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NO-donors. Part 17[1]: Synthesis and antimicrobial activity of novel ketoconazole–NO-donor hybrid compounds

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ABSTRACT

Novel hybrid compounds combining the antifungal drug ketoconazole with a diazen-1-ium-1,2-diolate or an organic nitrate moiety and the corresponding NO-donors without ketoconazole were synthesized and their activities against a broad variety of fungal strains were tested. Hybridization modifies the spectrum of antimicrobial activities and generally, the ketoconazole–NO-donor hybrids are more potent than ketoconazole. The NO-donors alone show insufficient effectiveness.

1. Introduction

Ketoconazole (**1a**, KT) is a synthetic broad spectrum antifungal drug that was released in the early 1980s and is used to prevent and treat mycotic infections systemically, but also externally. Due to its variable bioavailability, liver toxicity and inhibition of steroid biosynthesis, the role of KT as an orally administered drug has been limited to few indications such as candida-oesophagitis of patients with acquired immune deficiency syndrome or to suppress glucocorticoid synthesis in the treatment of Cushing's disease, for example.[2] Pharmacodynamically, all azole-antimycotics prevent the fungal synthesis of ergosterole, which is a major constituent in fungal cell membranes, by enzyme inhibition of the cytochrome P450 dependent 14-α-demethylase.[2] The inhibitory effect of KT against bacterial growth in mycobacteria and streptomycetes is based on the interaction with cytochrome P450 as well.[3]

Previous studies have demonstrated the antimicrobial activity of endogenously derived nitric oxide (NO) from the inducible NO synthase isoform (iNOS) as well as from exogenously applied NO-donors.[4–8] Nitrogen bound diazen-1-ium-1,2-diolates (diazeniumdiolates, NONOates, 'solid NO') represent a commonly used tool in nitric oxide related research and are NO-donating compounds with the general structure $R_1R_2N[N(O)NO]^-$. The predictable and exclusive release of the reactive nitrogen species (RNS) NO· in a temperature, pH and structure dependent manner with half-lives reaching from a few seconds to many hours, depending on the compound used, are the most mentionable advantages of this class of NO-donor.[9–12] In order to prevent the immediate decomposition when dissolved in aqueous solution and further modulate the liberation of NO, NONOates can be derivatized to prodrugs that require enzymatic and/or chemical activation to yield in the corresponding NO-releasing diazeniumdiolate. Recent reports show that not only the NONOate DETA/NO itself exerts inhibitory effects against *Candida* species, but also in synergy when applied in combination with azole antimycotics such as KT, fluconazole and miconazole.[13]

Contrary to NONOates, organic nitrates require reduction of the nitrogen in order to deliver NO. Glutathione reductase, cytochrome P450, xanthine oxidoreductase and mitochondrial aldehyde dehydrogenase were recognized to be mediators in the bioactivation of organic nitrates.[14–18] Furthermore, organic nitrates are supposed to induce NO-like biological effects without generating free NO radicals and may be addressed as NO-mimetics rather than NO-donors. Therapeutically, organic nitrates are used for prophylaxis and acute treatment of angina pectoris, whereas most of the nitrate hybrid drugs introduced up to now possess a dihydropyridine or NSAID structure as a second active principle.[19,20]

We now report on the synthesis of a series of phenylpiperazines—which is a part of the ketoconazole (KT) structure—and ketoconazole derivatives bearing a diazen-1-ium-1,2-diolate or an organic nitrate moiety and the NO-liberation profile of the NONOate compounds. Furthermore, the NO-donors and

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KT–NO-donor hybrid compounds were tested for their in vitro antimicrobial activity against a series of fungal strains by measuring the minimum inhibitory concentration (MIC).

2. Methods

2.1. Chemistry

For the conversion of KT (**1a**) into a NO-donating hybrid compound, we focused on the phenylpiperazine substructure as pointed out in Scheme 1. In order to make **1a** accessible for derivatization, the acetyl residue was cleaved from the phenylpiperazine moiety with NaOH solution. As mentioned above, the time profile of NO liberation from NO-donors (e.g., NONOates) depends significantly on their individual chemical structure. To differentiate whether biologic effects rise from the released NO or from the synergy of KT and NO, all of the chemical modifications of the deacetylated KT (**2a**) were performed with phenylpiperazine (**2b**) as well.

