Cheletropic Traps for the Fluorescence Spectroscopic Detection of Nitric Oxide (Nitrogen Monoxide) in Biological Systems

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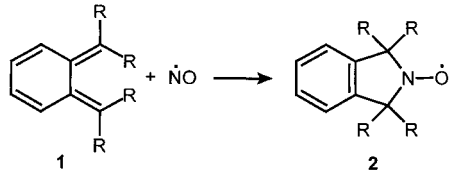
Abstract: The concept of nitric oxide cheletropic traps (NOCT), which relies on the ESR spectroscopic detection of nitroxide radicals formed on trapping of NO by the NOCTs, has been extended to NOCTs that incorporate a fluorophoric system and form a new fluorophore on reaction with NO (FNOCT = fluorescent NOCT). The synthesis of the acetoxymethylesters of two FNOCTs, which can be loaded into cells, is described. Inside the cells the esters are enzymatically hydrolysed, thus allowing intracellular trapping of NO. The primary nitroxides formed on reaction with NO are reduced to the corresponding hydroxylamines under physiological conditions, thus remedying the fluorescence-quenching properties of nitroxide radicals. It is demonstrated that NO formed by lipopolysaccharide(LPS)-activated alveolar macrophages can easily be detected by these FNOCTs on a single cell basis.

Keywords: alveolar macrophages · cheletropic traps · fluorescence spectroscopy · nitrogen oxides · quinodimethanes

Introduction

Nitric oxide (nitrogen monoxide) is a molecule of great biological interest. In recent years it has been and still is the subject of active research.[1, 2] Since its identification as the endothelium-derived relaxing factor (EDRF)[3–6] an almost exponential growth of the literature has taken place in this area. Several methods for the detection and quantitation of nitric oxide are available. Their characteristics have been reviewed in recent reference books.[7, 8] Each method has its merits, but none is without disadvantages and limitations in terms of specificity, sensitivity, or versatility. Only fluorescence methods seem to be ideally suited for the monitoring of NO production in biological samples with both temporal and spacial resolution. Recently developed fluorescence approaches[9, 10] have been applied for the detection of NO in single cells;[9] however, they require the in-situ transformation of NO into nitrosation agents and are, therefore, not direct sensors for NO. Procedures that mimic the biological reaction of NO with heme proteins use either synthetic iron complexes[11] or cytochrome *c*[12] labeled with fluorescent dyes. So far, they cannot be applied intracellularly and their sensitivity is limited to micromolar concentrations of NO.

In recent years we have been involved in the development of a family of molecules that specifically trap nitric oxide by means of a formal cheletropic reaction. By the use of reactive *o*-quinodimethanes of type **1** as biradical equivalents persistent nitroxide radicals (**2**) are produced that can be easily monitored by ESR spectroscopy.[13–15] The first members of



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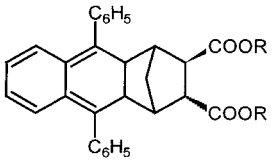
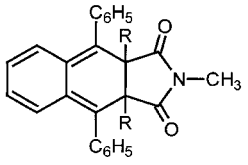


3a R = H
3b R = CH_3



3c R = H
3d R = CH_3

the family, which were based on the parent *o*-quinodimethane, are characterized by short lifetimes due to secondary reactions and/or low preparative yields. Nevertheless, their use for

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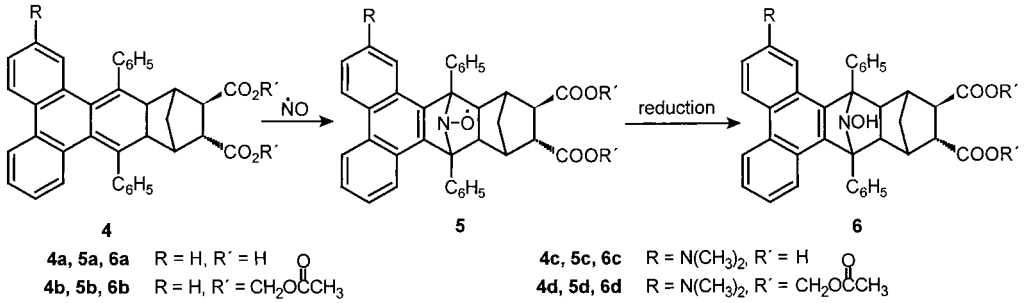


Scheme 1. Reaction scheme for the formation of **5** and **6**.

the detection of NO could be demonstrated by scavenging NO produced from rat-liver macrophages (Kupffer cells).[13] Further developments led to the synthesis of polycyclic systems containing the *o*-quinodimethane skeleton; these proved to be more stable and could be made water soluble by suitable substitution, for example, **3a**.[16] Although they showed high reactivity towards nitric oxide ($k_2 = 10^3 - 10^5\,\text{M}^{-1}\text{s}^{-1}$ in water at 22 °C) the rather short lifetime of the corresponding nitroxides put a limit to their application in physiological environment. Nevertheless, some successful biological applications have been demonstrated.[17, 18]

In light of this situation a new concept was developed that still relies on the same type of reaction but now uses fluorescence spectroscopy as sensitive detection method (FNOCT = fluorescent nitric oxide cheletropic trap). The principle has been published in preliminary communications.[19,20] It is based on the fact that the reaction of *o*-quinodimethanes of type **4** with nitric oxide generates nitroxide radical products **5** with an aromatic phenanthrene unit as the active fluorophore (Scheme 1).

The parent compounds **4** should be either nonfluorescent or should show fluorescence at different excitation and emission wavelengths than products **5**. Previous studies revealed that the fluorescence intensity increased dramatically when the nitroxide radicals **5** were reduced to the corresponding hydroxyl amines **6**. This results from the fact that nitroxide radicals are fluorescence quenchers.[21]

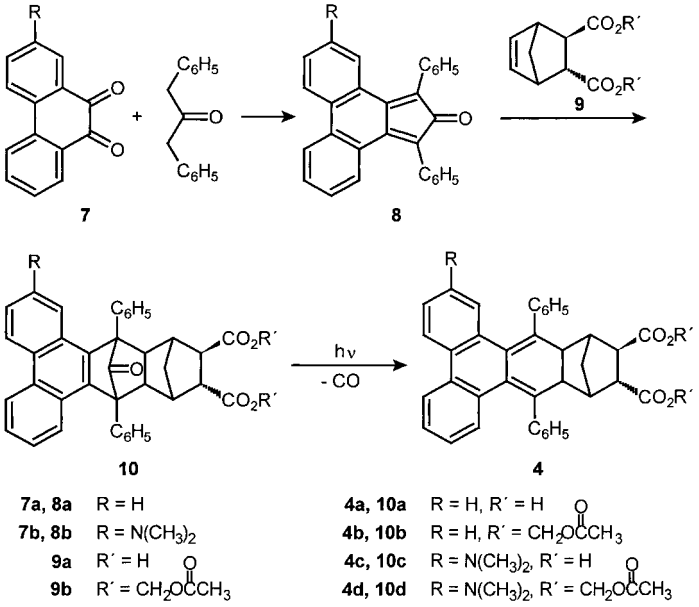
In this contribution we report on the synthesis of suitable FNOCTs and a first application as scavenger of nitric oxide produced by alveolar macrophages. The production of nitric oxide in single cells is monitored by means of digital fluorescence microscopy, following the build up of the fluorescence of the NO trapping product.

