New NO-Donors with Antithrombotic and Vasodilating Activities, Part 21

Pseudonitrosites and Other Azodioxides with Vicinal Electron Acceptors☆

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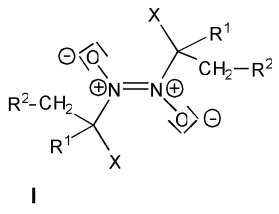
Key Words: *Vicinal nitro-nitroso compounds; antiplatelet activities; antithrombotic activities; NO formation; N_2O formation*

Summary

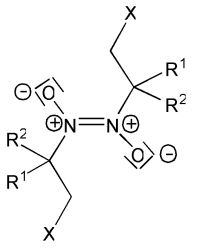
Twelve vicinally substituted nitro-nitroso compounds (pseudonitrosites) were synthesized, nine of them for the first time. In the solid state the dimeric azodioxides are present. In the class of the pseudonitrosites **2a**–**h,** all compounds exhibited comparatively strong antiplatelet activity *in vitro* (Born test, collagen). Four of them showed an IC_{50} below 10 μM, **2a** being the most active substance with an IC_{50} = 2.1 μM. When administered orally to rats (60 mg/kg) small antithrombotic effects were observed. The pseudonitrosite **6d** was the most active compound (18% inhibition in arterioles). The *in vitro* decomposition of **2a** at 37 °C gave NO and N_2O, indicating that the above pharmacological effects were mediated by an NO-dependent mechanism. The replacement of the nitro group in the pseudonitrosite partial structure by other electron acceptors i.e. acetyl, carboxyl, or acetyloxy groups leads to inactive (**10a**) or less active compounds (**10b**, **c**).

Introduction

Recently we reported on antithrombotic azodioxides (**I**) which were activated by electron acceptors geminal to the nitroso group[1,2]. The rationale was that the acceptor X should decrease the electron density of the C-NO bond and thereby make those compounds prone to the release of nitric oxide. With participation of the adjacent activated methylene group the expulsion of nitrosohydrogen which is the reduced congener of NO, should become favorable.



I



II

The success of this idea prompted us to investigate whether an electron acceptor vicinal to the nitroso group (**II**) could also "activate" azodioxides to release the above NO species. We were then further encouraged by the report of Wieland[3] who had already observed the formation of dinitrogen oxide from styrene pseudonitrosite (**II**: X = NO_2; R^1 = ph; R^2 = H; see also compound **2a** of this paper). Compounds **2a–c** were designed according to the suggestions of Topliss.

Chemistry

The type **2** azodioxides (see Fig. 1) which are trivially called pseudonitrosites were obtained according to Klamann et al.[4] by reaction of alkenes with dinitrogen trioxide. This reagent forms spontaneously when a mixture of nitric oxide and air is passed through the solution of the alkene. Six of the pseudonitrosites (**2c**–**h**) were prepared for the first time. The substituents R^1 were chosen on the basis of previous experience concerning suitable lipophilia and (platelet) membrane affinity. The compounds form colorless crystals and hence are type **2** azodioxides. This is backed up by the IR spectra where the valence vibration for the NO group is found between 1200–1209 cm^{-1}, i.e. in the N-O single bond region. This confirms simultaneously that the *E*-isomer is present[4a]. When dissolved in chloroform the compounds form colorless solutions. In the NMR spectra a single set of signals is seen. Both observations indicate that only the dimers **2** and not the monomeric form **3** are present. The azodioxide structure is so stable that in the (+)-FAB mass spectrum the pseudomolecular ion $[M+H]^+$ is seen for the dimer, e.g. **2e** = 293 with high intensity (50%). The nitromethylene group gives rise to a prochiral moiety so that an AMX spin system with the vicinal proton (**2a–c**) is observed (see Fig. 2).

In **2d–h** a typical $AMXY_2$ pattern is seen. In acetone at 20 °C the pseudonitrosites are time dependently rearranged to the nitrooximes **4**. This reaction follows a first order kinetic. After 6 h only 40% of the pseudonitrosite **2c** are left. This can be followed easily by the ^{1}H-NMR where at 5.83 ppm the singlet for the methylene group of **4c** is observed. The small peak at 5.62 ppm shows that a small portion of the *Z*-oxime (6 h: ≈1%) has formed, too. The 2-nitro-1-phenylethanone oxime **4a** was isolated in pure state to test its activity for comparison with the vinylogous nitrooxime FK 409 (v.i.) for which the release of nitric oxide has been reported[5].

When reacting methylacrylic acid esters (**5**) with N_2O_3 the azodioxides **6** are obtained[6]. Formally these are pseudonitrosites with an additional carboxylic acid ester partial structure. This electron-withdrawing group was incorporated in order to activate further the azodioxide for the release of NO. In the solid state **6a–d** form colorless crystals indicating the azodioxide structure. In the (+)-FAB mass spectrum the pseudomolecular ion is recorded with high intensity (e.g. **6a**: $[M+H]^+$ = 353 (65)). Chloroform solutions of these compounds are colorless. When gently warmed (50 °C) they become blue i.e. the monomeric **7** are formed. The ^{1}H-NMR spectrum shows that a mixture of **6** and **7** is present. For example, the ratio **6a/7a** is 8:1.

[1)] Part of the PhD thesis M. Herpel, FU Berlin 1997.