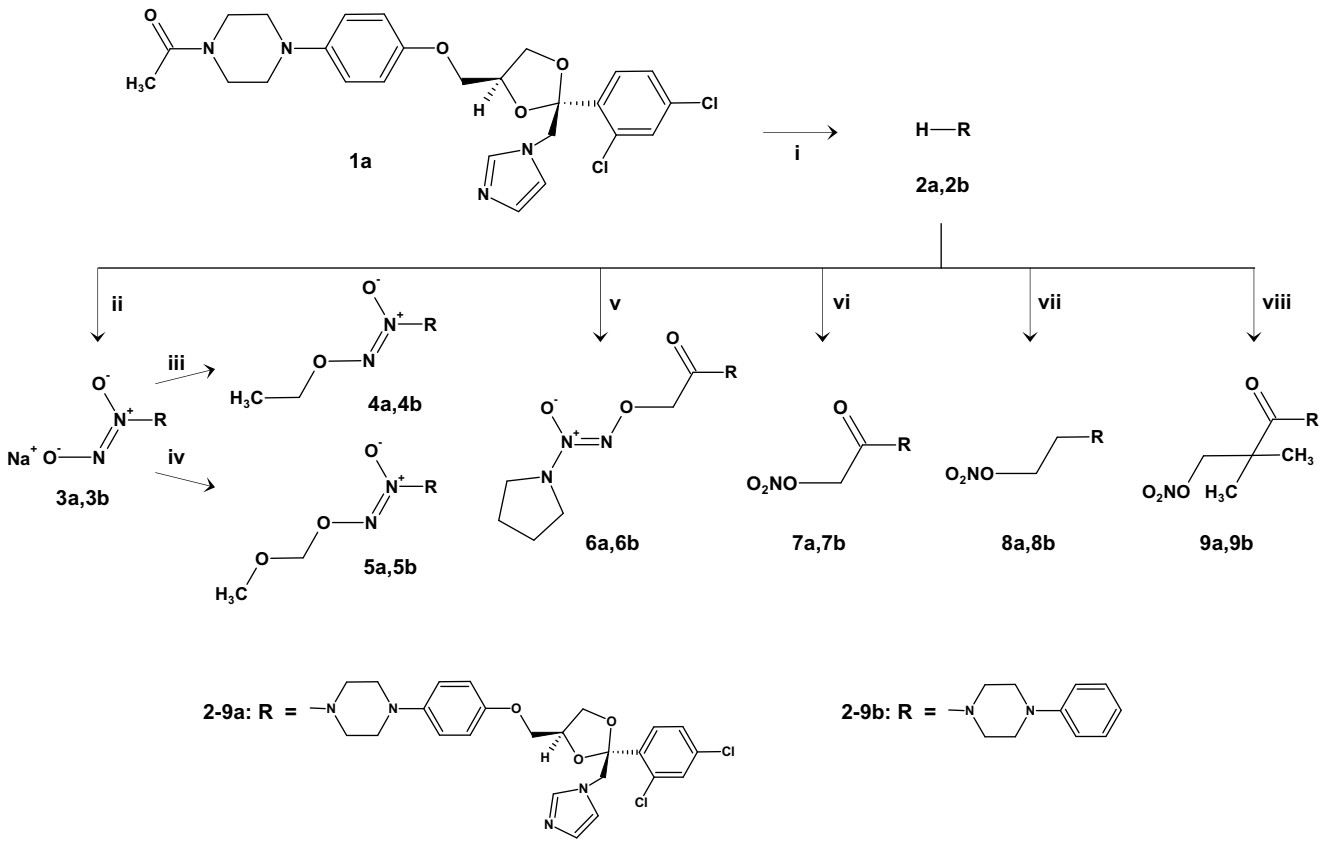
The sodium salt NONOates **3a** and **3b** were produces by exposing the respective amines to 5 bar of NO under exclusion of oxygen and water in 1:1 methanol/THF for **3a** and dry methanol for **3b**.[21] We used the NOtizer apparatus as described previously.[12,22] The half-lives ($t_{1/2}$) of the NONOates were determined both via UV-spectroscopy monitoring decomposition of the diazeniumdiolate group and by direct NO measurement by using a nitric oxide sensitive electrode as reported previously.[12,23] The half-life of NO release for **3a**/**3b** was approximately $t_{1/2} = 4.5$ min with no significant difference in kinetics. The alkylation of **3a**/**3b** with bromoethane and chlormethyl methylether yielded **4a**/**4b** and **5a**/**5b**, respectively, which do not release NO until the residues attached to the O^2-atom of the diazen-1-ium-1,2-diolate group are cleaved. While the metabolism of **4a**/**4b** in vitro is enzyme related, compounds **5a**/**5b** can be hydrolyzed chemically to the respective NONOate, formaldehyde and methanol.

The NONOate derivatives **6a**/**6b**, resulting from the acylation of **2a**/**2b** with chloroacetyl chloride followed by the reaction with sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (PYRRO/NO), are reliant on bond cleavage as well in order to produce the NO-liberating NONOate and the hydroxyacetamide derivative. The half-life of the resulting PYRRO/NO is approximately $t_{1/2} = 3–5$ s under physiologic conditions.[24] In contrast to **4a**/**4b** and **5a**/**5b**, this type of prodrug synthesis strategy advantageously allows the design of drugs with any kinetic of NO release just by appropriate modification on the NONOates structure. For instance, the NO release from **6a**/**6b** can be slowed down easily by changing the amine backbone linked to the diazeniumdiolate group to a bulky alkylamine or polyamine.[12,23] Additionally, the amide carbonyl function attached to the phenylpiperazine moiety yields in a more KT-like structure.

The organic nitrates **7a**/**7b** differ from **8a**/**8b** only through the carbonyl group, introduced by acylation of **2a**/**2b** with carbonyldiimidazole (CDI) activated nitratoacetic acid, whereas **8a**/**8b** was prepared by alkylation of **2a**/**2b** with 2-bromoethyl nitrate. These two sets of organic nitrates were designed to assess the relevance of the amide moiety in association with the NO mimetic group. Furthermore, the nitratopivalinic acid amides **9a**/**9b** were synthesized to recognize the effect of structural changes within the organic nitrate moiety on the biological activities.