Abstract in German: *Das Konzept cheletroper Spinfänger (Nitric Oxide Cheletropic Trap = NOCT), welches auf dem ESR-spektroskopischen Nachweis von Nitroxid-Radikalen beruht, die beim Abfang von NO gebildet werden, wurde auf fluoreszenzfähige cheletrope Fänger (FNOCT = Fluorescent NOCT) ausgedehnt. Bei der Reaktion mit NO entsteht aus dem fluoreszierenden FNOCT ein neuer Fluorophor mit Fluoreszenz bei anderen Wellenlängen. Sowohl die Abnahme der Fluoreszenz der FNOCTs als auch die Zunahme der Fluoreszenz des nach Abfang von NO durch Reduktion des primären Nitroxid-Radikals gebildeten Hydroxylamin-Derivates können für die Erfassung von NO eingesetzt werden. Es wird die Synthese der Acetoxymethylester von zwei FNOCTs beschrieben, mit denen Zellen beladen werden können. Diese Ester werden in den Zellen enzymatisch gespalten, so daß die Säureanionen der FNOCTs nach Zugabe eines Anionenkanalhemmers in den Zellen verbleiben und zum Nachweis des NO genutzt werden können. Die primär entstehenden Nitroxid-Radikale werden unter den physiologischen Bedingungen zu den entsprechenden Hydroxylamin-Derivaten reduziert. Dadurch wird die fluoreszenzlöschende Eigenschaft der Nitroxid-Radikale behoben. Es wird gezeigt, daß NO, gebildet von Alveolar-Makrophagen nach Stimulation der Zellen mit Lipopolysacchariden (LPS), in einfacher Weise mit diesen FNOCTs nachgewiesen werden kann.*

Results

Synthesis and characterization of the fluorescent nitric oxide cheletropic traps: A biologically useful nitric oxide trap should show excitation and emission wavelengths that allow fluorescence microscopy of living cells. If the excitation wavelength is < 340 nm cells might be damaged by irradiation. Furthermore, most optical systems do not operate at these wavelengths. We thus reasoned that FNOCT **4a**, which has been described in the preliminary communication, might not be suited for our purpose owing to its rather short excitation wavelength of $\lambda_{exc} = 315$ nm and its major emission between 360 and 420 nm. Therefore, we introduced a dimethylamino group in **4a** in order to shift fluorescence excitation and emission to longer wavelengths.

Starting materials for the synthesis were the corresponding phenanthrene-9,10-quinones **7a** and **7b**. Condensation of **7a** and **7b** with 1,3-diphenylpropane-2-one gave **8a** and **8b** in 90% and 45% yield, respectively, (Scheme 2). Both underwent smooth Diels–Alder addition with norborn-5-ene-2,3-(*E*)-dicarboxylic acid (**9a**) and its diacetoxymethylester **9b** to give the polycyclic ketones **10a** and **10b**. A mixture of two adducts in a ratio of 2:1 was obtained from the reactions of **8b** with **9a** and **9b**. As the cycloaddition of **8a** to **9a** or **9b** produced only one isomer, for which we derived the displayed stereochemistry from spectroscopic data and semiempirical quantumchemical calculations, we concluded that a mixture of regioisomers of **10c** and **10d** is obtained in the cycloadditions of **8b** to **9a** and **9b.** These isomers differ in the orientation of the dimethylamino group relative to the



Scheme 2. Reaction scheme for the formation of **4**.

substituents at the norbornane skeleton. As a result of their similarity, the isomers could not be separated by conventional techniques. This, however, does not hamper their use. The NO scavengers **4a–d** finally were obtained by quantitative photolytic decarbonylation of **10a–d** and were fully characterized by spectroscopic techniques (Scheme 2).

The parent ketones **10a–d** exhibit the same fluorophoric π system as the NO adducts **5a–d** of the corresponding FNOCTs **4a–d**. Because nitroxides are fluorescence quenchers,[21] reduction of the nitroxides to the corresponding hydroxylamines **6a–d** is required to obtain the maximum fluorescence level. With this prerequisite ketones **10a–d** can serve as spectroscopic models for the reduced NO-trapping products **6a–d**. Figure 1 shows the fluorescence excitation and emission spectra of **10a** (Figure 1a) and **10c** (Figure 1b). As anticipated, the dimethylamino substituent in **10c** shifts the bands to longer wavelengths, into a region that is well suited for biological applications.

The *o*-quinoid π systems of **4a** and **4c** were analyzed with regard to their fluorescence properties. The fluorescence excitation and emission spectrum of **4c** in phosphate buffer is displayed in Figure 2. The fluorescence spectrum of **4a** is of negligible intensity compared with that of **4c** and therefore not shown. It is interesting to note that the fluorescence intensitiy of **4c** depends on pH; it decreases with increasing pH and disappears completely at pH ≥ 9. The fluorescence intensity is at a maximum at pH 4–5. The analysis of the fluorescence intensity as a function of pH yielded a pK_a of 6.7, which is attributed to the protonation equilibrium of the dimethylamino group. The fluorescence is highest in the fully protonated form and is lost in the free amino form. At a physiological pH of 7.2 (phosphate buffer) about 24% of the dimethylamino group is protonated.

In Figure 3 the ESR spectrum is shown that was recorded after addition of a solution of NO in THF to a solution of **4c** in the same solvent. The spectrum is easily interpreted in terms of nitroxide radical **5c**. The additional fine structure of the

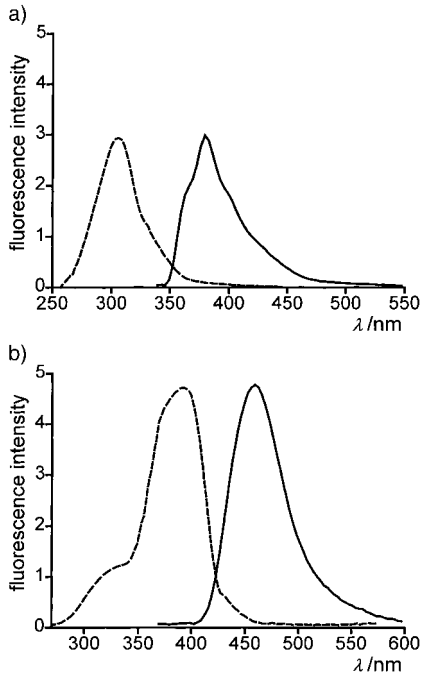


Figure 1. Fluorescence spectra of **10a** and **10c**. Excitation (broken line) and emission spectra (continuous line) of a) **10a** ($c = 50\,\mu M$, $\lambda_{exc} = 315$ nm for emission spectrum, $\lambda_{em} = 380$ nm for excitation spectrum) and b) **10c** (*B*, $c = 50\,\mu M$, $\lambda_{exc} = 380$ nm for emission spectrum, $\lambda_{em} = 460$ nm for excitation spectrum) in sodium phosphate buffer pH 7.2.

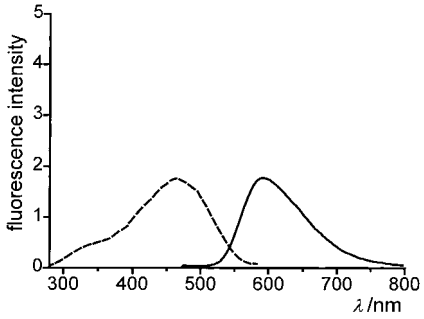


Figure 2. Fluorescence spectra of **4c**. Excitation (broken line) and emission spectrum (continuous line) of **4c** ($c = 50\,\mu M$, $\lambda_{exc} = 460$ nm for emission spectrum, $\lambda_{em} = 600$ nm for excitation spectrum) in sodium phosphate buffer pH 7.2.

