullet -} \rightarrow OONO^- \quad (1)$$

$$RS^- + RS^{\bullet} \rightarrow RSSR^{\bullet -} \quad (2)$$

$$RSSR^{\bullet -} + O_2 \rightarrow RSSR + O_2^{\bullet -} \quad (3)$$

In the vasorelaxation experiments, a remarkable effect of **2a** was found without addition of thiols. Addition of glutathione did not lead to a significant increase of the IC_{50} value. As already discussed, liberation of NO is assumed resulting from the reaction of DD with intracellular thiols. This explains that, due to the relatively high concentration of thiols within cells (approximately 10^{-3} M),[10] the vasorelaxation is only slightly influenced by addition of thiol to the medium. Taking into account that there is an optimum thiol concentration for NO formation, addition of thiol could even decrease vasodilation. It cannot be excluded that other species, such as hydroxylamine or nitroxyl anion, contribute to the biological effects of DD.[3c] However, effects of these species would also support the importance of the reaction of DD with thiols for their vasorelaxant activity. The vasorelaxant action of **2a** found at concentrations around 2 μM is in the same order of magnitude as compared to data for the most active members of other classes of nitrovasodilators.[3,11]

Cytotoxic effects of DD were observed at concentrations ≥ 0.1 mM; diazetines with higher toxicity possess higher rate constants of the reaction with thiols. Thus, toxicity of these compounds is likely to result from the toxicity of the reaction products (**5**, **6**) or from the formation of thiyl radicals and nitric oxide. However, thiyl radicals are formed continuously by various reactions of thiols in biological systems, and their generation is associated with metabolic pathways.[12] In addition, an "indirect toxicity" of DD could be considered, resulting from thiol depletion due to the reaction of diazetines within the cell.

Conclusions

The results of this study resolve the contradiction between the high vasorelaxant activity of 3-halogeno-3,4-dihydrodiazete 1,2-dioxides and the low rate of their spontaneous NO release. The data obtained indicate that the formation of nitric oxide due to the reaction of 3-halogen-substituted DD with thiols can play a key role in their biological activity. Consistent with the experimental results, a mechanism of reaction was proposed which can be the basis for the design of new compounds with desired properties. This work encourages further investigations on both chemistry and biological activity of diazetine derivatives.

Experimental Section

Reagents and Instruments. Compounds **1a**,[1] **2a**,[1] **3**,[13] **4**,[2b] **5a**,[14] **5b**,[14] and **7**[1] were synthesized according to literature procedures. Purity of the DD samples used for measurement was checked by HPLC (see below). Dulbecco's phosphate-buffered saline (D-PBS), pH 7.4 (Biochrom, Germany), and Milli-Q water were used for preparation of the solutions; in several experiments transition-metal ions were removed by stirring with Chelex 100 resin (Bio-Rad). Because of the low solubility of DD **1a**,**b** and **2a**,**b**, concentrations of stock solutions were determined by absorption measurements at 267−273 nm (Beckman DU 640 spectrophotometer). All commercial reagents were of highest purity available. IR spectra of **1b**, **2b**, and **5a** were recorded in KBr pellets (Impact 400 instrument, Nicolet), and UV spectra of the compounds

were measured in ethanol (Specord UV/VIS spectrophotometer). ^{1}H NMR spectra (δ, ppm) were recorded using 1.4% solutions on a 200-MHz Varian Gemini 200 H/C spectrometer (**1b**, **5a**, and **6**) or 600-MHz Bruker DMX 600 spectrometer (**2b**). Reaction of **1a** with cysteine was investigated by recording ^{1}H NMR spectra of the reaction mixture on a Varian Gemini 200 H/C spectrometer in D_2O at different reaction times; starting reagent concentrations were **1a**, 10 mM; cysteine·HCl, 40 mM; K_2CO_3, 30 mM.