2.2. Biological testing

Antifungal activities of the compounds were studied qualitatively by agar diffusion tests according to the literature[25,26] and



Scheme 1. Reagents and conditions: (i) 20% aq NaOH, 80 °C, 24 h; (ii) 5 bar NO, n_{eq} CH_3ONa, rt; (iii) bromoethane, 72 h; (iv) chlormethyl methylether, 72 h; (v) 1—chloroacetyl chloride, 4 °C, 2—PYRRO/NO, rt, 72 h; (vi) CDI activated nitratoacetic acid, rt, 24 h; (vii) 2-bromoethyl nitrate, rt, 72 h; (viii) CDI activated nitratopivalinic acid, rt, 24 h.

quantitatively by determination of minimal inhibitory concentrations (MIC) according to the NCCLS guidelines using the broth microdilution method.[27] Fifty microliters of a 8 mM compound solution in DMSO/methanol (1:10) were serially diluted by factor two with the culture medium (RPMI 1640 with L-glutamine, MOPS and without sodium bicarbonate, LONZA Verviers SPRL, Belgium). Exceptionally, compounds **3a**/**3b** were diluted in 0.1 M NaOH solution, only the last dilution step was performed in the given media and used immediately. Then the wells were inoculated with 50 μL of test organisms to give a final concentration of 6×10^3 CFU/mL. After microtiter plates were incubated at 37 °C (*Aspergillus fumigatus, Aspergillus terreus*) or at 30 °C (*Aspergillus niger*, *Candida, Sporobolomyces*, and *Penicillium* strains) for 24 h, the MIC-values were read with a Nepheloscan Ascent 1.4 automatic plate reader (Labsystems, Vantaa, Finland) as the lowest dilution of compound allowing no visible growth.

The MIC for *Fusarium oxysporum*were determined by the agar diffusion method. Fifty microliters of each of the 12 serial twofold dilutions were filled in agar (malt extract agar from Roth, Karlsruhe, Germany; seeded with 0.5 mL of a pretested mycelial solution) holes of 9 mm in diameter. After incubation at 30 °C for 24 h the MIC was read as the lowest concentration giving an inhibition zone.

3. Results and discussion

Prescreening in the agar diffusion assay showed significant differences in the diameters of inhibition zones for compounds **1a**–**9a** tested on fungal strains of *A. fumigatus, A. terreus*, *A. niger*, *Fusarium oxysporum*, *Candida albicans*, *Candida glabrata*, *Sporobolomyces salmonicolor* and *Penicillium notatum* (data not shown). For quantitative evaluation the MIC-values were used. Table 1 displays the MIC of the KT–NO-donor hybrids **3a**–**9a** compared to KT (**1a**) and *N*-deacetyl-KT (**2a**) for the different fungal strains. For better comparison, the respective fractional inhibitory concentration (FIC = MIC compound/ MIC KT) was calculated, values under 1 represent higher antifungal activity than KT alone. The *N*-deacetyl-ketoconazole **2a** is a major metabolite of KT and its activity has been tested on *Plasmodium falciparum*, *Candida albicans* and *Candida tropicalis* previously.[28,29] Literature data reporting no significant differences between **1a** and **2a** in activity against these *Candida* isolates were confirmed by us for *C. glabrata* as well. However, major loss of antifungal activity for **2a** against *Aspergillus* isolates and *P. notatum* can be recognized in our study. The MIC of **2a** is 30- to 60-fold higher for some of the strains compared to the *N*-acetylated **1a**. The direct NO-donor derivative of KT, carrying a diazeniumdiolate moiety (**3a**), shows similar antifungal potency against all strains tested compared to **1a**. NO-donor **3a** exclusively releases the redox-species NO· with a half-life of approximately $t_{1/2}$ = 4.5 min at pH 7.4 and 37 °C. Complete NO liberation from **3a** yields the secondary amine equivalent to structure **2a**. Obviously the NO released from **3a** is beneficial for the antifungal activity since **3a** shows higher activity than **2a**, but not synergistic enough to pursue **1a**. Synthesizing diazeniumdiolate-prodrugs **4a** and **5a** with an ethyl- and methoxymethyl moiety bound to the O^2-atom of the NONOate-group, respectively, enhances the antifungal properties tremendously compared to the sodium salt NONOate **3a**. Both of these compounds are more active than KT, especially against the tested *Aspergillus* strains, but no significant differences were found against *C. albicans*. A possible explanation is that this species lacks the means necessary to metabolize **4a** and **5a** into its active NO-donor. No difference in efficacy is observed when comparing compound **6a** to KT. Cleavage of **6a** results, amongst other, in the fast releasing NONOate PYRRO/NO with $t_{1/2}$ = 3 s for NO release. Perhaps the low synergistic effect of this KT–NO-donor hybrid is due to lower permeability through membranes compared to the NONOate-prodrugs **4a**/**5a** or maybe due to the very short half-life of the NO-donor.

The synergistic antimicrobial effects of organic nitrates with KT have not been investigated before. Looking at this class of NO-donors it is difficult to distinguish if the observed activity results from the synergy of KT with NO, with another nitrogen species or results from changes in a receptor-related interaction. The organic nitrates **7a** and **9a**, which are bound to the KT structure as amides, showed to be more potent than KT alone, especially compound **9a** against the tested *Aspergillus* strains. It can be recognized clearly that the spectrum of antifungal activity of the tested KT–NO-donors is different from the spectrum of KT, mainly resulting in a higher potency against *Aspergillus* species. Unexpectedly, the alkyl nitrate residue incorporated in compound **8a** turned out to reduce the activity, suggesting that the combination of amide linkage and organic nitrate is obligatory for antifungal properties.

The NO-donor compounds **2b**–**9b** lacking the KT structure showed no inhibition of test organism growth in all agar diffusion tests on a broad spectrum of bacterial and fungal strains at a concentration of 0.2 mM. The NONOate DETA/NO has been previously reported to have a MIC-value of about 2000 μg/mL (12.5 mM) on *Candida* species, DPTA/NO even up to 5000 μg/mL (26.2 mM).[13,30] Therefore, as expected, the NONOate, NONOate-prodrugs and organic nitrates derived from phenylpiperazine (**2b**–**9b**) showed no reckonable antimicrobial activity at a concentration of 2000 μM if applied exclusively. This demonstrates the necessity of the antifungal KT-backbone for synergistic effects with NO-donor moieties.