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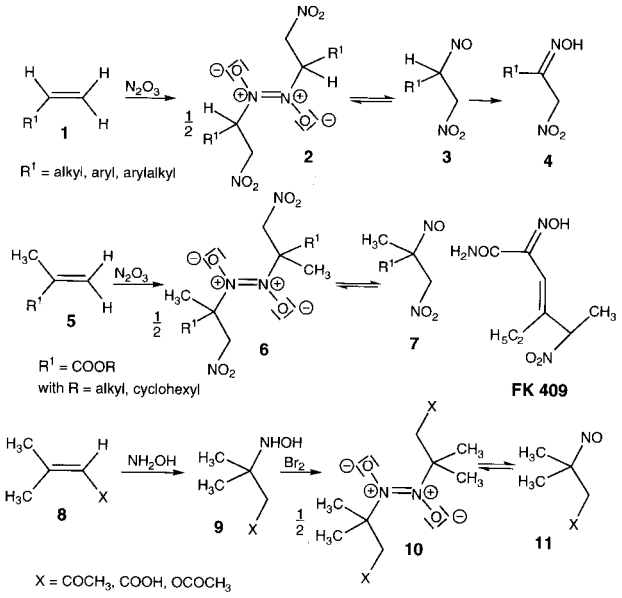


Fig. 1: Synthesis of pseudonitrosites and other azodioxides with vicinal electron acceptors

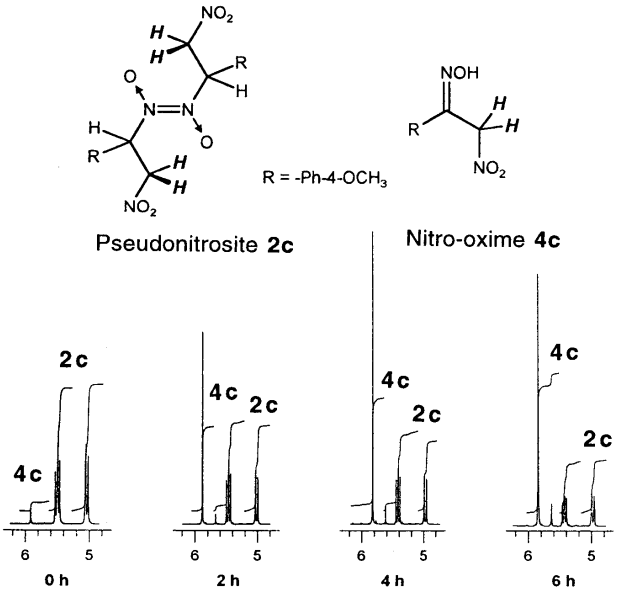


Fig. 2: Rearrangement of **2c** to **4c** in acetone at 20°C. Part of the ^{1}H-NMR spectrum (δ = 5–6 ppm) which shows the signals of the methylene groups.

In the type **10** azodioxides the electron-withdrawing nitro group is replaced by other electron acceptors i.e. by an acetyl, carboxyl, or acetyloxy group. These compounds were obtained from the hydroxylamino derivatives **9** by oxidation with bromine. Starting material were type **8** olefins for **10a**[7] and **10b**. Compound **9c** was obtained from 2-methyl-2-nitropropanol by acetylation and subsequent reduction with Zn/NH_4Cl[8].

The compounds **10a–c** form colorless crystals. The infrared absorption between 1246 and 1259 cm^{-1} supports the azodioxide structure. When dissolved in chloroform immediately a mixture of **10** and **11** is observed (^{1}H-NMR). The solutions are slightly blue. After 150 min the blue color is much deeper and the ^{1}H-NMR spectrum shows that the equilibrium is dominated by the type **11** monomers.

Biology

a) Inhibition of blood platelet aggregation (in vitro, Born test)

The platelet aggregation experiments were carried out as usual[9]. In short platelet rich plasma (PRP) was prepared from freshly drawn citrated human blood by mild centrifugation. Platelet aggregation was induced by collagen. The ensuing increase in light transmission is recorded in an Elvi aggregometer (control). Then the PRP is incubated with different concentrations of the test compound and the influence of collagen on the light transmission is recorded again. The concentration which inhibits the platelet aggregation half-maximally (IC_{50}) is determined graphically from the percentage of inhibition at various concentrations of the test compound. The results are compiled in the fifth column of Table 1.

In the class of the pseudonitrosites **2a–h** all compounds exhibited rather strong antiplatelet activity. Four of them showed an IC_{50} below 10 μM, **2a** being the most active substance with an IC_{50} = 2.1 μM. Neither a decrease (**2b**) nor an increase (**2c**) of electron density in the aromatic ring of R^1 could further improve this result. In the series of aliphatic substituents peak activity was found for a pentyl group (**2e**). This agrees with earlier observations indicating that this group has a good affinity for the platelet membrane. The arylalkyl derivatives **2g** and **2h** also exhibited antiplatelet effects at almost the same concentration (≈ 9 μM). The rearrangement product of **2a,** i.e. **4a** (R^1 = ph) showed no antiplatelet activity. This contrasts with FK 409, which is a vinylogous nitrooxime for which an IC_{50} = 4 μM has been reported[5a].

In the pseudonitrosites with an additional carboxylic ester group (**6a–d**) the antiplatelet effects covered a wide range from 4.2–38 μM. The more lipophilic esters **6c** and **6d** showed the best results.

The replacement of the nitro group in X was not favorable. The compounds were either inactive (**10a**) or showed poor antiplatelet effects (**10b**, **c**).

b) Inhibition of thrombus formation

The influence of the test compounds on the formation of thrombi was assayed as usual in a laser thrombosis model[10]. The title compounds were administered orally to rats (60 mg/kg). After 2 h the formation of thrombi in mesenteric arterioles and venules by the beam of an argon laser via a microscope (35 mW, 50 ms) was observed. The number of exposures (”shots”) necessary to form a thrombus of defined size is counted. From the average shot number the percentage of inhibition of thrombosis is calculated[11]. The results are compiled in the last columns of Table 1.

In the row of the pseudonitrosites **2a**–**h** five of eight compounds showed small antithrombotic activities in the same order of magnitude, i.e. ≈10% inhibition of thrombus formation in arterioles and 5% in venules. No correlation with the *in vitro* results (Born test) could be observed. This suggests that the results are dominated by parameters such as stability

in the gastrointestinal tract or different affinity for enzymes involved in the metabolism of the nitric oxide species.

In the group of compounds with R^1 being an carboxylic acid ester the antithrombotic effects range from none (**6a**) to medium in **6d** which is the most active compound tested.