3,4-Dihydro-3-chloro-3,4,4-trimethyldiazete 1,2-Dioxide (1b) and 2a,3,4,5,6,6a-Hexahydro-2a-chloro-6a-methylbenzodiazete 1,2-Dioxide (2b). A solution of 45 mL of sodium hypochlorite (Merck, Germany) containing 12% active chlorine (0.07 mol) was saturated with a mixture of sodium dihydrogen phosphate and disodium hydrogen phosphate to pH 8 and cooled to a temperature of −4 to −8 °C. A solution of 12 mmol of 2-(hydroxyamino)-2-methylbutan-3-one oxime (**5a**) acetate or 2-(hydroxyamino)-2-methylcyclohexanone oxime (**5b**) acetate and 600 mg of $KHCO_3$ in 20 mL of water was added dropwise within 30 min under vigorous stirring maintaining the temperature of the reaction mixture below −3 °C. Reaction products were extracted with chloroform (3 × 30 mL); the extract was dried with $MgSO_4$. The solvent was removed under vacuum, and the residue was purified by column chromatography (silica gel, $CHCl_3$). **1b**: yield 20%, mp 140−142 °C dec (from ethanol); IR 1560 (i, N=N); UV (ethanol, nm) 267 (ϵ = 9000); ^{1}H NMR ($CDCl_3$) 1.75 (3H, s), 1.85 (3H, s) (gem. CH_3), 2.30 (3H, s, CBr−CH_3). Anal. ($C_5H_9N_2O_2Cl$) C, H, N; Cl: calcd, 21.5; found, 22.8. **2b**: yield 7%, mp 127−129 °C dec (from ethanol); IR 1560 (i, N=N); UV (ethanol, nm) 265 (ϵ = 8200); ^{1}H NMR ($CDCl_3$) 1.73 (3H, s, CH_3), 1.61 (2H, m, 5-CH_2), 1.77 (2H, m, 4-CH_2), 1.90 (1H, m), 2.25 (1H, m) (6-CH_2), 2.27 (1H, m), 2.67 (1H, m) (3-CH_2). Anal. ($C_7H_{11}N_2O_2$-Cl).

Reaction of 3-Bromo-3,4-dihydro-3,4,4-trimethyldiazete 1,2-Dioxide (1a) with Cysteine. A. A suspension of **1a** (105 mg/0.5 mmol) in 20 mL of a solution containing 300 mg of sodium dihydrogen phosphate and 245 mg (2 mmol) of cysteine was stirred at room temperature overnight; the resulting solution was filtered to remove cystine precipitate and extracted with ethyl acetate. The extract was dried with sodium sulfate. After the solvent was removed, a solid residue (60 mg) was obtained; recrystallization from ethyl acetate gave 2-(hydroxyamino)-2-methylbutan-3-one oxime (**5a**): yield 80%, mp 100−104 °C (no mp depression was observed for mixed probe with the sample obtained according to literature data);[14a] ^{1}H NMR ($(CD_3)_2SO$) 1.17 (6H, s), 1.73 (3H, s), 5.50 (1H, s, br), 6.95 (1H, s), 10.30 (1H, s). The sample synthesized according to the literature[14a] had an identical spectrum.

B. Solid cysteine was occasionally added by portions (0.5−1 mg/h) during 120 h to a stirred suspension of **1a** (105 mg, 0.5 mmol) in an aqueous solution of Na_2HPO_4 (300 mg in 10 mL). The reaction mixture was treated as described above, and the residue obtained after extract evaporation was separated by chromatography on a preconditioned TLC plate (Kieselgel 60, Merck, Germany), eluent chloroform/methanol (10:1). The products were washed out with methanol yielding 40 mg of **5a** (60%, identified as described above) and 12 mg (20%) of **6**: mp 84−87 °C; ^{1}H NMR ($CDCl_3$) 1.38 (6H, s), 1.91 (3H, s) (^{1}H NMR data and melting point are in agreement with literature data[15]); ^{13}C NMR ($CDCl_3$) 10.5, 28.5, 73.3, 162.7.

Spin-Trapping Experiments. EPR spectra were recorded using a Bruker ECS 106 spectrometer. A solution of glutathione (2 mM), **2a** (1 mM), and DMPO (200 mM) was placed into a flat quartz cell; EPR spectra were recorded after 5 and 10 min. Instrument settings were modulation frequency, 100 kHz; modulation amplitude, 0.1 mT; field set, 347.5 mT; scan range, 10 mT; microwave power, 10 mW. SOD (Boehringer Mannheim, 50 U/mL) and catalase (Sigma, 1 kU/mL) were added to prevent formation of the hydroxyl radical adduct. Spectrum simulation was performed using the EPR simulation program, version 1.4 (Bruker, Germany).