The difference in antimicrobial activity of the KT–NO-donors in comparison to KT can be explained through several possible mechanisms that can vary in the respective pathogen species. For instance, from a chemical point of view the entry of the hybrids into microbial cells can differ through properties such as lipophilicity or molecular weight of the compounds. Microbial cellular targets for NO such as proteins, lipids or DNA can be altered or

Table 1
Antifungal activities in vitro of ketoconazole and the ketoconazole–NO-donor hybrid compounds **1a**–**9a**: MIC-values (μM)

Compound	*Aspergillus fumigatus*	*Aspergillus terreus*	*Aspergillus niger*	*Fusarium oxysporum*	*Candida albicans*	*Candida glabrata*	*Sporobolomyces salmonicolor*	*Penicillium notatum*
1a = KT	62.5	31.25	62.5	500	25	12.5	1.56	8
2a	2000 (32)	2000 (64)	2000 (32)	2000 (4)	25 (1)	50 (4)	25 (16)	250 (31.25)
3a	200 (3.2)	100 (3.2)	100 (1.6)	200 (0.4)	100 (4)	12.5 (1)	1.25 (0.8)	20 (2.5)
4a	12.5 (0.2)	3.13 (0.1)	1.56 (0.025)	50 (0.1)	50 (2)	3.13 (0.25)	0.16 (0.1)	1.25 (0.16)
5a	8 (0.13)	8 (0.26)	4 (0.06)	250 (0.5)	25 (1)	3.13 (0.25)	2 (1.3)	1.56 (0.2)
6a	125 (2)	62.5 (2)	62.5 (1)	1000 (2)	25 (1)	12.5 (1)	2 (1.3)	8 (1)
7a	25 (0.4)	12.5 (0.4)	12.5 (0.2)	100 (0.2)	25 (1)	1.56 (0.12)	0.16 (0.1)	2.5 (0.31)
8a	1000 (16)	1000 (32)	250 (4)	1000 (2)	12.5 (0.5)	6.25 (0.5)	15.6 (10)	62.5 (7.81)
9a	3.13 (0.05)	1.56 (0.05)	1.56 (0.025)	25 (0.05)	6.25 (0.25)	3.13 (0.25)	0.6 (0.38)	8 (1)
1–9b	>2000	>2000	>2000	>2000	>2000	>2000	>2000	>2000

(FIC = MIC compound/MIC KT).

detoxified by pathogen specific defense mechanisms such as NO-scavengers like homocysteine or glutathione, detoxifying enzymes or DNA repair mechanisms.[6] Concerning the synergistic effect of KT with NO, it is also conceivable that NO interacts with the hemoprotein CYP450 of the 14-α-demethylase, and hence amplifies the antimicrobial target of KT.

4. Conclusion

Ketoconazol–NO-donor hybrid drugs represent a useful approach in antifungal treatment. For KT carrying a diazeniumdiolate moiety, derivatization to prodrugs is obligatory to ensure stability of the compounds. Hybrid drugs of KT with an organic nitrate group are highly active against the tested strains as well, whereas variation in structure seems to have greater influence on in vitro efficacy compared to the NONOate-hybrids. This study demonstrates that it is not regardless of which type of NO-donor is used to display synergy with KT, but also that the spectrum of susceptibility against different strains can differ from the one of the antimycotic agent alone. The KT–NO-donor hybrids may advantageously ensure a better drug targeting for NO and other RNS in comparison to fixed combinations of KT and NO-donor, which will have different distribution properties in vitro and in vivo. It is also considerable that the antiinfective properties of these compounds in vivo could be much greater, since amongst the versatile NO activities is its important regulatory role in the immune system or in inflammatory processes that would indirectly contribute against microbial pathogens.

5. Experimental

5.1. General methods and materials

Chemicals (including **2b**) were purchased from VWR International Merck KGaA, Darmstadt and Linde AG, Unterschleissheim, Germany (NO gas 2.5 UN–Nr. 1660) and used without further treatment unless noted otherwise. Ketoconazole (**1a**) was purchased from Caesar & Loretz GmbH Hilden, Germany (Art. Nr. 5270-25G). Melting points are uncorrected and were measured in open capillary tubes, using a Gallenkamp melting point apparatus. ^{1}H and ^{13}C NMR spectral data were obtained from a Bruker Advance 250 spectrometer (250 MHz) and Advance 400 spectrometer (400 MHz), respectively. Elemental analysis was performed on a Hereaus Vario EL apparatus and TLC on silica gel F254 plates (Merck). MS data were determined by GC/MS, using a Hewlett Packard GCD-Plus (G1800C) apparatus (HP-5MS column; J&W Scientific) or a mass spectrometer type SSQ-710 from Finnigan MAT (EI and FAB).

5.2. General procedures for synthesis of compounds in Scheme 1

5.2.1. (i) Deacetylation of 1a to 2a

KT (**1a**) was purchased from Caesar & Loretz GmbH Hilden, Germany (Art. Nr. 5270-25G) and 8 g (15 mmol) was dissolved in 175 mL methanol under reflux. Afterwards, 20% aqueous NaOH solution (40 mL) was slowly added and kept under reflux until completion of the reaction (TLC controlled). Chopped ice was added to the solution and the precipitated product was filtered and dried under vacuum to give **2a** in quantitative yield.