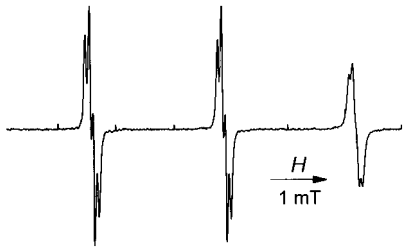


Figure 3. ESR spectrum of **5c**. Radical **5c** was generated in THF solution by reaction of **4c** with NO at 23 °C. ESR parameters: $g = 2.00610$, $a(^{14}N) = 2.287$ mT, $a(exo\text{-}H_\gamma) = 0.076$ mT (2H), $a(H) = 0.048$ mT (1H), $a(H) = 0.014$ mT (4H).

three nitrogen hyperfine lines results from hyperfine splittings by the hydrogen atoms of the norbornane moiety. A virtually identical spectrum was observed for **5a** under similar conditions.[19] The same ESR spectra were also obtained in

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phosphate buffer. In aqueous solution, however, the radicals are not very persistent, decaying within 10–30 min to non-radical products and accompanied by the growth of the typical fluorescence of the phenanthrene chromophore.

The efficiency of compounds **4** to trap nitric oxide was determined by the stopped-flow technique by monitoring the disappearance of the characteristic UV/Vis absorption of the *o*-quinoid π system at about 460 nm. The second-order rate constant for the reaction of **4a** with NO is $k_2 = 930 \pm 70\,\mathrm{M^{-1}s^{-1}}$ in benzene at 23 °C and $60 \pm 8\,\mathrm{M^{-1}s^{-1}}$ in phosphate buffer pH 7.4. The dimethylamino-substituted derivative **4c** reacts at similar rates, namely, $k_2 = 600 \pm 42\,\mathrm{M^{-1}s^{-1}}$ at room temperature in THF (21 °C) and $170 \pm 10\,\mathrm{M^{-1}s^{-1}}$ in phosphate buffer. Slightly reduced rate constants in aqueous solution compared with organic solvents have been observed previously for other NOCTs too.[19]

In order to allow the FNOCT molecules to permeate cell membranes the acetoxymethylesters **4b** and **4d** were synthesized. Inside living cells these esters should be cleaved enzymatically to produce the free acids **4a** and **4c**, respectively; this hinders the trap as well as its NO adduct from leaving the cell. To check on the enzymatic hydrolysis, pig-liver esterase (20 units $\mathrm{mL^{-1}}$) was added to a solution of either **4b** or **4d** in phosphate buffer at 37 °C in the presence of 2% DMSO and 0.2% Pluronic®. The progress of ester cleavage was followed by thin-layer chromatography (on silica gel with toluene/ethylacetate 3:1). Comparison with authentic samples of the free acids **4a** and **4c**, respectively, revealed that for both esters hydrolysis was complete after 1 h. In the absence of the enzyme **4b** as well as **4d** were stable for at least 24 h under these conditions.

The effect of the controlled addition of ascorbate on the fluorescence intensity has been described for radical **5a**.[19] The result of a similar trapping experiment with **4c** in the presence of ascorbate is shown in Figure 4. Excitation at

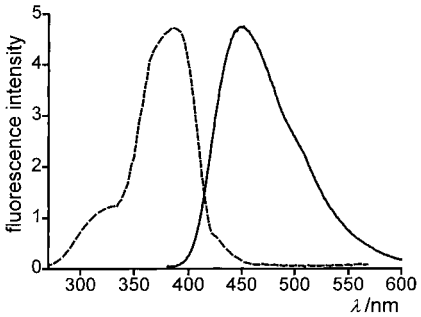

Figure 4. Fluorescence spectra of **6c**. Excitation (broken line) and emission spectrum (continuous line) of **6c** ($c = 50\,\mu\mathrm{M}$, $\lambda_{exc} = 380$ nm for emission spectrum, $\lambda_{em} = 460$ nm for excitation spectrum) in sodium phosphate buffer pH 7.2.

$\lambda_{exc} = 380$ nm yielded an emission spectrum for **6c** ($\lambda_{max} = 460$ nm) that is very similar to the emission spectrum of ketone **10c** (see Figure 1). The fluorescence quantum yields (Q_F) of **6a** and **6c** in phosphate buffer were measured relative to fluorescein as standard ($Q_F = 0.91$[22]), giving Q_F (**6a**) = 0.03 and Q_F (**6c**) = 0.27. As for **4c**, the fluorescence of **6c** is pH dependent, the highest intensity is found at pH 8–9. From the pH dependence of the fluorescence intensity a pK_a of 6.7 was determined, confirming that the protonation of the dimethylamino group is responsible for the pH effect. Even in the absence of a reducing agent nitroxide **5c** has a rather short lifetime in aqueous solution. The same fluorescence intensity, as found in the presence of a reducing agent, was reached 10–30 min after the addition of NO. This fact demonstrates that the degradation of the nitroxide radical does not affect the fluorophoric system.

The effect of a reducing agent on the fate of nitroxides **5b** and **5d** was studied in THF by the use of acetaldehyde in place of ascorbate. Thus, a freshly prepared solution of **4b** or **4d** containing an equimolar amount of acetaldehyde was treated with an equimolar amount of NO solution and analysed immediately by ^{1}H NMR and MS spectroscopy. The resulting spectra were fully consistent with **6b** or **6d**, respectively, being the only products formed (40% conversion). As expected, the ^{1}H NMR spectra of **6b** and **6d** are virtually identical to those of ketones **10b** and **10d**, respectively.

Before FNOCTs **4** were applied in a biological system, further tests were carried out in order to ensure that the specificity of the reaction with nitric oxide was high compared with possible reactions with other biologically relevant reactive molecules. Figure 5 displays the behavior of FNOCT

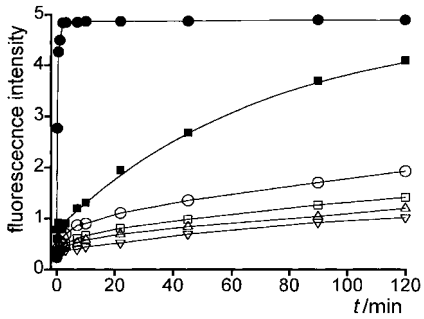

Figure 5. Reactivity of FNOCT **4c** towards NO and other biologically relevant reactive oxygen species. Time dependence of the fluorescence intensity at 23 °C monitored from **4c** ($c = 50\,\mu\mathrm{M}$, $\lambda_{exc} = 380$ nm, $\lambda_{em} = 460$ nm) in pure sodium phosphate buffer pH 7.2 (▽) and in the presence of 100 μM NO-solution + 100 μM ascorbate (●), 20 μM spermine NONOate (■), 100 μM H_2O_2 (□), 100 μM H_2O_2 + 50 μM Fe^{2+} (○) and 100 μM $O_2^{\bullet-}$ (△).

4c in phosphate buffer (pH 7.2, 23 °C, $\lambda_{exc} = 380$ nm, $\lambda_{em} = 460$ nm) against hydrogen peroxide, hydrogen peroxide in the presence of Fe^{II} (Fenton system), and superoxide. No significant increase in fluorescence intensity was observed in the presence of these additives compared with the growth observed in pure buffer solution (which we attribute to a slow thermal transformation of FNOCT **4c** into products also incorporating the phenanthrene unit). The fastest reaction was observed with nitric oxide in the presence of ascorbate, in which the maximum of the fluorescence intensity was reached within a few seconds.