The type **10** derivatives showed no antithrombotic activity at all. This might be due to the high stability of these compounds. At least in $CDCl_3$ solution after 150 min nearly no decomposition was seen (^{1}H-NMR). For **6b** this was as well true in D_2O/NaOD solution. Compound **4a** showed no antithrombotic effect.

c) Influence on blood pressure

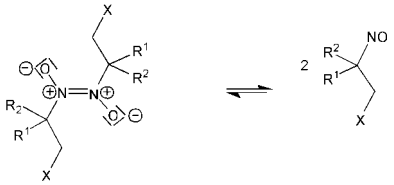
The pattern of pharmacological activities of NO donors comprises more or less pronounced decrease in blood pressure[11a, 11b]. We therefore assayed as usual[16] compounds **2a**, **4a**, **10a,** and **10b** which had been rather effective in the thrombosis model in spontaneously hypertensive rats (SHR). We did not observe a significant decrease in blood pressure (2 h after p.o. administration of 60 mg/kg). Type **6** compounds which are very unstable could not be tested as we were unable to obtain a sufficient amount for the blood pressure experiments. The pseudonitrosites are to our knowledge the second class of NO donors where the desired antithrombotic effect could be separated from the unwanted hypotonous properties[1] . This result suggests that the endothelium at least of SHR is not able to convert pseudonitrosites to nitric oxide.

Decomposition Experiments

From the pattern of antiplatelet and antithrombotic activities (see Table 1) we assumed that these pharmacological effects are mediated by nitric oxide or its reduced congener nitrosohydrogen. In principle the formation of both from the pseudonitrosite **2a** has already been demonstrated by Wieland[3] and Jonkman et al.[12]. However highly unphysiological conditions were used. Wieland observed the formation of dinitrogen oxide in concentrated sulfuric acid. Jonkman et al. investigated the formation of NO at 60 °C in styrene. We tried to approach more physiological conditions, namely aqueous media and 37 °C as reaction temperature.

For our decomposition experiments we selected the pseudonitrosite **2a** which was the most active compound *in vitro* (IC_{50} = 2.1 μM). Because of its poor solubility in phosphate buffer the decomposition experiments were also carried out in ethanol. The results are summarized in Table 2. For comparison the data for the well-known NO donor SIN 1 are given in Table 3.

Table 1: *In vitro* (Born test) antiplatelet and *in vivo* antithrombotic properties of pseudonitrosites **2a–h** and other azodioxides with vicinal electron acceptors after p.o. adminstration to rats (60 mg/kg). Man Whitney U-test[12]. n.s.= not significant. In the Born test the deviation from the mean is about 10%. The IC_{50} of acetylsalicylic acid is 175 μM.



Compound	X	R^1	R^2	Born test IC_{50} [μM]	Inhibition of thrombus formation arterioles % ± s_x	$p \geq$	venules % ± s_x	$p \geq$
2a	NO_2	ph	H	2.1	10 ± 1	0.002	5 ± 1	0.02
2b	NO_2	4-Cl-ph	H	10.5	7 ± 1	0.01	5 ± 2	0.05
2c	NO_2	4-CH_3O-ph	H	19.5	8 ± 1	0.01	7 ± 1	0.01
2d	NO_2	C_3H_7	H	18.0	n.s.		n.s.	
2e	NO_2	C_5H_{11}	H	9.4	11 ± 1	0.002	n.s.	
2f	NO_2	C_7H_{15}	H	15.0	7 ± 2	0.02	4 ± 1	0.05
2g	NO_2	CH_2-ph	H	8.7	n.s.		n.s.	
2h	NO_2	$(CH_2)_2$-ph	H	9.6	n.s.		n.s.	
6a	NO_2	$COOCH_3$	CH_3	38.0	n.s.	n.s.		
6b	NO_2	$COOC_2H_5$	CH_3	24.0	13 ± 1	0.002	9 ± 1	0.002
6c	NO_2	$COOC_4H_9$	CH_3	4.2	4 ± 2	0.2	n.s.	
6d	NO_2	COOCyclhex	CH_3	10.0	18 ± 1	0.002	9 ± 1	0.002
10a	$COCH_3$	CH_3	CH_3	62.5	n.s.		n.s.	
10b	COOH	CH_3	CH_3	39.0	n.s.		n.s.	
10c	$OCOCH_3$	CH_3	CH_3	46.0	n.s.		n.s.	

Table 2: NO formation (chemiluminescence) and N_2O formation (gas chromatography) of the pseudonitrosite **2a** as a function of the solvent (T = 37 °C) and its pH after 1 h.

Solvent	NO-formation [% ± SD]	N_2O-formation [% ± SD]
none	not detected	< 0.03 (n = 5)
0.1 M phosphate buffer pH 7.4 (suspension)	not detected	1.01 ± 0.10 (n = 5)
Ethanol	0.18 ± 0.01 (n = 5)	3.81 ± 1.62 (n = 5)

Table 3: NO formation (chemiluminescence) and N_2O formation (gas chromatography) of the NO donor SIN 1 as a function of the solvent (T = 37 °C) and its pH after 1 h.

Solvent	NO-formation [% ± SD]	N_2O-formation [% ± SD]
none	not detected	0.01 ± 0.001 (n = 5)
0.1 M phosphate buffer pH 7.4	2.7 ± 0.2 (n = 5)	0.03 ± 0.003 (n = 5)
Ethanol	0.9 ± 0.05 (n = 5)	not detected

In ethanol only small amounts of nitric oxide (0.18%) could be detected. In the solid state and in suspension (phosphate puffer) no decomposition to nitric oxide was seen. In contrast considerable amounts of NO were released from SIN 1 especially in phosphate buffer. On the other hand large amounts of N_2O in phosphate buffer (1%) and ethanol (3.8%) have been generated indicating that nitrosohydrogen primarily had been formed ($2\ HNO \rightarrow H_2N_2O_2 \rightarrow N_2O + H_2O$). Obviously the release of nitrosohydrogen is facilitated by the strong electron withdrawing nitro group in vicinal position to the NO moiety. SIN 1 did form nearly no N_2O under these conditions.