5.2.2. (ii) General procedure for synthesis of 3a/3b

Equimolar amounts of elementary sodium were dissolved in dry methanol (75 mL) and the solution filtered and added to **2a** (5 mmol) and **2b** (50 mmol), respectively. Additionally, dry tetrahydrofuran (75 mL) was only poured to the mixture containing **2a**. The solutions were transferred into the reaction chamber of the NOtizer® apparatus and closed tightly. The chamber was evacuated and flushed with dry nitrogen for four consecutive times, then evacuated again. An initial pressure of 5 bar nitric oxide was applied and the solutions stirred magnetically at 700 rpm. The pressure was raised back to 5 bar until remaining constant. The precipitated products after solvent concentration were filtered, washed with ether and dried in a desiccator under reduced pressure over night.

5.2.3. (iii, iv) General procedure for synthesis of 4a/4b and 5a/5b

A mixture of triturated anhydrous Na_2CO_3 (2 g) and **3a/3b** suspended in 50 mL hexamethylphosphoric triamide (HMPA) was stirred at 4 °C and an equimolar amount of the respective alkyl halide added dropwise. After ice bath removal, the mixtures were stirred at room temperature for 72 h. Insoluble solids were removed by filtration and the solution poured into $NaHCO_3$ saturated water. The products were extracted with ethyl acetate (2× 30 mL) and washed with water (5× 30 mL). The residues after solvent evaporation were purified by column chromatography on silica gel using the given eluent to furnish **4a**, **4b**, **5a** or **5b**, respectively.

5.2.4. (v) General procedure for synthesis of 6a/6b

A mixture of triturated anhydrous Na_2CO_3 (2 g) and 1.5 molar excess chloroacetyl chloride in 20 mL dichloromethane (DCM) was stirred at 4 °C and **2a**/**2b** dissolved in 40 mL DCM added dropwise and stirred until completion of the reaction (TLC controlled), respectively. Solids were filtered off and the filtrate was washed with saturated $NaHCO_3$ solution (1× 50 mL) and water (3× 50 mL). The organic phase was dropped to a freshly prepared suspension of Na_2CO_3 and equimolar amount of sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (PYRRO/NO) in 50 mL HMPA at room temperature. A stream of dry nitrogen was passed through the mixture until the DCM was evaporated and stirred for 72 h afterwards. The mixture was filtered into 60 mL of $NaHCO_3$ saturated water, extracted with ethyl acetate (2× 50 mL), washed with water (5× 75 mL) and dried over Na_2SO_4. The residues after solvent evaporation were purified by column chromatography on silica gel using the given eluent to furnish **6a** or **6b**, respectively.

5.2.5. (vi, viii) General procedure for synthesis of 7a/7b and 9a/9b

Compounds **2a**/**2b** (1 mmol) dissolved in 30 mL dichloromethane were slowly added via a syringe through a septum to a solution of CDI activated nitratoacetic acid or nitratopivalinic acid (1.5 mmol) in 20 mL dichloromethane under dry nitrogen atmosphere and stirred for 24 h at room temperature. The solutions were washed with $NaHCO_3$ saturated water (3× 40 mL) and the organic phase evaporated to yield residues of **7a**, **7b**, **9a** or **9b** that were purified through silica gel column chromatography or recrystallization, respectively.

Note that the much simpler acetylation of the secondary nitrogen atom with bromoacetyl bromide and subsequent nitration using silver nitrate in acetonitrile to produce compounds **7a**/**7b** only worked for the preparation of **7b**. The acetal group incorporated in the KT molecule was cleaved upon workup preventing this reaction to furnish **7a**.

5.2.6. (vii) General procedure for synthesis of 8a/8b

A given amount of **2a**/**2b** dissolved in dichloromethane (10 mL) was slowly added to a twofold excess of 2-bromoethyl nitrate and stirred at 60 °C for 48 h. The organic phase was washed with $NaHCO_3/H_2O$ (3× 20 mL) and evaporated to give brown fluids that required column chromatography purification on silica gel to yield **8a**/**8b**.

5.3. Experimental data for compounds shown in Scheme 1

5.3.1. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine (2a)

White powder (99% yield); mp 173 °C; ^{1}H NMR: 400 MHz ($CDCl_3$): δ 3.03 (s, 8H), 3.32–3.36 (dd, J = 9.6, 6.7 Hz, 1H), 3.72–3.77 (dd, J = 8.2, 4.9 Hz, 2H), 3.86–3.90 (dd, J = 6.7, 6.6 Hz, 1H), 4.31–4.37 (quin, J = 5.7 Hz, 1H), 4.39–4.53 (dd, J = 40.7, 14.8 Hz, 2H), 6.76 (d, J = 9.1 Hz, 2H), 6.88 (d, J = 9.1 Hz, 2H), 6.97 (d, J = 13.6 Hz, 2H), 7.25 (d, J = 8.1 Hz, 1H), 7.48 (d, J = 14.7 Hz, 2H), 7.57 (d, J = 8.4 Hz, 1H); ^{13}C NMR: 400 MHz ($CDCl_3$): δ 46.3, 51.3, 51.8, 67.6, 67.8, 74.9, 108.0, 115.3, 118.1, 121.1, 127.2, 128.6, 129.5, 131.3, 133.0, 134.7, 135.9, 138.8, 146.8, 152.3; EI-MS m/z (%): 83 (15), 173 (20), 183 (39), 432 (28), 445 (61), 460 (100), 471 (32), 489 (16, $[M+H]^+$) Anal. Calcd for $C_{24}H_{26}Cl_2N_4O_3$: C, 58.90; H, 5.35; N, 11.45. Found: C, 58.90; H, 5.36; N, 11.38.