In another experiment (also shown in Figure 5) the NO donor spermine NONOate was added to a solution of FNOCT **4c**. A first-order increase of the fluorescence intensity was observed, which reflects the release of NO by this molecule. The half-life of fluorescence growth

($t_{1/2} = 54$ min at 21 °C) corresponds to the decay rates of spermine NONOate as reported in the literature.[20, 23, 24]

Aromatic π systems tend to aggregate at higher concentrations in aqueous solution.[25] This would lead to a nonlinear dependence of the fluorescence intensity on the concentration of the NO trap. However, in the nanomolar to 100-micromolar concentration range, that is, in the concentration range relevant for cellular systems, the fluorescence intensity of **6c** was found to be strictly linearily correlated ($r^2 = 0.996$) with the concentration of **4c**. This is different to **4a**/**6a** system, in which a deviation from the linear behavior was found for concentrations of **4a** above 20 μM.

The sensitivity for NO detection was determined by adding increasing amounts of NO to a 50 μM solution of **4c** in phosphate buffer. As is shown in Figure 6 the fluorescence of **6c** can be detected already at nM concentrations of NO. Strict linearity of the fluorescence intensity versus the NO concentration is observed up to the μM range, indicating that all of the NO added is trapped by **4c.**

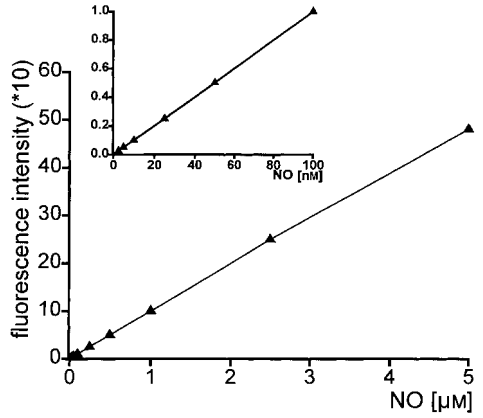

Figure 6. Dependency of the fluorescence intensity of **6c** on the NO concentration after reaction with **4c** ($c = 50$ μM, $\lambda_{exc} = 380$ nm, $\lambda_{em} = 460$ nm) for the micromolar and nanomolar range in sodium phophate buffer pH 7.2.

Biological applications of FNOCT 4d: Alveolar macrophages are known to produce relatively large amounts of NO after activation with lipopolysaccharides and, therefore, were used to test the applicability of the FNOCT concept in a biological system. As we aimed at NO measurements on the single-cell level using digital fluorescence microscopy, conditions for loading the cells with FNOCT **4d** were first investigated. The best loading was achieved at a FNOCT **4d** concentration of 50 μM (dissolved in presence of 0.1% Pluronic® and 1% DMSO) and a loading time of 90 min at 37 °C. Higher concentrations or longer loading times led to increased cell death, lower concentrations or shorter loading times yielded only suboptimal fluorescence intensities as assessed at $\lambda_{exc} = 488 \pm 10$ nm, $\lambda_{em} > 520$ nm. In the presence of probenecid (1.4 mM) no dye leakage could be detected, whereas in the absence of probenecid dye leakage occurred. This indicated that the expected intracellular hydrolysis of ester **4d** to the corresponding acid **4c** had taken place. Fluorescence microscopy demonstrated that the dye was distributed almost homogeneously over the cytosol and the organelles, with only the cell nuclei being spared. Cell viability after measurements was >85%; this was comparable with the respective solvent control, but was somewhat lower than the viability of untreated cells (≥90%). Photobleaching of the fluorescence of **6c** was negligible.

The approximate cellular concentration of FNOCT **6c** was determined by fluorescence spectroscopy after lysis of the cells with triton X-100 (1%). A mean intracellular FNOCT concentration of 100 ± 5 pmol 10^{-6} cells was determined corresponding to an intracellular concentration of 83 ± 4.2 μM, assuming an average cell volume of 1.2 pL cell^{-1}.[26]

For unstimulated, FNOCT-loaded alveolar macrophages, fluorescence at $\lambda_{exc} = 380 \pm 10$ nm and $\lambda_{em} = 460 - 490$ nm was almost identical to the autofluorescence of the cells (as assessed by an unloaded control incubation; Figure 7). Upon addition of 10 μM of the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), that is, subjecting an excess amount of NO to the FNOCT-loaded cells, there was a rapid increase in fluorescence at these wavelengths. The final fluorescence level obtained should reflect complete consumption of the NO trap. When alveolar macrophages had been activated with LPS for 11 h prior to loading with FNOCT **4d**, the fluorescence at $\lambda_{exc} = 380 \pm 10$ nm and $\lambda_{em} = 460 - 490$ nm had increased to the same level after the loading period of 90 min. This increase in fluorescence was observed for all cells with little cell-to-cell variation. No further increase was monitored with duration of the experiment, thus, at the present intracellular concentration the dye had been completely consumed within the loading time of 90 min. Cells treated with the NO synthase inhibitor NMA, regardless if activated or not, did not show a fluorescence intensity exceeding the level of autofluorescence, confirming that the foregoing signal was indeed due to trapping of NO from the NO synthase-catalysed reaction. For comparison, the Griess and DAN tests were

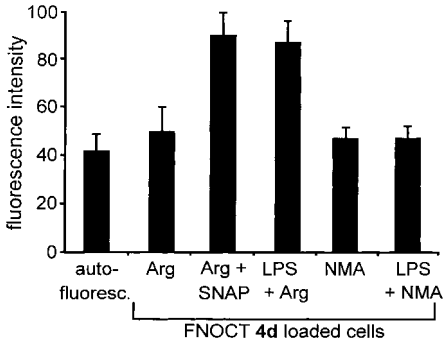

Figure 7. Effect of NO on the fluorescence of FNOCT-loaded alveolar macrophages. Rat alveolar macrophages were loaded with FNOCT **4d** (50 μM, 90 min) and fluorescence was monitored by digital fluorescence microscopy at $\lambda_{exc} = 380 \pm 10$ nm, $\lambda_{em} = 460 - 490$ nm. For release of NO either the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP, 10 μM) was added to the incubation medium (Krebs–Henseleit buffer containing 2 mM L-glutamine and 10 mM D-glucose) and supplemented when necessary with L-arginine (Arg, 0.5 mM), or cells were activated by preincubation with lipopolysaccharides (LPS, 0.5 μg mL^{-1}, 11 h). N^G-monomethyl-L-arginine (NMA, 0.1 mM) was added to some incubations in order to inhibit NO formation. Cellular autofluorescence (autofluoresc.) in the absence of FNOCT **4d** is shown for comparison. Data shown represent mean ± S. D. of 20–50 cells from 4 preparations.

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carried out under identical conditions of cell preparation and stimulation. While FNOCT **4c** allowed easy detection of NO formed 11–12 hours after activation, neither of the two other methods provided such evidence under these conditions. The DAN test was positive 12 hours after stimulation when all NO formed during the first 12 hours was accumulated; for the Griess method 16 h of accumulation were necessary.