Reaction Kinetic Measurements with HPLC. Reaction mixtures were analyzed by HPLC using a Shimadzu LC-10A chromatograph, equipped with a Supelcosil LC-18S column filled with 50 mM phosphate buffer in methanol/water (3:1) as eluent running at 1 mL/min. Absorption at 210 nm was measured using a SPD-10A UV−vis detector to determine the concentrations of the compounds. In this system, typical retention times were 13 min (**2a**), 10 min (**2b**), 7 min (**1a**), 5 min (**1b**), and 3 min (*N*-acetylcysteamine). 2,4-Dinitrophenol was added to the reaction mixtures as a standard to decrease the injection error.

Nitric Oxide Measurements. NO formation was monitored by ozone-mediated chemiluminescence (270 B NO chemiluminescence analyzer, Sievers, Boulder, CO). This method is based on the oxidation of NO released into the gas phase by ozone. For continuous measurements (Figures 6 and 7) Corning flasks (25 cm^2) with or without BAEC, containing 2 mL of D-PBS (with Ca^{2+}, Mg^{2+}), were placed into a Boekel shaker-incubator at 37 °C; the headspace gas was forced by vacuum directly into the reaction chamber. The measurements of NO release from BAEC were performed after the cells were washed five times with the buffer. To measure NO release under anaerobic conditions (Figure 4), a DD solution in D-PBS was placed into the purge vessel under constant He flow, the thiol solution was injected through a septum, and signal detector readings were registered at the plateau level. In several experiments, up to 10^{-7} M $CuSO_4$, $Fe_2(SO_4)_3$, or 0.1 mM neocuproine (Sigma) was added. Alternatively, NO production was measured under the same conditions by the oxyhemoglobin method[16] using a Beckman DU 640 spectrophotometer. Concentration of oxyhemoglobin was 5 μM, and absorbance was registered at 401 nm. A differential extinction coefficient of 39 mM^{-1} cm^{-1} was used for calculations. In separate experiments, the concentration of oxyhemoglobin was increased up to 8 μM or SOD (40 U/mL) was added.

Cell Cultivation and Toxicity Studies. Cells of the BAEC line BKEz-7,[17] passages 12−22, were cultivated in Corning flasks (25 cm^2) as previously described.[18] The cytotoxicity of DD **1a** and **2a**,**b** was tested in a concentration range of 0.05−6 mM. After 24 h of incubation with DD, percentages of viable cells were determined using the neutral red viability test.[19]

Studies of Vasorelaxation Properties of 2a. Diazetine dioxide **2a** was tested regarding its vasorelaxant action on rat mesenterial vessels in a concentration range from 2×10^{-8} to 1.5×10^{-4} M in an accumulative mode.[20] Briefly, ring segments of mesenteric small arteries (diameter, <300 μm; length, 2 mm) were dissected from male MOL-Wistar rats (Møllegaard, Denmark) and mounted in a dual myograph for small vessels (J.P. Trading, Denmark). The vessels were equilibrated for 30 min at 37 °C with incubation medium (gassed with 5% CO_2/95% O_2, pH 7.4) containing (mM): NaCl, 119; KCl, 4.7; KH_2PO_4, 1.18; $MgSO_4$, 1.17; $NaHCO_3$, 25; $CaCl_2$, 2.5; EDTA, 0.026; glucose, 5.5. The arteries were precontracted with 10 μM $PGF_{2\alpha}$ prior to addition of DD. In separate experiments, glutathione (0.5 mM) was added to the incubation medium.

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References

(1) (a) Ullman, E. F.; Singh, P. 3,3,4,4-Tetramethyl-1,2-diazetine 1,2-dioxide, a useful low-energy triplet quencher. *J. Am. Chem. Soc.* **1972**, *94*, 5077−5078. (b) Volodarsky, L. B.; Tikhonova, L. A. Formation of furoxanes and 1,2-diazetine-1,2-dioxides upon α-hydroxylaminooximes oxidation. *Khim. Geterotsykl. Soedin.* **1975**, *6*, 748−752 (in Russian).