5.3.2. Sodium 1-(4-{[2-(2,4-dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)-4-(diazen-1-ium-1,2-diolat)piperazine (3a)

Crude white solid (82% yield); mp 165–170 °C dec; ^{1}H NMR: 400 MHz (DMSO-d_6): δ 3.05 (m, 4H), 3.28–3.41 (m, 5H), 3.72–3.77 (dd, J = 8.2, 4.9 Hz, 2H), 3.86–3.90 (dd, J = 6.7, 6.6 Hz, 1H), 4.31–4.37 (quin, J = 5.7 Hz, 1H), 4.39–4.53 (dd, J = 40.7, 14.8 Hz, 2H), 6.76 (d, J = 9.1 Hz, 2H), 6.88 (d, J = 9.1 Hz, 2H), 6.97 (d, J = 13.6 Hz, 2H), 7.25 (d, J = 8.1 Hz, 1H), 7.48 (d, J = 14.7 Hz, 2H), 7.57 (d, J = 8.4 Hz, 1H); Anal. Calcd for $C_{24}H_{25}Cl_2N_6NaO_5$: C, 50.45; H, 4.41; N, 14.71. Found: C, 50.54; H, 4.06; N, 14.37.

5.3.3. Sodium 1-(phenylpiperazin-1-yl)diazen-1-ium-1,2-diolate (3b)

White powder (86% yield); mp 148 °C dec; ^{1}H NMR: 250 MHz (NaOD): δ 3.10–3.31 (m, 8H), 6.82–6.94 (m, 3H), 7.15–7.21 (m, 2H); Anal. Calcd for $C_{10}H_{13}N_4NaO_2$: C, 49.18; H, 5.37; N, 22.94. Found: C, 49.22; H, 5.37; N, 22.88.

5.3.4. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)-4-(O^2-ethyl-diazen-1-ium-1,2-diolat)piperazine (4a)

From 1.4 mmol **3a** a brown solid after column purification with ethanol:acetone (1:1) as eluent (62% yield); mp 181 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 1.39 (t, J = 7.0, 3H) 3.07 (m, 4H), 3.26–3.41 (m, 5H), 3.72–3.77 (dd, J = 8.2, 4.9 Hz, 2H), 3.86–3.90 (dd, J = 6.7, 6.6 Hz, 1H), 4.25–4.53 (m, 5H), 6.74 (d, J = 9.1 Hz, 2H), 6.88 (d, J = 9.1 Hz, 2H), 6.97 (d, J = 13.6 Hz, 2H), 7.25 (d, J = 8.1 Hz, 1H), 7.48 (d, J = 14.7 Hz, 2H), 7.59 (d, J = 8.4 Hz, 1H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 14.4, 46.3, 51.3, 51.8, 67.6, 67.8, 69.8, 74.9, 108.0, 115.3, 118.6, 121.1, 127.2, 128.6, 129.3, 131.7, 133.0, 134.7, 135.9, 138.8, 145.8, 152.9; Anal. Calcd for $C_{26}H_{30}Cl_2N_6O_5$: C, 54.08; H, 5.24; N, 14.55. Found: C, 53.98; H, 5.23; N, 14.22.

5.3.5. O^2-Ethyl-1-(phenylpiperazin-1-yl)diazen-1-ium-1,2-diolate (4b)

Brown plates (72% yield); mp 198 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 1.40 (t, J = 7.0 Hz, 3H), 3.37 (t, J = 5.0 Hz, 4H), 3.58 (t, J = 5.1 Hz, 4H), 3.31 (q, J = 7.1 Hz, 2H), 6.88–6.96 (m, 3H), 7.26–7.33 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 14.5, 48.8, 51.3, 69.8, 117.2, 120.6, 129.3, 150.3; Anal. Calcd for $C_{12}H_{18}N_4O_2$: C, 57.58; H, 7.25; N, 22.38. Found: C, 57.69; H, 7.28; N, 22.22.

5.3.6. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)-4-(O^2-methoxymethyl-diazen-1-ium-1,2-diolat)piperazine (5a)

From 1.0 mmol **3a** a beige solid after column purification with dichloromethane:methanol (9:1) as eluent (47% yield); mp 186 °C; ^{1}H NMR: 400 MHz ($CDCl_3$): δ 3.05 (m, 4H), 3.28–3.41 (m, 5H), 3.52 (s, 3H), 3.73–3.77 (dd, J = 8.2, 4.9 Hz, 2H), 3.83–3.95 (dd, J = 6.7, 6.6 Hz, 1H), 4.30–4.39 (quin, J = 5.7 Hz, 1H), 4.39–4.53 (dd, J = 40.7, 14.8 Hz, 2H), 5.24 (s, 2H), 6.73 (d, J = 9.1 Hz, 2H), 6.89 (d, J = 9.1 Hz, 2H), 6.97 (d, J = 13.6 Hz, 2H), 7.29 (d, J = 8.1 Hz, 1H), 7.48 (d, J = 14.7 Hz, 2H), 7.56 (d, J = 8.4 Hz, 1H); ^{13}C NMR: 400 MHz ($CDCl_3$): δ 49.3, 51.3, 51.7, 57.0, 67.7, 67.8, 74.7, 97.9, 108.0, 115.3, 118.7, 121.1, 127.2, 128.4, 129.5, 131.3, 133.0, 134.6, 135.9, 138.8, 145.8, 152.4; FAB-MS m/z (%): 255 (40), 257 (33), 307 (100), 432 (20), 446 (28), 459 (33), 487 (43), 518 (50), 593 (24, $[M+H]^+$); Anal. Calcd for $C_{26}H_{30}Cl_2N_6$ O_6: C, 52.62; H, 5.10; N, 14.16. Found: C, 52.95; H, 5.21; N, 13.82.