Discussion

A method is presented that permits the sensitive detection of nitric oxide. The procedure makes use of the fact that FNOCTs **4** react with NO in a formal cheletropic reaction to give bridged nitroxide radicals **5**. These nitroxides are immediately reduced under cellular conditions to hydroxylamine derivates **6**, thereby generating a phenanthrene-type fluorophore. This methodology is different to most of the other known fluorescence assays for NO in the sense that the fluorophore is produced directly by the reaction of the reagent with NO, and also leads to a permanent incorporation of the latter in the fluorescent product. The fluorescence methods proposed for biological applications so far[8, 9] produce fluorescence only indirectly and require prior transformation of NO into other more reactive nitrogen–oxygen species, for example, NO^+, NO_2, or N_2O_3. This, of course, affects the specificity of such methods, always leaving some ambiguities concerning the truly detected species.

The second-order rate constants for the reaction of **4a** and **4c** with NO are of the order of $100\,M^{-1}s^{-1}$. In terms of quantitation of NO production in biological systems it would be desirable to have rate constants close to diffusion control in order to compete efficiently with the physiological decay reactions of NO. Whereas autoxidation of NO should not interfere owing to the low steady-state level of O_2 in typical cell systems, NO would react rapidly with iron complexes[27, 28] or superoxide.[29–31] On the other hand, smaller k_2 values might be more advantageous for physiological studies considering that not all of the NO being produced will be trapped and, therefore, interference with NO-dependent cellular responses will be largely diminished. In other words, a simultaneous study of NO production and NO-dependent cellular responses remains possible.

The finite lifetimes of our nitroxides **5**, which would make ESR detection in cell systems difficult, were the reason for the development of the FNOCTs. On reaction with NO these systems retain the fluorophore regardless of whether the nitroxide radical decays or not. Even better, as nitroxides are known to be fluorescence quenchers (see above), it is desirable to generate nonradical products. This can be achieved by intentionally adding a suitable reducing agent, for example, acetaldehyde, or taking advantage of the fact that reducing agents like ascorbate, glucose, or glutathione are ubiquituous in living cells.

The specificity of our FNOCTS for the trapping of NO is demonstrated by the fact that the increase in fluorescence intensity in the presence of hydrogen peroxide, hydrogen peroxide + Fe^{II} (Fenton reaction), or superoxide is very similar to the increase in pure buffer solution, indicating insensitivity towards these reactive oxygen species. Peroxynitrite [oxoperoxonitrate(−1)], another putative reactive oxygen species[32] indeed reacts with FNOCT **4c**, as was noticed by an increase of fluorescence and the disappearance of the color of FNOCT **4c** after addition of a small excess of authentic peroxynitrite. A large excess of peroxynitrite led to a decrease of the fluorescence. Details of the reaction of FNOCT **4c** with peroxynitrite will be reported in a forthcoming publication.[33]

The applicability of the trap for NO detection in single, viable cells could be demonstrated by the use of isolated rat alveolar macrophages. FNOCTs **4c**/**4d** were nontoxic in the final concentration used. FNOCT **4d** could be loaded into the cells and was apparently hydrolyzed to the membrane-impermeable FNOCT **4c** which remained in the cells. The intracellular trap could be shown to respond in the same way as in the chemical system to externally applied as well as to endogeneously produced NO, while no signal was observed in unstimulated cells or in the presence of an inhibitor of cellular NO formation. By means of digital fluorescence microscopy, the signal could be detected on the single-cell level, underlining the sensitivity of the method. Together, these data demonstrate that the approach taken is possible. The method presented is likely to be useful for studying a broad range of NO-related questions in cellular systems.

Experimental Section

Instrumentation: NMR: Bruker AMX300, Varian Gemini200 (signals indicated with * are attributed to the minor isomer of the compound); ESR: Bruker ER420 with DigiS data acquisition system (GfS Aachen, Germany); IR: Perkin–Elmer Series 1600 FTIR; UV/Vis: Cary 219; fluorescence spectroscopy: Jobin Yvon JY3 with DigiS data acquisition system and FL3095 of J&M; MS: Fisons VG ProSpec 300; elemental analysis: Carlo Erba Model 1106; melting points: Büchi 510 (uncorrected); stopped-flow measurements: HiTech Scientific Ltd. SF41 Cryostopped-flow spectrometer (Salisbury, England).

Materials: Dulbecco's modified eagle medium (DMEM, low glucose) and gentamycin sulfate were purchased from GibcoBRL (Eggenstein, Germany); lipopolysaccharides, probenecid, and fetal calf serum were from Sigma (Steinheim, Germany); Pluronic® F-127, propidium iodide, and spermine NONOate ((*Z*)-1-{*N*-[3-aminopropyl]-*N*-[4-(3-aminopropylammonio)butyl]amino}-diazen-1-ium-1,2-diolate) were from Molecular Probes Europe BV (Leiden, The Netherlands); trypan blue, N^G-monomethyl-L-arginine monoacetate and esterase (pig liver, 200 units mg^{-1}) were from Aldrich (Steinheim, Germany); dimethyl sulfoxide and triton X-100 were from Merck (Darmstadt, Germany). Falcon 6-well cell-culture plates were obtained from Becton Dickinson (Heidelberg, Germany) and glass coverslips were from Assistent (Sondheim/Röhn, Germany).

2-Dimethylaminophenanthrene-9,10-quinone (7b): A suspension of 2-amino-phenanthrene-9,10-quinone[34, 35] (1.25 g, 5.6 mmol) and sodium carbonate (0.60 g, 5.6 mmol) in methanol (5.0 mL) was heated to 60 °C and dimethyl sulfate (1.1 mL, 1.41 g, 10.2 mmol) was added. After 1 h every 30 min sodium carbonate (0.30 g, 2.8 mmol) and dimethyl sulfate (0.55 mL, 5.1 mmol) were added to the reaction mixture five times. The reaction was stopped after 4 h by addition of chloroform (250 mL). After filtration the chloroform phase was washed several times with water until the water phase remained colorless. Removal of chloroform provided a dark-blue solid consisting of a mixture of mono- and dimethylated product. Chromatography (100 g silica gel 60, Merck, 200 μm) on a 70 cm column (diameter 2 cm) with toluene/ethylacetate 3:1 gave 0.80 g (3.2 mmol, 57%) **7b** together with 0.33 g (1.4 mmol, 25%) monomethylated product. M.p. 170 °C; ^{1}H NMR (200 MHz, $CDCl_3$, TMS): $\delta = 8.06$ (dd, ^3J(7-H, 8-H) = 7.8 Hz, ^4J(6-H, 8-H) = 1.6 Hz, 1H; 8-H), 7.78 (dd, ^3J(5-H, 6-H) = 7.8 Hz,

^4J(5-H, 7-H) = 1.0 Hz, 1H; 5-H), 7.76 (d, ^4J(3-H, 4-H) = 9.6 Hz, 1H; 4-H), 7.55 (ddd, ^3J(5-H, 6-H) = 7.8 Hz, ^3J(6-H, 7-H) = 7.8 Hz, ^4J(6-H, 8-H) = 1.4 Hz, 1H; 6-H): 7.37 (d, ^4J(1-H, 3-H) = 3.0 Hz, 1H; 1-H), 7.26 (ddd, ^3J(6-H, 7-H) = 7.5 Hz, ^3J(7-H, 8-H) = 7.6 Hz, ^4J(7-H, 5-H) = 1.0 Hz, 1H; 7-H), 6.93 (dd, ^3J(3-H, 4-H) = 9.4 Hz, ^4J(1-H, 3-H) = 3.0 Hz, 1H; 3-H), 3.06 (s, 6H; CH_3); ^{13}C NMR (50 MHz, $CDCl_3$, TMS): δ = 180.8 (*C*=O), 150.2 (C-2), 137.4, 131.5, 129.5, 123.3 (C-1a, C-4a, C-5a, C-8a), 135.8, 130.1, 127.2, 125.2, 122.6, 118.5, 112.2 (C-1, C-3, C-4, C-5, C-6, C-7, C-8), 39.91 (CH_3); IR (KBr): $\tilde{\nu}$ = 3065 (=C−H), 2885 (−CH_3), 2801 (N−CH_3), 1667 (C=O)$_{quinone}$, 1594 cm^{-1} (C=C)$_{quinone}$; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{16}H_{13}N_1O_2$: 251.095536; found 251.094629; difference −3.6 ppm; UV/Vis (THF): λ_{max} (lg ε) = 550 (3.28), 330 (4.02, shoulder), 290 nm (4.55); R_F = 0.54 (toluene/ethylacetate 3:1).