(2) (a) Severina, I. S.; Ryaposova, I. K.; Volodarsky, L. B.; Mazhukin, D. G.; Tikhonov, A. Ya.; Schwartz, G. Ya.; Granik, V. G.; Grigoryev, D. A.; Grygoryev, N. B. Derivatives of 1,2-diazetidine-1,2-di-*N*-oxides − a new class of soluble guanylate cyclase activators with vasodilatory properties. *Biochem. Mol. Biol. Int.* **1993**, *30*, 357−366. (b) Utepbergenov, D. I.; Khramtsov, V. V.; Vlassenko, L. P.; Markel, A. L.; Mazhukin, D. G.; Tikhonov, A. Ya.; Volodarsky, L. B. Kinetics of nitric oxide liberation by 3,4-dihydro-1,2-diazete 1,2-dioxides and their vasodilatory properties *in vitro* and in vivo. *Biochem. Biophys. Res. Commun.* **1995**, *214*, 1023−1032. (c) Severina, I. S.; Belushkina, N. N.; Grigoryev, N. B. Inhibition of ADP-induced human platelet aggregation by a new class of soluble guanylate cyclase activators capable of nitric oxide generation. *Biochem. Mol. Biol. Int.* **1994**, *33*, 957−967. (d) Ryaposova, I. K.; Grigoryev, N. B.; Severina, I. S. A new class of soluble guanylate cyclase activators, generating nitric oxide. *Biokhimiya (Moscow)* **1994**, *59*, 537−542 (in Russian). (e) Belushkina, N. N.; Grigoryev, N. B.; Severina, I. S. Inhibition of human platelet aggregation by a new class of soluble guanylate cyclase inhibitor, generating nitric oxide. *Biokhimiya (Moscow)* **1994**, *59*, 1689−1697 (in Russian).

(3) (a) Feelisch, M.; Schönafinger, K.; Noack, E. Thiol-mediated generation of nitric oxide accounts for the vasodilator action of furoxans. *Biochem. Pharmacol.* **1992**, *44*, 1149−1155. (b) Fukuyama, S.; Hirasawa, Y.; Cox, D.; Koda, S.; Kita, Y. Acceleration of nitric oxide (NO) release from FK409, a spontaneous NO releaser, in the presence of sulfhydryl-bearing compounds. *Pharm. Res.* **1995**, *12*, 1948−1952. (c) Feelisch, M.; te Poel, M.; Zamora, R.; Deussen, A.; Moncada, S. Understanding the controversy over the identity of EDRF. *Nature* **1994**, *368*, 62−65.

(4) Tikhonova, L. A.; Volodarsky, L. B. Properties of 3-bromo-1,2-diazetine-1,2-dioxides. *Izv. Sib. Otd. Akad. Nauk SSSR, Ser. Khim. Nauk* **1977**, *1*, 103−106 (in Russian).

(5) (a) Gorren, A. C. F.; Schrammel, A.; Schmidt, K.; Mayer, B. Decomposition of *S*-nitrosoglutathione in the presence of copper ions and glutathione. *Arch. Biochem. Biophys.* **1996**, *330*, 219−228. (b) Dicks, A. P.; Swift, H. R.; Williams, D. L. H.; Butler, A. R.; Al-Sa'doni, H. H.; Cox, B. G. Identification of Cu^{+} as the effective reagent in nitric oxide formation from *S*-nitrosothiols (RSNO). *J. Chem. Soc., Perkin Trans. 2* **1996**, 481−487.

(6) Kharitonov, V. G.; Sundquist, A. R.; Sharma, V. S. Kinetics of nitrosation of thiols by nitric oxide in the presence of oxygen. *J. Biol. Chem.* **1995**, *270*, 28158−28164.

(7) Winterbourn, C. C.; Metodiewa, D. Reaction of superoxide with glutathione and other thiols. *Methods Enzymol.* **1995**, *251*, 81−86.