5.3.7. O^2-Methoxymethyl-1-(phenylpiperazin-1-yl)diazen-1-ium-1,2-diolate (5b)

Yellow solid (61% yield); mp 187 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 3.36 (mc, 4H), 3.51 (s, 3H), 3.64 (mc, 4H), 5.24 (s, 2H), 6.89–6.97 (m, 3H), 7.25–7.29 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 48.3, 51.0, 57.0, 97.9, 117.2, 121.2, 129.3, 150.3; Anal. Calcd for $C_{12}H_{18}N_4O_3$: C, 54.12; H, 6.81; N, 21.04. Found: C, 54.25; H, 6.78; N, 20.86.

5.3.8. O^2-(1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine-4-carboxymethyl)-1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (6a)

Column purification on silica gel with ethyl acetate:acetonitrile (1:1) then methanol washout to give orange solid (38% yield); mp 237 °C; ^{1}H NMR: 400 MHz ($CDCl_3$): δ 1.93 (m, 4H), 3.02–3.13 (m, 8H), 3.32–3.36 (m, 1H), 3.55 (mc, 4H), 3.72–3.77 (m, 2H), 3.86–3.90 (dd, J = 6.7, 6.6 Hz, 1H), 4.31–4.37 (quin, J = 5.7 Hz, 1H), 4.39–4.53 (dd, J = 40.7, 14.8 Hz, 2H), 4.80 (s, 2H), 6.76 (d, J = 9.1 Hz, 2H), 6.90 (m, 2H), 6.97 (m, 2H), 7.25 (d, J = 8.1 Hz, 1H), 7.49 (d, J = 14.7 Hz, 2H), 7.57 (d, J = 8.3 Hz, 1H); ^{13}C NMR: 400 MHz ($CDCl_3$): δ 22.9, 36.8, 51.3, 51.7, 53.5, 67.6, 67.7, 71.4, 74.8, 108.0, 115.3, 118.8, 121.2, 127.2, 128.4, 129.5, 131.3, 133.0, 134.6, 135.9, 138.8, 145.6, 152.9, 166.7; FAB-MS m/z (%): 202 (48), 217 (55), 235 (50), 255 (100), 432 (38), 446 (51), 459 (70), 471 (63), 489 (64), 517 (39), 547 (82), 600 (69), 660 (42, M^++H), 682 (19, $[M+Na]^+$), 698 (17, M^++K); Anal. Calcd for $C_{30}H_{35}Cl_2N_7$ O_6: C, 54.55; H, 5.34; N, 14.84. Found: C, 54.17; H, 5.43; N, 14.78.

5.3.9. O^2-[(1-Phenylpiperazine-4-carboxymethyl)-1-(pyrrolidin-1-yl)]diazen-1-ium-1,2-diolate (6b)

Column purification with ethanol/acetone (1:1) as eluent gave orange oil (58% yield); ^{1}H NMR: 250 MHz ($CDCl_3$): δ 1.80 (m, 4H), 2.58 (m, 4H), 3.16 (mc, 4H), 3.37 (s, 2H), 3.76 (mc, 4H), 6.87–6.95 (m, 3H), 7.25–7.31 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 23.8, 41.7, 43.4, 49.4, 49.9, 53.9, 58.5, 116.5, 120.4, 129.3, 151.0, 168.6; Anal. Calcd for $C_{16}H_{23}N_5O_3$: C, 57.64; H, 6.95; N, 21.01. Found: C, 57.53; H, 6.99; N, 21.17.

5.3.10. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine-4-carboxymethylnitrate (7a)

Crude light orange solid after column purification with methanol on silica gel (86% yield); mp > 250 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 3.03 (m, 4H), 3.27 (m, 5H), 3.47 (s, 2H), 3.72–3.78 (m, 2H), 3.83–3.90 (dd, J = 6.6, 6.6 Hz, 1H), 4.30–4.57 (m, 3H), 6.76 (d, J = 9.0 Hz, 2H), 6.88–6.98 (m, 4H), 7.25 (mc, 1H), 7.48 (d, J = 14.4 Hz, 2H), 7.49 (d, J = 8.4 Hz, 1H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 39.6, 50.1, 50.7, 51.2, 51.7, 67.5, 67.6, 74.7, 108.0, 115.3, 119.2, 121.2, 127.2, 128.5, 129.5, 131.4, 133.0, 134.6, 135.9, 138.8, 145.1, 153.3; Anal. Calcd for $C_{26}H_{27}Cl_2N_5$ O_7: C, 52.71; H, 4.59; N, 11.82. Found: C, 53.01; H, 4.53; N, 11.68.

5.3.11. 1-Phenylpiperazine-4-carboxymethylnitrate (7b)

Red crystals after recrystallization with ethanol/ether (74% yield); mp 243 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 3.21 (m, 4H), 3.70 (mc, 4H), 5.07 (s, 2H), 6.91–6.97 (m, 3H), 7.26–7.34 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 42.0, 44.5, 49.4, 67.7, 116.9, 121.0, 129.3, 150.6, 162.9; Anal. Calcd for $C_{12}H_{15}N_3O_4$: C, 54.33; H, 5.70; N, 15.84. Found: C, 54.01; H, 5.56; N, 15.56.