2,5-Diphenyl-3,4-(2,2′-biphenylene)cyclopenta-2,4-diene-1-one (8a): This compound was prepared by means of the procedure given in ref. [36].

2,5-Diphenyl-3,4-(4-dimethylamino-2,2′-biphenylene)cyclopenta-2,4-diene-1-one (8b): Compound **7b** (0.55 g, 2.2 mmol) and 1,3-diphenylpropane-2-one (0.46 g, 2.2 mmol) in ethanol (6.0 mL) were treated with a 20% solution of potassium hydroxide in ethanol (0.6 mL) at 55 °C according to the literature.[36] The violet suspension changed to a yellow solution, from which a black solid precipitated. For completion the reaction mixture was kept at 0 °C over night. Filtration gave 0.43 g (1.0 mmol, 45%) of **8b**. M.p. 200–205 °C; ^{1}H NMR (300 MHz, $CDCl_3$, TMS): δ = 7.61 (dd, ^3J(5″-H, 6″-H) = 7.6 Hz, ^4J(4″-H, 6″-H) = 1.4 Hz, 1H; 6″-H), 7.60 (d, ^4J(5′-H, 6′-H) = 9.0 Hz, 1H; 6′-H), 7.45 (dd, ^3J(3″-H, 4″-H) = 7.7 Hz, ^4J(3″-H, 5″-H) = 1.0 Hz, 1H; 3″-H), 7.43–7.35 (m, 10H; C*H*-phenyl), 7.16 (ddd, ^3J(3″-H, 4″-H) = 7.6 Hz, ^3J(4″-H, 5″-H) = 7.4 Hz, ^4J(4″-H, 6″-H) = 1.5 Hz, 1H; 4″-H), 6.84 (d, ^4J(3′-H, 5′-H) = 3.0 Hz, 1H; 3′-H), 6.77 (ddd, ^3J(4″-H, 5″-H) = 7.9 Hz, ^3J(3″-H,4″-H) = 7.6 Hz, ^4J(3″-H, 5″-H) = 1.0 Hz, 1H; 5″-H), 6.60 (dd, ^3J(5′-H, 6′-H) = 9.0 Hz, ^4J(3′-H, 5′-H) = 3.0 Hz, 1H; 5′-H), 2.60 (s, 6H; CH_3); ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 200.6 (*C*=O), 149.6 (C-4′), 149.3, 148.7 (C-2, C-5), 134.6, 132.9, 132.5, 128.7, 127.0, 122.9, 122.7, 121.9 (C-3, C-4, C-1′, C-1″, C-2′, C-2″, *C-ipso*-phenyl), 131.3, 130.1, 130.0, 129.4, 128.5, 128.4, 127.9, 127.8, 126.2, 125.4, 123.1, 115.3, 112.3 (C$H_{arom.}$), 39.91 (CH_3); IR (KBr): $\tilde{\nu}$ = 3055 (=C−H), 2886 (−CH_3), 2790 (N−CH_3), 1697 (C=O), 1599 (C=C), 1368 cm^{-1} (C−N); MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{31}H_{23}N_1O_1$: 425.185262; found 425.177965; difference −7.2 ppm; UV/Vis (THF): λ_{max} (lg ε) = 655 (2.91), 282 (4.49), 320 (4.37, shoulder), 270 nm (4.45).

5-Norbornene-2-*endo*-3-*exo*-dicarboxylic acid diacetoxymethylester (9b): First sodium hydride (2.4 g, 100 mmol) and then chloromethyl acetate[38] (5.52 g, 50 mmol) were added to a mixture of 5-norbornene-2,3-(*E*)-dicarboxylic acid (**9a**)[37] (4.55 g, 25 mmol) in absolute dimethyl formamide (50 mL) at 0 °C, in analogy to the literature.[39] The mixture was converted in 38% to the diacetoxymethylester **9b** at 40 °C after 4 h. B.p. 130 °C (10 Pa); ^{1}H NMR (200 MHz, $CDCl_3$, TMS): δ = 6.24 (dd, ^3J(5-H, 6-H) = 5.8 Hz, ^3J(5-H, 4-H) = 3.4 Hz, 1H; 5-H), 6.01 (dd, ^3J(6-H, 3-H) = 5.8 Hz, ^3J(6-H, 1-H) = 3.0 Hz, 1H; 6-H), 5.74–5.70 (m, 2H; OCH_2O), 5.68–5.61 (m, OCH_2O), 3.40 (d, ^3J(2-H, 3-H) = 4.2 Hz, 1H; 3-H), 3.26 (brs, 1H; 1-H/4-H), 3.11 (brs, 1H; 1-H/4-H), 2.69 (dd, ^3J(2-H, 3-H) = 4.6 Hz, ^4J(7′-H, 2-H) = 1.6 Hz, 1H; 2-H), 2.07 (s, 3H; CH_3), 2.06 (s, 3H; CH_3), 1.57 (d, ^2J(7-H, 7′-H) = 8.6 Hz, 1H; 7-H), 1.41 (dd, ^2J(7′-H, 7-H) = 8.8 Hz, ^4J(2-H, 7′-H) = 1.6 Hz, 1H; 7′-H); ^{13}C NMR (50 MHz, $CDCl_3$, TMS): δ = 172.8, 171.6, 169.4 (*C*=O), 137.6, 134.8 (C-5, C-6), 79.49, 79.24 (OCH_2O), 47.66, 47.43, 47.08, 45.74, 45.63 (C-1, C-2, C-3, C-4, C-7), 20.53 (CH_3); IR (film): $\tilde{\nu}$ = 3067 (=C−H), 2998, 2954, 2878 (C−H), 1762 (C=O), 1371 (CH_3), 1225, 1157 cm^{-1} (C−O); MS (70 eV, EI): *m*/*z* (%): = 328 (20) [M^++2], 296 (5) [$M^+ - 2\,CH_3$], 267 (5) [$M^+ - OCOCH_3$], 254 (10) [$M^+ - CHOCOCH_3$], 237 (4) [$M^+ - OCH_2OCOCH_3$], 207 (15) [$M^+ - 2\,OCOCH_3$], 194 (8) [$M^+ - C_5H_8O_4$], 164 (60) [$C_9H_8O_3$], 66 (100) [C_5H_6]; $C_{15}O_8H_{18}$ (326.3) calcd C 55.2, H 5.52; found C 54.9, H 5.41.