The results suggest that the observed antiplatelet and antithrombotic effects (Table 1) might be mediated by an NO dependent mechanism. The low IC_{50} of **2a** in the Born test indicates that the release of $NO^{\bullet}$ and/or NO^- is supported by the metabolic capacity of the PRP. Preferentially and primarily **2a** functions as releaser of nitrosohydrogen which however under aerobic conditions can be oxidized to nitric oxide.

Experimental Part

Chemistry[1)]

Mp (uncorr.), Lindström.– Elemental analysis: Elementar Vario EL.– IR: ATI Mattson Genenis Serie FTIR.– UV/VIS: Kontron Instruments UVIKON 930.– NMR: Bruker AC 300 and DPX 400.– EI-MS: Varian MAT CH7 A and Kratos MS 25 RF.– FAB-MS: Varian MAT CH 5-DF.

General procedure for the synthesis of **2a–h**[4]

Into a stirred solution of 0.1 mol alkene in 80 ml diethyl ether/petroleum ether (1+1) a mixture of nitric oxide and air is introduced at 0–5 °C for 30 min. Then the solution is stirred for 30 min and kept at –16 °C for 24 h. Crystals are formed. They are filtered off under suction and washed with a little cold petroleum ether.

*(E)-1,1′-Azobis-(2-nitro-1-phenyl-ethane) N,N′-dioxide (***2a***)*

From 10.4 g styrene (0.1 mol) in 100 ml toluene at 60 °C[4]. Colorless crystals, mp 113 °C (ref.[4] 112 °C), yield 2.1 g (11%).– Anal. $C_{16}H_{16}N_4O_6$.– IR (KBr): ν = 1561 cm^{-1} (NO_2); 1201 (NO dim.).– ^{1}H-NMR ([D$_6$] acetone): δ = 5.04 (dd, J = 16.0/2.9 Hz, 2H, CH_2-NO_2, M-part), 5.43 (dd, J = 16.0/11.1 Hz, 2H, CH_2-NO_2, A-part), 6.97 (dd, J = 11.1/2.9 Hz, 2H, H-C-NO, X-part), 7.46 (m, 6H, ph-3-H, 4-H, 5-H), 7.53 (m, 4H, ph-2-H and 6-H).– ^{13}C-NMR ([D$_6$] acetone): δ = 68.7 (H-C-NO), 74.2 (CH_2-NO_2), 128.7 (ph-C-3 and C-5), 129.9 (ph-C-2 and C-6), 130.4 (ph-C-4), 131.2 (ph-C-1).– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): m/z (%) = 361 (0.7) [$(M+H)^+$].

*(E)-1,1′-Azobis-[2-nitro-1-(4-chlorophenyl)-ethane] N,N′-dioxide (***2b***)*

From 5.0 g 4-chlorostyrene (36.2 mmol). Colorless crystals, mp 118 °C (ref.[14] 104 °C), yield 1.2 g (15%).– Anal. $C_{16}H_{14}Cl_2N_4O_6$.– IR (KBr): ν = 1561 cm^{-1} (NO_2); 1208 (NO dim.).– ^{1}H-NMR ([D$_6$] acetone): δ = 5.09 (m, 2H, CH_2-NO_2, M-part), 5.47 (m, 2H, CH_2-NO_2, A-part), 6.99 (dd, J = 11.0/2.7 Hz, 2H, H-C-NO, X-part), 7.51 (d, J = 8.5 Hz, 4H, ph-3-H and 5-H), 7.58 (d, J = 8.5 Hz, 4H, ph-2-H and 6-H) all diastereomer A; 5.09 (m, 2H, CH_2-NO_2, M-part), 5.47 (m, 2H, CH_2-NO_2, A-part), 6.90 (dd, J = 11.0/2.7 Hz, 2H, H-C-NO, X-part), 7.25 (d, J = 8.4 Hz, 4H, ph-3-H and 5-H), 7.39 (d, J = 8.4 Hz, 4H, ph-2-H and 6-H) diastereomer B. Ratio A/B = 55:45.– ^{13}C-NMR ([D$_6$] acetone): δ = 67.5 (H-C-NO), 73.3 (CH_2-NO_2), 128.8 (ph-C-1), 129.5, 130.2, 135.6 (ph-C-4) all diastereomer A; 67.9 (H-C-NO), 73.0 (CH_2-NO_2), 128.8 (ph-C-1), 129.4, 130.1, 135.6 (ph-C-4) diastereomer B.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): m/z (%) = 429 (2) [$(M+H)^+$], 184 (59) [mon^+-NO], 138 (100) [$C_8H_7Cl^+$].

*(E)-1,1′-Azobis-[2-nitro-1-(4-methoxyphenyl)-ethane] N,N′-dioxide (***2c***)*

From 5.0 g 4-methoxystyrene (37.3 mmol). Colorless crystals, mp 109 °C, yield 1.0 g (13%).– Anal. $C_{18}H_{20}N_4O_8$.– IR (KBr): ν = 1563 cm^{-1} (NO_2); 1200 (NO_{Dim}).– ^{1}H-NMR ([D$_6$] acetone): δ = 3.83 (s, 6H, OCH_3), 5.00 (dd, J = 15.9/2.9 Hz, 2H, CH_2-NO_2, M-part), 5.44 (dd, J = 15.9/11.1 Hz, 2H, CH_2-NO_2, A-part), 6.90 (dd, J = 11.1/2.9 Hz, 2H, H-C-NO, X-part), 6.99 (d, J = 8.7 Hz, 4H, ph-3-H and 5-H), 7.46 (d, J = 8.7 Hz, 4H, ph-2-H and 6-H).– ^{13}C-NMR ([D$_6$] acetone): δ = 55.2 (OCH_3), 67.4 (H-C-NO), 73.7 (CH_2-NO_2), 114.6 (ph-C-3 and C-5), 122.4 (ph-C-1), 129.7 (ph-C-2 and C-6), 161.1 (ph-C-4).– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): m/z (%) = 421 (0.2) [$(M+H)^+$], 180 (40) [mon^+-NO], 134 (76) [$C_9H_{10}O^+$].