(8) Khlestkin, V. K.; Mazhukin, D. G.; Tikhonov, A. Ya.; Bagryanskaya, I. Yu.; Gatilov, Yu. V.; Utepbergenov, D. I.; Khramtsov, V. V.; Volodarsky, L. B. Unexpected transformation of 1,2-bis(N-methoxy-*N*-nitrosoamino)cycloalkanes: First synthesis of 4,5-dihydro-1,2,3-triazole 2-oxides. *Tetrahedron Lett.* **1996**, *37*, 5997−6000.

(9) Gilchrist, T. L. Nitroso-alkynes and nitroso-alkenes. *Chem. Soc. Rev.* **1983**, *12*, 53−73.

(10) Maragos, C. M.; Morley, D.; Wink, D. A.; Dunams, T. M.; Saavedra, J. E.; Hoffman, A.; Bove, A. A.; Isaak, L.; Hrabie, J. A.; Keefer, L. K. Complexes of ·NO with nucleophiles as agents for the controlled biological release of nitric oxide. Vasorelaxant effects. *J. Med. Chem.* **1991**, *34*, 3242−3247.

(11) Meister, A.; Anderson, M. E. Glutathione. *Annu. Rev. Biochem.* **1983**, *52*, 711−760.

(12) Yu, B. P. Cellular defenses against damage from reactive oxygen species. *Physiol. Rev.* **1994**, *74*, 139−162.

(13) Greene, F. D.; Gilbert, K. E. Cyclic azo dioxides. Preparation, properties and consideration of azodioxide-nitrosoalkane equilibria. *J. Org. Chem.* **1975**, *40*, 1409−1415.

(14) (a) Volodarsky, L. B.; Putsykin, Yu. G. Chemistry of α-hydroxylaminooximes. XI. Synthesis of alicyclic α-hydroxylaminooximes. *Zh. Org. Khim.* **1967**, *3*, 1686−1694 (in Russian). (b) Putsykin, Yu. G.; Volodarsky, L. B. Synthesis of aliphatic α-hydroxylaminooximes from olefine nitrosochlorides. *Izv. Sib. Otd. Akad. Nauk SSSR, Ser. Khim. Nauk.* **1968**, *4*, 101−105 (in Russian).

(15) Boswell, G. A. Reaction of nitrosyl fluoride and selected steroid enes. A new synthesis of δ5-keto steroids. *J. Org. Chem.* **1968**, *33*, 3699−3713.

(16) Murphy, M. E.; Noack, E. Nitric oxide assay using hemoglobin method. *Methods Enzymol.* **1994**, *233*, 240−246.

(17) Halle, W.; Siems, W. E.; Jentsch, K. D.; Teuscher, E.; Göres, E. Die in vitro kultivierte Aorten-Endothelzelle in der Wirkstoffforschung − Zellphysiologische Charakterisierung und Einsatzmöglichkeiten der Zellinie BKEz-7**.** *Pharmazie* **1984**, *39*, 77−81.

(18) Haseloff, R. F.; Zöllner, S.; Kirilyuk, I. A.; Grigor'ev, I. A.; Reszka, R.; Bernhardt, R.; Mertsch, K.; Roloff, B.; Blasig, I. E. Superoxide-mediated reduction of the nitroxide group can prevent detection of nitric oxide by nitronyl nitroxides. *Free Radical Res.* **1997**, *26*, 7−17.

(19) Mertsch, K.; Grune, T.; Siems, W. G.; Landhoff, A.; Saupe, N.; Blasig, I. E. Hypoxia and reoxygenation of brain endothelial cells in vitro: A comparison of biochemical and morphological response. *Cell. Mol. Biol.* **1995**, *41*, 243−253.

(20) (a) Rohde, E.; Furkert, J.; Fechner, K.; Beyermann, M.; Mulvany, M. J.; Richter, R. M.; Denef, K.; Bienert, M.; Berger, H. Corticotropin-releasing hormone (CRH) receptors in the mesenteric small arteries of rats resemble the (2)-subtype. *Biochem. Pharmacol.* **1996**, *52*, 829−833. (b) Mulvany, M. J.; Halpern, W. Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.* **1977**, *41*, 19−26.

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