3,5-Diphenyl-3,5-(9,10-phenanthrylene)tricyclo[5.2.1.0^{2,6}]decane-4-one-8-*exo*-9-*endo*-dicarboxylic acid (10a): Compounds **8a** (0.50 g, 1.30 mmol) and **9a** (0.27 g, 1.50 mmol) were heated in chlorobenzene (12.0 mL) for 15 h under reflux in the dark. After the removal of chlorobenzene (6 mL) by destillation and addition of benzene (10 mL), the precipitated solid was collected by filtration, dissolved in THF (80 mL) and treated with active carbon at room temperature for 30 min. The product was precipitated from the filtrate by addition of *n*-pentane. Further purification was achieved by dissolving in THF at 50 °C and precipitating at room temperature by addition of water. Yield 0.64 g (1.0 mmol, 84%); m.p. 282 °C; ^{1}H NMR (300 MHz, [D$_8$]THF, TMS): δ = 11.28 (brs, 2H; COO*H*), 8.79 (d, ^3J = 8.8 Hz, 2H; 4′ H, 5-′H), 8.08 (brd, ^3J = 7.5 Hz, 1H; *o*′-H or *o*″-H), 7.90 (brd, ^3J = 7.8 Hz, 1H; *o*′-H or *o*″-H), 7.65 (dd, ^3J = 6.5 Hz, 7.5 Hz, 1H; *m*′-H or *m*″-H), 7.59 (dd, ^3J = 7.8 Hz, 7.5 Hz, 1H; *m*′-H or *m*″-H), 7.47–7.52 (m, 2H; 3′-H, 6 ′H), 7.39–7.45 (m, 2H; *p*-H), 7.30–7.36 (m, 2H; *m*-H), 7.10–7.19 (m, 6H; 2′-H, 7′-H), 3.24 (brs, 2H; 2-H, 6-H), 3.14 (dd, ^3J = 5.2 Hz, 4.5 Hz, 1H; 8-H), 3.05 (brd, ^3J = 5.2 Hz, 1H; 9-H), 2.87 (d, ^3J = 3.9 Hz, 1H; 7-H), 2.82 (brs, 1H; 1-H), 0.47 (d, ^2J = 10.9 Hz, 1H; 10b-H), −0.51 (d, ^2J = 10.0 Hz, 1H; 10a-H); ^{13}C NMR (75 MHz, [D$_8$]THF, TMS, CH-COSY, COLOC): δ = 198.99 (*C*=O)$_{ketone}$, 173.96, 174.65 (*C*=O)$_{acid}$, 137.94, 138.29 (*C-ipso*-phenyl), 135.57, 135.73 (C-9′, C-10′), 132.44 (C-*o*), 131.88, 131.92 (C-4′a, C-5′a), 129.56, 129.73 (C-*m*′, C-*m*″), 128.98, 129.06 (C-*m*), 128.67 (C-1′a, C-8′a), 128.56, 128.73 (C-*o*′, C-*o*″), 128.11, 128.19 (C-*p*), 127.11 (C-2′, C-7′), 126.89, 126.93 (C-3′, C-5′), 126.14 (C-1′, C-8′), 124.23 (C-4′, C-5′), 63.96, 64.42 (C-3, C-5), 51.65 (C-8), 51.01 (C-9), 48.06 (C-6), 45.39 (C-1), 44.16 (C-2), 43.00 (C-7), 34.60 (C-10); IR (KBr): $\tilde{\nu}$ = 3350–3490 (−OH), 3088, 3058, 3031 (=CH), 2969, 2926 (−CH), 1785 (C=O$_{ketone}$), 1710 cm^{-1} (C=O$_{acid}$); UV/Vis (MeOH): λ_{max} (lg ε) = 253 (4.45), 260 (4.53), 271 (4.19), 285 (3.80), 297 (3.82), 310 nm (3.82); Fluorescence (phosphate buffer pH 7.4): λ_{exc} = 315 nm, λ_{em} = 365, 380, 405 nm; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{38}H_{28}O_5$: 564.1937; found: 564.1951; difference 2.5 ppm; $C_{38}H_{28}O_5$ (564.6): calcd C 80.83, H 4.99; found C 80.01, H 4.68.

3,5-Diphenyl-3,5-(9,10-phenanthrylene)tricyclo[5.2.1.0^{2,6}]decane-4-one-8-*exo*-9-*endo*-dicarboxylic acid diacetoxymethylester (10b): Compounds **9b** (2.20 g, 12.0 mmol) and **8a** (3.83 g, 10.0 mmol) were heated in chlorobenzene (100 mL) for 15 h at 130 °C. After removal of the solvent, the residue was dissolved in dichloromethane and precipitated by addition of *n*-heptane. Recrystallization from ethanol gave 5.24 g (7.40 mmol, 74%) of **10b**. M.p. 225 °C; ^{1}H NMR (300 MHz, [D$_8$]THF, TMS): δ = 8.80 (d, ^3J = 8.2 Hz, 2H; 4′-H, 5′-H), 7.97 (brd, ^3J = 7.5 Hz, 1H; *o*′-H or *o*″-H), 7.90 (brd, ^3J = 7.5 Hz, 1H; *o*′-H or *o*″-H), 7.70 (dd, ^3J = 7.5 Hz, ^3J = 7.5 Hz, 1H; *m*′-H or *m*″-H), 7.68 (dd, ^3J = 7.5 Hz, ^3J = 7.5 Hz, 1H; *m*′-H or *m*″-H), 7.52 (dd, ^3J = 7.5 Hz, ^3J = 7.5 Hz, 2H; 3′-H, 6′-H), 7.45 (dd, ^3J = 7.4 Hz, ^3J = 7.4 Hz, 2H; *p*-H), 7.35–7.32 (m, 2H; *m*-H), 7.21–7.12 (m, 6H; 1′-H, 2′-H, 7′-H, 8′-H, *o*-H), 5.95 (d, ^2J = 10 Hz, 1H; OCH_2O), 5.92 (d, ^2J = 10 Hz, 1H; OCH_2O), 5.65 (d, ^2J = 13 Hz, 1H; OCH_2O), 5.63 (d, ^2J = 13 Hz, 1H; OCH_2O), 3.29 (d, ^3J = 9 Hz, 1H; 2-H or 6-H), 3.24 (brs, 2H; 8-H, 9-H), 3.18 (d, ^3J = 8.5 Hz, 1H; 2-H or 6-H), 2.99 (brs, 1H; 7-H), 2.82 (brs, 1H; 1-H), 2.01 (s, 3H; CH_3), 1.97 (s, 3H; CH_3), 0.50 (brd, ^2J (10a-H, 10b-H) = 11.7 Hz, 1H; 10a-H), −0.5 (brd, ^2J (10a-H, 10b-H) = 13 Hz, 1H; 10b-H); ^{13}C NMR (75 MHz, [D$_8$]THF, TMS): δ = 198.7 (*C*=O)$_{ketone}$, 172.4, 171.6, 169.8, 169.5 (*C*=O)$_{ester}$, 138.0, 137.8, 135.8, 135.5, 132.1, 132.0, 128.8, 128.7 (C-1′a, C-4′a, C-5′a, C-8′a, C-9′, C-10′, *C-ipso*-phenyl), 132.5, 132.4, 130.1, 129.9, 129.2, 129.2, 128.7, 128.6, 128.4, 128.3 (*C*H-phenyl), 127.3, 127.1, 126.2 (C-2′, C-3′, C-4′, C-5′, C-6′, C-7′), 124.4, 124.3 (C-1′, C-8′), 80.71, 80.58 (OCH_2O), 64.45, 64.07 (C-3, C-5), 51.68, 51.01, 47.89, 45.17, 44.23, 43.16 (C-1, C-2, C-6, C-7, C-8, C-9), 34.76 (C-10), 20.50, 20.28 (CH_3); IR (KBr): $\tilde{\nu}$ = 3029 (=CH), 2924 (CH), 1781 (C=O$_{ketone}$), 1762 (C=O$_{ester}$), 1614 (C=C), 1368 (O−CO−CH_3), 1228, 1168, 1006 cm^{-1} (C−O); UV/Vis (THF): λ_{max} (lg ε) = 360 (282), 350 (2.55), 342 (2.77), 335 (2.62), 320 (4.08), 300 (4.09), 253 (4.79), 245 nm (4.71); Fluorescence (THF): λ_{exc} = 315 nm, λ_{em} = 361, 382, 404, 425 nm; MS (70 eV, EI): *m*/*z* (%): = 710 (2) [M^++2], 680 (100) [$M^+ - CO$]; $C_{44}H_{36}O_9$ (708.8): calcd C 74.6, H 5.08; found C 73.9, H 4.98.