*(E)-2,2′-Azobis-(1-nitro-pentane) N,N′-dioxide (***2d***)*

From 7.0 g 1-pentene (0.1 mol). Colorless crystals, mp 78 °C, yield 0.5 g (3%).– Anal. $C_{10}H_{20}N_4O_6$.– IR (KBr): ν = 1562 cm^{-1} (NO_2); 1204 (NO dim.).– ^{1}H-NMR ([D$_6$] acetone): δ = 0.94 (t, J = 7.3, 6H, CH_3), 1.42 (m, 4H, CH_2-CH_3), 1.81 (m, 4H, CH_2-C_2H_5), 5.01 (dd, J = 15.6/2.9, 2H, CH_2-NO_2, M-part), 5.17 (dd, J = 15.5/10.5, 2H, CH_2-NO_2, A-part), 6.07 (m, 2H, H-C-NO, X-part).– ^{13}C-NMR ([D$_6$] acetone): δ = 13.4 (CH_3), 18.2 ($\underline{C}H_2$-CH_3), 30.5 ($\underline{C}H_2$-C_2H_5), 64.1 (H-C-NO), 73.6 (CH_2-NO_2).– MS ([+]-FAB, aceton/m-nitrobenzyl alcohol): m/z (%) = 293 (50) [$(M+H)^+$], 116 (49) [mon^+-NO], 69 (100), 41 (66).

*(E)-2,2′-Azobis-(1-nitro-heptane) N,N′-dioxide (***2e***)*

From 9.8 g 1-heptene (0.1 mol). Colorless crystals, mp 71 °C, yield 0.7 g (4%).– Anal. $C_{14}H_{28}N_4O_6$.– IR (KBr): ν = 1558 cm^{-1} (NO_2); 1206 (NO dim.).– ^{1}H-NMR ([D$_6$] acetone): δ = 0.89 (t, J = 6.9, 6H, CH_3), 1.30–1.42 (m, 12H, (CH_2)$_3$-CH_3), 1.80–1.86 (m, 4H, CH_2-C_4H_9), 5.01 (dd, J = 15.6/2.8, 2H, CH_2-NO_2, M-part), 5.18 (dd, J = 15.6/10.5, 2H, CH_2-NO_2, A-part), 6.07 (m, 2H, H-C-NO, X-part).– ^{13}C-NMR ([D$_6$] acetone): δ = 13.6 (CH_3), 22.4, 24.5, 28.5, 31.5, 64.3 (H-C-NO), 73.6 (CH_2-NO_2).– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): m/z (%) = 349 (23) [$(M+H)^+$], 144 (33) [mon^+-NO], 55 (100), 41 (43).

[1)] The full set of data is given in the PhD thesis of M. Herpel, FU Berlin 1997.

*(E)-2,2′-Azobis-(1-nitro-nonane) N,N′-dioxide (**2f**)*

From 12.6 g 1-nonene (0.1 mol). Colorless crystals, mp 73 °C, yield 0.4 g (2%).– Anal. $C_{18}H_{36}N_4O_6$.– IR (KBr): ν = 1560 cm^{-1} (NO_2); 1201 (NO dim.).– ^{1}H-NMR ([D$_6$] acetone): δ = 0.89 (t, *J* = 7.0 Hz, 6H, CH_3), 1.29–1.42 (m, 20 H, (CH_2)$_5$-CH_3), 1.80–1.86 (m, 4H, CH_2-C_6H_{13}), 5.01 (dd, *J* = 15.6/2.9, 2H, CH_2-NO_2, M-part), 5.18 (dd, *J* = 15.6/10.6, 2H, CH_2-NO_2, A-part), 6.07 (m, 2H, H-C-NO, X-part).– ^{13}C-NMR ($CDCl_3$): δ = 14.0 (CH_3), 22.5, 24.9, 28.5, 28.8, 29.0, 31.6, 63.9 (H-C-NO), 72.9 (CH_2-NO_2).– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 405 (59) [$(M+H)^+$], 172 (24) [mon^+-NO], 69 (97), 55 (100), 41 (99).

*2,2′-Azobis-(1-nitro-3-phenyl-propane) N,N′-dioxide (**2g**)*

From 11.8 g 3-phenyl-propene (0.1 mol). Colorless crystals, mp 125 °C, yield 0.5 g (3%).– Anal. $C_{18}H_{20}N_4O_6$.– IR (KBr): ν = 1552 cm^{-1} (NO_2).– ^{1}H-NMR ([D$_6$] acetone): δ = 2.92 (dd, *J* = 13.8/7.8 Hz, 2H, CH_2-ph, B′-part), 3.07 (m, 2H, CH_2-ph, A′-part), 4.84 (dd, *J* = 15.6/2.8 Hz, 2H, CH_2-NO_2, M-part), 5.13 (dd, *J* = 15.5/10.1, 2H, CH_2-NO_2, A-part), 6.15 (m, 2H, H-C-NO, X-part), 7.26–7.35 (m, 10 H, ph) all diastereomer A; 3.07 (dd, *J* = 13.9/7.9 Hz, 2H, CH_2-ph, B′-part), 3.21 (dd, *J* = 13.9/5.3 Hz, 2H, CH_2-ph, A′-part), 4.89 (dd, *J* = 15.7/2.8 Hz, 2H, CH_2-NO_2, M-part), 5.15 (dd, *J* = 15.7/10.5, 2H, CH_2-NO_2, A-part), 6.15 (m, 2H, H-C-NO, X-part), 7.26–7.35 (m, 10 H, ph) diastereomer B. Ratio A/B = 1:5.– ^{13}C-NMR ([D$_6$] acetone): δ = 34.6 (CH_2-ph), 65.9 (H-C-NO), 73.2 (CH_2-NO_2), 128.4 (ph-C-4), 129.6, 130.4, 135.4 (ph-C-1) all diastereomer A; 34.0 (CH_2-ph), 66.5 (H-C-NO), 73.1 (CH_2-NO_2), 128.4 (ph-C-4), 129.7, 130.3, 135.4 (ph-C-1) diastereomer B.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 389 (19) [$(M+H)^+$], 164 (39) [mon^+-NO], 117 (69), 91 (100) [$C_7H_7^+$].