5.3.12. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)piperazine-4-ethylnitrate (8a)

Yellow plates after column purification with methanol on silica gel (62% yield); mp > 250 °C; ^{1}H NMR: 400 MHz ($CDCl_3$): δ 2.64–2.81 (m, 6H), 3.22 (mc, 4H), 3.35 (mc, 1H), 3.72–3.91 (m, 3H), 4.30–4.37 (quin, J = 5.7 Hz, 1H), 4.42–4.65 (m, 4H), 6.76–6.91 (m, 4H), 6.97 (d, J = 13.6 Hz, 2H), 7.25–7.53 (m, 4H); ^{13}C NMR: 400 MHz ($CDCl_3$): δ 49.1, 52.9, 53.4, 57.7, 67.6, 67.8, 70.4, 74.6, 108.0, 115.3, 117.9, 121.0, 127.2, 128.6, 129.5, 131.3, 133.2, 134.7, 136.0, 138.8, 146.7, 152.3; Anal. Calcd for $C_{26}H_{29}Cl_2N_5O_6$: C, 53.99; H, 5.05; N, 12.11. Found: C, 54.02; H, 4.78; N, 12.14.

5.3.13. 1-Phenylpiperazine-4-ethylnitrate (8b)

Yellow solid after column chromatography first with methanol, then with dichloromethane/*n*-hexane (2:8) as eluent (55% yield); mp > 250 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 2.65–2.79 (m, 6H), 3.50 (mc, 4H), 4.62 (t, J = 6.2 Hz, 2H), 6.85–6.95 (m, 3H), 7.25–7.30 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 49.0, 52.9, 55.0, 70.7, 116.2, 119.9, 129.0, 151.1; Anal. Calcd for $C_{12}H_{17}N_3O_3$: C, 57.36; H, 6.82; N, 16.72. Found: C, 57.47; H, 7.09; N, 16.63.

5.3.14. 1-(4-{[2-(2,4-Dichlorophenyl)-2-(1*H*-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy}phenyl)-4-(2,2-dimethyl-3-oxopropylnitrate)piperazine (9a)

White crude product after column purification with dichloromethane/ethanol (9:1) as eluent (70% yield); mp > 250 °C; ^{1}H NMR: 400 MHz ($CDCl_3$): δ 1.41 (s, 6H), 3.03–3.20 (mc, 8H), 3.28 (m, 1H), 3.67–3.90 (m, 3H), 4.34–4.53 (m, 3H), 4.59 (s, 2H), 6.74–6.88 (mc, 4H), 6.99 (d, J = 13.6 Hz, 2H), 7.25 (m, 1H), 7.48–7.55 (m, 3H); ^{13}C NMR: 400 MHz ($CDCl_3$): δ 18.4, 22.4, 42.1, 45.0, 50.8, 51.3, 58.4, 67.6, 67.8, 74.8, 108.0, 115.3, 118.6, 121.2, 127.2, 128.4, 129.5, 131.4, 133.0, 134.6, 135.9, 138.8, 145.5, 153.0, 172.7; EI-MS m/z (%): 203 (24), 215 (29), 245 (46), 255 (100), 257 (74), 432 (38), 446 (56), 459 (30), 487 (32), 488 (21), 556 (48), 587 (33), 634 (45, $[M+H]^+$); Anal. Calcd for $C_{29}H_{33}Cl_2N_5O_7$: C, 54.89; H, 5.24; N, 11.04. Found: C, 54.91; H, 5.61; N, 10.94.

5.3.15. 1-Phenyl-4-(2,2-dimethyl-3-oxopropylnitrate)piperazine (9b)

Colourless crystals after recrystallization from dichloromethane/*n*-hexane (1:1) (92% yield); mp > 250 °C; ^{1}H NMR: 250 MHz ($CDCl_3$): δ 1.42 (s, 6H), 3.19 (t, J = 5.0 Hz, 4H), 3.80 (t, J = 5.0 Hz, 4H), 4.60 (s, 2H), 6.89–6.95 (m, 3H), 7.26–7.33 (m, 2H); ^{13}C NMR: 250 MHz ($CDCl_3$): δ 22.4, 42.1, 44.9, 49.5, 79.2, 116.5, 120.6, 129.3, 150.8, 172.7; Anal. Calcd for $C_{15}H_{21}N_3O_4$: C, 58.62; H, 6.89; N, 13.67. Found: C, 58.75; H, 6.89; N, 13.62.

5.3.16. Intermediate compounds for synthesis of 6a/6b, 7a/7b, 8a/8b and 9a/9b

Sodium 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate, nitratoacetic acid, 2-bromoethyl nitrate and nitratopivalinic acid were synthesized as described in the literature and analytical data were confirmed.[12,24,31–33]

5.4. NO release measurements

NO release was measured using the Apollo 4000 free radical analyzer system with an ISO-NOP nitric oxide selective sensor built in a multi-port measurement chamber by World Precision Instruments Inc., USA. The measurement chamber was tempered with a Lauda M3 circulating thermostat at 37 °C and stirred at 800 rpm. All experiments were started after the initial background signal was below 7000 pA at a channel range of 10 nA for the electrode. Calibration was conducted daily with 2.0 mL of 0.1 M KI/0.1 M H_2SO_4 solution in the chamber and an increasing amount of 50 μM $NaNO_2$ solution to produce nitric oxide by reduction. The nitrite solution was applied with an Eppendorf pipette (10, 20, 30, 40, 60 μL) resulting in 249, 493, 728, 952 and 1388 nM solutions of NO, respectively. The dissociation of the diazeniumdiolates was measured by injecting 20 μL of a 50 μM NONOate/0.01 M NaOH solution into 2.0 mL of phosphate buffer, pH 6.4, resulting in a 495 nM solution of NONOate. Since 1 mol NONOate produces 2 mol NO, the redox current flow of a 990 nM NO solution was measured. Note that the nitric oxide sensor measurement is not accumulative and only measures the arising current at the given time. For this reason a phosphate buffer pH 6.4 was used to ensure a sharper signal.[12]

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