*2,2′-Azobis-(1-nitro-4-phenyl-butane) N,N′-dioxide (**2h**)*

From 13.2 g 4-phenyl-1-butene (0.1 mol). Colorless crystals, mp 121 °C, yield 0.4 g (2%).– Anal. $C_{20}H_{24}N_4O_6$.– IR (KBr): ν = 1558 cm^{-1} (NO_2).– ^{1}H-NMR ([D$_6$] acetone): δ = 2.17 (m, 4H, CH_2-CH_2-ph), 2.74 (m, 4H, CH_2-ph), 5.11 (dd, *J* = 15.6/2.8 Hz, 2H, CH_2-NO_2, M-part), 5.28 (dd, *J* = 15.6/10.6, 2H, CH_2-NO_2, A-part), 6.19 (m, 2H, H-C-NO, X-part), 7.18–7.31 (m, 10 H, ph).– ^{13}C-NMR ([D$_6$] acetone): δ = 31.0 and 31.5 ($\underline{C}H_2$-$\underline{C}H_2$-ph), 64.8 (H-C-NO), 74.0 (CH_2-NO_2), 127.1 (ph-C-4), 129.2, 129.3, 141.4 (ph-C-1).– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 417 (14) [$(M+H)^+$], 208 (6) [mon^+], 178 (7) [mon^+-NO], 91 (100) [$C_7H_7^+$].

*2-Nitro-1-phenyl-ethan-1-on-oxim (**4a**)*

1.0 g (2.8 mmol) **2a** is refluxed in 20 ml ethanol for 1 h. After cooling the solvent is removed in vacuo. The remaining oil in kept at 5 °C until crystals have formed. They are filtered off under suction and recrystallized. Colorless crystals, mp 88 °C, yield 0.3 g (31%).– Anal. $C_8H_8N_2O_3$.– IR (KBr): ν = 3231 (OH) cm^{-1}; 2899; 1633; 1560 (NO_2); 1447; 1380; 1284; 1075; 981; 935; 762; 689.– ^{1}H-NMR/400 MHz ([D$_6$]DMSO): δ (ppm) = 5.81 (s, 2H, CH_2-NO_2), 7.39 (br, s, 3H, ph-3H, 4-H and 5-H), 7.64 (br, s, 2H, ph-2-H and 6-H), 12.24 (s, 1H, =NOH, exchangeable).– ^{13}C-NMR/100 MHz ([D$_6$]DMSO): δ (ppm) = 68.7 (CH_2-NO_2), 125,7 (ph-C-3 and C-5), 128.6 (ph-C-2 and C-6), 129.3 (ph-C-4), 133.9 (ph-C-1), 147.2 (C=NOH).– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 181 (100) [$(M+H)^+$], 135 (51) [M^+-NO_2], 117 (25), 103 (26), 79 (57).

General procedure for the synthesis of **6a-d**[6]

A stirred solution of 50 mmol methylacrylic acid ester in 10 ml diethyl ether is treated with 10 ml 40% H_2SO_4. The mixture is cooled to 0 °C and 5 g solid $NaNO_2$ are added over a period of 15 min while maintaining the temperature below 5 °C. After 30 min the diethyl ether phase is separated, washed three times with 10 ml H_2O and dried over Na_2SO_4. Then the diethyl ether is removed *in vacuo*. The residue is dissolved in little diethyl ether/petroleum ether and kept at –16 °C for 2 weeks. Crystals are formed. They are quickly filtered off under suction off and washed with cold petroleum ether.

*(E)-2,2′-Azobis-(2-methyl-3-nitro-propanoic acid methyl ester) N,N′-dioxide (**6a**)*

From 5.0 g methyl methacrylate (50.0 mmol). Colorless crystals, mp 98 °C (blue) (ref.[6] 100–110 °C), yield 0.5 g (6%).– Anal. $C_{10}H_{16}N_4O_{10}$.– IR (KBr): ν = 1752 cm^{-1} (CO); 1561 (NO_2).– UV/VIS ($CHCl_3$): λ_{max} = 655 nm.– ^{1}H-NMR ([D$_6$] acetone): δ = 1.83/1.87 (s, 6H, CH_3), 3.80/3.83 (s, 6H, OCH_3), 5.22 (m, 2H, CH_2-NO_2, B-part), 5.48 (m, 2H, CH_2-NO_2, A-part) all dimer; 1.45 (s, 3H, CH_3), 3.89 (s, 3H, OCH_3), 5.22 (m, 1H, CH_2-NO_2, B-part), 5.48 (m, 1H, CH_2-NO_2, A-part) monomer. Ratio dim/mon = 8:1.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 353 (65) [$(M+H)^+$], 246 (14), 206 (18) [$C_5H_8N_3O_6^+$], 178 (69), 146 (96) [mon^+-NO], 101 (100), 69 (86), 41 (76), 30 (71) [NO^+].

*(E)-2,2′-Azobis-(2-methyl-3-nitro-propanoic acid ethyl ester) N,N′-dioxide (**6b**)*

From 5.7 g ethyl methacrylate (50.0 mmol). Colorless crystals, mp 63 °C (blue), yield 0.5 g (5%).– Anal. $C_{12}H_{20}N_4O_{10}$.– IR (KBr): ν = 1743 cm^{-1} (CO); 1560 (NO_2).– UV/VIS ($CHCl_3$): λ_{max} = 657 nm.– ^{1}H-NMR ([D$_6$] acetone): δ = 1.24–1.33 (m, 6H, CH_2-CH_3), 1.83/1.87 (s, 6H, CH_3), 4.15–4.41 (m, 4H, OCH_2), 5.18–5.27 (m, 2H, CH_2-NO_2, B-part), 5.39–5.50 (m, 2H, CH_2-NO_2, A-part) all dimer; 1.24–1.33 (m, 3H, CH_2-CH_3), 1.45 (s, 3H, CH_3), 4.15–4.41 (m, 2H, OCH_2), 5.18–5.27 (m, 1H, CH_2-NO_2, B-part), 5.39–5.50 (m, 1H, CH_2-NO_2, A-part) monomer. Ratio dim/mon = 6:1.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 381 (37) [$(M+H)^+$], 220 (13) [$C_6H_{10}N_3O_6^+$], 192 (44), 160 (74) [mon^+-NO], 115 (100), 87 (40), 69 (93), 30 (67), 29 (84) [$C_2H_5^+$].

*(E)-2,2′-Azobis-(2-methyl-3-nitro-propanoic acid butyl ester) N,N′-dioxide (**6c**)*

From 7.1 g butyl methacrylate (50.0 mmol). Colorless crystals, mp 42 °C (blue), yield 0.6 g (5%).– Anal. $C_{16}H_{28}N_4O_{10}$.– IR (KBr): ν = 1750 cm^{-1} (CO); 1561 (NO_2).– UV/VIS ($CHCl_3$): λ_{max} = 655 nm.– ^{1}H-NMR ([D$_6$] acetone): δ = 0.87–0.96 (m, 6H, CH_2-CH_3), 1.36–1.49 (m, 4H, CH_2-CH_3), 1.65–1.74 (m, 4H, CH_2-C_2H_5), 1.83/1.87 (s, 6H, CH_3), 4.15–4.34 (m, 4H, OCH_2), 5.17–5.28 (m, 2H, CH_2-NO_2, B-part), 5.37–5.49 (m, 2H, CH_2-NO_2, A-part) all dimer; 0.87–0.96 (m, 3H, CH_2-CH_3), 1.36–1.49 (m, 3H, CH_2-CH_3 and CH_3), 1.65–1.74 (m, 2H, CH_2-C_2H_5), 4.15–4.34 (m, 2H, OCH_2), 5.17–5.28 (m, 1H, CH_2-NO_2, B-part), 5.37–5.49 (m, 1H, CH_2-NO_2, A-part) monomer. Ratio dim/mon = 7:1.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 437 (14) [$(M+H)^+$], 248 (6) [$C_8H_{14}N_3O_6^+$], 220 (19), 188 (34) [mon^+-NO], 143 (54), 114 (33), 87 (53), 57 (100) [$C_4H_9^+$], 41 (84), 29 (64).

*(E)-2,2′-Azobis-(2-methyl-3-nitro-propanoic acid cyclohexyl ester) N,N′-dioxide (**6d**)*

From 8.4 g cyclohexyl methacrylate (50.0 mmol). Colorless crystals, mp 70 °C (blue), yield 0.5 g (4%).– Anal. $C_{20}H_{32}N_4O_{10}$.– IR (KBr): ν = 1735 cm^{-1} (CO); 1562 (NO_2).– UV/VIS ($CHCl_3$): λ_{max} = 657 nm.– ^{1}H-NMR ([D$_6$] acetone): δ = 1.27–1.56 (m, 20 H, cyclohexyl-2-H, 3-H, 4-H, 5-H, 6-H), 1.87 (s, 6H, CH_3), 4.82–4.91 (m, 2H, cyclohexyl-1-H), 5.16–5.49 (m, 4H, CH_2-NO_2) all dimer; 1.27–1.56 (m, 10 H, cyclohexyl-2-H, 3-H, 4-H, 5-H, 6-H), 1.45 (s, 3H, CH_3), 4.98 (m, 1H, cyclohexyl-1-H), 5.16–5.49 (m, 2H, CH_2-NO_2) monomer. Ratio dim/mon = 4:1.– MS ([+]-FAB, acetone/m-nitrobenzyl alcohol): *m/z* (%) = 489 (2) [$(M+H)^+$], 325 (30), 214 (6) [mon^+-NO], 132 (22), 83 (100) [$C_6H_{11}^+$], 69 (30), 55 (90), 41 (92).

*(E)-2,2′-Azobis-(2-methyl-pentan-4-one) N,N′-dioxide (**10a**)*

To a stirred solution of 2.0 g (15.2 mmol) 4-hydroxylamino-4-methylpentan-2-one (**9a**) in 40 ml H_2O bromine is added until no decolorization of bromine is observed. Then the reaction mixture is extracted three times with 40 ml diethyl ether. The collected extracts are washed with H_2O, dried over Na_2SO_4 and the diethyl ether is removed *in vacuo*. The residue is purified on a clay plate and washed with little cold petroleum ether.

Colorless crystals, mp 73 °C (ref.[16] 75–76 °C), yield 0.5 g (25%).– Anal. $C_{12}H_{22}N_2O_4$.– IR (KBr): ν = 1716 cm^{-1} (CO); 1257 (NO dim.).– UV/VIS ($CHCl_3$): λ_{max} ("ε") = 666 (16.2) nm.– ^{1}H-NMR ($CDCl_3$): δ = 1.59 (s, 12H, CH_3), 2.15 (s, 6H, $COCH_3$), 3.25 (s, 4H, CH_2) all dimer; 1.31 (s, 6H, CH_3), 2.15 (s, 3H, $COCH_3$), 3.05 (s, 2H, CH_2) monomer. Ratio dim/mon = 2:1.– ^{13}C-NMR ($CDCl_3$): δ = 24.9 (CH_3), 30.8 ($CO\underline{C}H_3$), 51.1 (CH_2), 76.5 (C-NO), 204.5 ($\underline{C}OCH_3$) all dimer; 21.8 (CH_3), 31.6 ($CO\underline{C}H_3$), 49.9 (CH_2), 96.5 (C-NO), 205.2 ($\underline{C}OCH_3$) monomer.– MS ([+]-FAB, $CHCl_3$/m-nitrobenzyl alcohol): *m/z* (%) = 259 (9) [$(M+H)^+$], 161 (25), 99 (60) [mon^+-NO], 43 (100) [CH_3CO^+].

*(E)-3,3′-Azobis-(3-methyl-butanoic acid) N,N′-dioxide (***10b***)*

To a stirred solution of 2.0 g (15.0 mmol) 3-hydroxylamino-3-methyl-butyric acid (**9b**) in 20 ml 20% HCl bromine is added until no decolorization of bromine is observed. Then the reaction mixture is extracted three times with 20 ml diethyl ether. The diethyl ether phase is washed with H_2O and extracted three times with 20 ml 20% NaOH. The collected aqueous NaOH extracts are acidified with 20% HCl (pH ≈ 2) and extracted three times with 20 ml diethyl ether. The extracts are washed with little H_2O and dried over Na_2SO_4. The diethyl ether is removed *in vacuo* and the residue is purified on a clay plate.

Light blue crystals, mp 110 °C, yield 0.6 g (30%).– Anal. $C_{10}H_{18}N_2O_6$.– IR (KBr): ν = 1702 cm^{-1} (CO); 1547 (NO mon.); 1246 (NO dim.).– UV/VIS (CH_3OH): λ_{max} ("ε") = 666 (17.5) nm.– ^{1}H-NMR ([D_6] DMSO): δ = 1.51 (s, 12H, CH_3), 3.03 (s, 4H, CH_2), 12.22 (s, 2H, D_2O exchange, COOH) all dimer; 1.18 (s, 6H, CH_3), 3.08 (s, 2H, CH_2), 12.22 (s, 1H, D_2O exchange, COOH) monomer. Ratio dim/mon = 3:1.– ^{13}C-NMR ([D_6] DMSO): δ = 23.5 (CH_3), 41.2 (CH_2), 75.5 (C-NO), 170.8 (COOH) all dimer; 20.7 (CH_3), 40.9 (CH_2), 96.1 (C-NO), 171.3 (COOH) monomer.– MS ([+]-FAB, DMSO/glycerol): *m/z* (%) = 263 (8) [$(M+H)^+$], 163 (17), 101 (66) [mon^+-NO], 83 (43), 59 (100) [$C_2H_3O_2^+$], 43 (28).

*(E)-2,2′-Azobis-[1-acetoxy-2-methyl-propane] N,N′-dioxide (***10c***)*

From 10.0 g 2-methyl-2-nitro-propan-1-ol (84.0 mmol)[8]. Instead of $K_2Cr_2O_7$ bromine was used for oxidation. Colorless crystals, mp 63 °C (ref.[8] 67–69 °C), yield 0.9 g (7%).– Anal. $C_{12}H_{22}N_2O_6$.– IR (KBr): ν = 1742 cm^{-1} (CO); 1259 (NO dim.).– UV/VIS ($CHCl_3$): λ_{max} ("ε") = 675 (22.2) nm.– ^{1}H-NMR ($CDCl_3$): δ = 1.60 (s, 12H, CH_3), 2.07 (s, 6H, $OCOCH_3$), 4.59 (s, 4H, CH_2) all dimer; 1.15 (s, 6H, CH_3), 1.98 (s, 3H, $OCOCH_3$), 4.80 (s, 2H, CH_2) monomer. Ratio dim/mon = 1:3.– ^{13}C-NMR ($CDCl_3$): δ = 20.7 ($OCO\underline{C}H_3$), 21.3 (CH_3), 65.9 (CH_2), 77.8 (C-NO), 170.3 ($O\underline{C}OCH_3$) all dimer; 18.2 (CH_3), 20.5 ($OCO\underline{C}H_3$), 66.8 (CH_2), 97.3 (C-NO), 170.5 ($O\underline{C}OCH_3$) monomer.– MS ([+]-FAB, $CHCl_3$/m-nitrobenzyl alcohol): *m/z* (%) = 291 (7) [$(M+H)^+$], 115 (100) [mon^+-NO], 43 (97) [CH_3CO^+].

Biology

Born test[9] and thrombosis experiments[10] were carried out as usual.

N_2O determination

Device for N_2O determination[16]

Head space vials: Perkin Elmer, volume 22 ml.
Gas chromatograph: Perkin Elmer Autosystem with headspace sampler HS 40, Column: Stainless steel, 12 feet, inner diameter 2 mm, filled with Porapak Q (Supelco, Deisenhofen), 80–100 mesh. N_2 flow: 10 ml min^{-1}; electron capture detector range 1, attenuation 64; make up gas: argon/methane (95+5), 60 ml min^{-1}. Inlet temp. 120 °C, detector temp 350 °C, oven temp. 50 °C for 5 min, then rise in temp. 15 °C min^{-1} up to 110 °C. This temp. is held for 2 min. Integrator Perkin Elmer Nelson 1020 (calibration curve cubic and origin).

Generation of the calibration curve[16]

A gas sampling bulb (volume 123 ml) is flushed with pure nitrous oxide. The molar amount of N_2O is corrected for *p* and *T*. By means of a gas tight syringe 100–300 µl in steps of 20 µl are transferred to a headspace sampler and analyzed. The peak area is determined and correlated with the amount of nitrous oxide present in the vials. Hereby calibration values between 4.13–12.39 µmol N_2O are obtained. Each standard deviation (σ = 0.4–2.3% rel.) was obtained from 4 experiments.

Determination of N_2O from decomposition experiments

A sample of about 3 µmol **2a** was weighed exactly into a headspace vial. The vial was sealed gas tight. By means of two cannulas the vial was flushed with argon (100 ml min^{-1}) for 20 min. The cannulas were removed and 3 ml of the solvent (see Table 2) which was also flushed with argon was injected with a gas tight GC-syringe through the septum into the headspace vial. The vial was placed in an incubator at 37 °C for 1h. Then the vial was placed in the headspace sampler and assayed for N_2O. The peak at t_R = 4.5 min was matched with the calibration curve and the percentage of N_2O formation was calculated.

NO determination

Device for NO determination

Head space vials: Perkin Elmer, volume 22 ml. NO/NO_x
Analyzer: Antechnika AC 30 M, Karlsruhe.

Generation of the calibration curve

The calibration curve was generated as described in ref.[17].

Determination of NO from decomposition experiments

The samples were prepared as described above (N_2O determination) and assayed for NO by the chemiluminescence method (described in detail in ref.[17]).

References

☆ Dedicated to Prof. Dr. P. Weyerstahl on the occasion of his 65th birthday.

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