3,5-Diphenyl-3,5-(2-dimethylamino-9,10-phenanthrylene)tricyclo[5.2.1.0^{2,6}]-decane-4-one-8,9-(*E*)-dicarboxylic acid (10c): A suspension of **8b** (100 mg, 0.235 mmol) was treated with **9a** (43 mg, 0.235 mmol) in chlorobenzene (2.0 mL) at 130 °C for 8 h. The white precipitate was removed by filtration, washed with little chlorobenzene, dissolved in THF and precipitated by addition of *n*-heptane. Recrystallization from ethanol yielded 103 mg (0.17 mmol, 72%) of **10c**. M.p. (decomp) 289–295 °C; ^{1}H NMR (300 MHz, [D$_8$]THF, TMS): δ = 11.0 (brs, 2H; COO*H*), 8.58–8.51 (m, 2H; 4′-H, 5′-H), 8.14–8.05 (m, 1H; *o*′-H or *o*″-H), 7.97–7.86 (m, 1H; *o*′-H or *o*″-H), 7.68–7.53 (m, 2H; *m*′-H, *m*″-H), 7.38–6.96 (m, 10H; 1′-H, 6′-H, 7′-H, 8′-H, *o*-H, *m*-H, *p*-H), 6.40 (dd, ^4J = 2.7 Hz, ^4J = 2.7 Hz, 1H; 3′-H), [3.19–3.11 (m, 3H), 3.04–3.01 (m, 1H), 2.93–2.91 (m, 1H), 2.87, 2.80* (brs, 1H), 1-H, 2-H, 6-H, 7-H, 8-H, 9-H], 2.61, 2.61* (s, 6H; NCH_3), 0.47 (brd, ^2J(10a-H, 10b-H) = 11.8 Hz, 1H; 10a-H), −0.34 (brd, ^2J(10a-H, 10b-H) = 11.5 Hz, 1H; 10b-H); ^{13}C NMR (75 MHz, [D$_8$]THF, TMS): δ = 199.3 (*C*=O)$_{ketone}$, 174.9, 174.3 (*C*=O)$_{acid}$, 149.4 (C-2′), 139.2*, 138.9, 138.7, 138.3*,135.7*, 135.5, 134.8, 134.6*, 130.2, 130.1*, 127.1*, 127.1, 122.6 (C-1′a, C-4′a, C-5′a,

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C-8′a, C-9′, C-10′, *C-ipso*-phenyl), 132.9, 132.6, 129.8*, 129.7, 129.6, 129.5*, 129.2, 129.0*, 129.0, 128.9, 128.8*, 128.7, 128.6*, 128.1*, 128.0, 128.0, 127.9*, 126.9, 126.2, 125.0, 124.9, 124.8*, 123.2, 115.0, 114.9*, 107.2, 107.1* ($CH_{arom.}$), 64.47*, 64.36, 64.00*, 63.90, (C-3, C-5), 51.97*, 51.86, 51.25, 48.47, 48.24*, 45.44, 44.42*, 44.22, 43.09 (C-1, C-2, C-6, C-7, C-8, C-9), 40.01 (NCH_3), 34.84 (C-10); IR (KBr): $\tilde{\nu}$ = 3068, 3030 (=CH), 2976, 2922, 2801 (CH), 2786 (N−CH_3), 1780 (C=O_{ketone}), 1710 (C=O_{acid}), 1614 (C=C), 1365 (C−N), 1007 cm^{-1} (C−O); UV/Vis (phosphate buffer pH 7.2): λ_{max} (lg ε) = 465 (3.48), 325 (4.13), 295 nm (4.37); Fluorescence (phosphate buffer pH 7.2): λ_{exc} = 380 nm, λ_{em} = 480 nm; MS (70 eV, EI): *m*/*z* (%): = 607 (10) [M^+], 579 (100) [M^+ − CO]; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{40}H_{33}N_1O_5$: 607.232730; found 607.235874; difference 5.2 ppm.

3,5-Diphenyl-3,5-(2-dimethylamino-9,10-phenanthrylene)tricyclo[5.2.1.0^{2,6}]-decane-4-one-8,9-(*E*)-dicarboxylic acid diacetoxymethylester (10d): A suspension of **8b** (100 mg, 0.235 mmol) and **9b** (76.7 mg, 0.235 mmol) in chlorobenzene (2.0 mL) was heated to 110 °C. When the dark-green solution had turned yellow (4 h) the solvent was removed, the solid was dissolved in dichloromethane, and the product precipitated by addition of *n*-heptane. Recrystallization from ethanol gave 150 mg (0.20 mmol, 85 %) of **10d** as a mixture of isomers. M.p.(decomp) 220–230 °C; ^{1}H NMR (300 MHz, [D_8]THF, TMS): δ = 8.58 (d, ^3J = 7.6 Hz, 1H; 4′-H or 5′-H), 8.55 (d, ^3J = 8.0 Hz, 1H; 4′-H or 5′-H), 8.01–7.86 (m, 2H; *o*′-H, *o*″-H), 7.72–7.62 (m, 2H; *m*′-H, *m*″-H), 7.46–6.98 (m, 10H; 1′-H, 6′-H, 7′-H, *o*-H, *m*-H, *p*-H), 6.40 (dd, ^4J = 3.0 Hz, ^4J = 3.0 Hz, 1H; 3′-H), 5.95–5.90 (m, 2H; OCH_2O), 5.68–5.61 (m, 2H; OCH_2O), 3.27–3.19 (m, 3H; 1-H or 7-H, 2-H, 6-H), 3.15, 3.12* (br d, ^4J = 3.0 Hz, 1H; 8-H or 9-H), 3.03*, 2.96 (br d, ^3J = 3.0 Hz, 1H; 8-H or 9-H), 2.86, 2.80* (br s, 1H; 1-H or 7-H), 2.63 (s, 6H; NCH_3), 2.01, 1.98*, 1.97*, 1.95 (s, 6H; CH_3), 0.53 (br d, ^2J(10a-H, 10b-H) = 12.0 Hz, 1H; 10a-H), −0.31 (br d, ^2J(10a-H, 10b-H) = 11.7 Hz, 1H; 10b-H); ^{13}C NMR (75 MHz, [D_8]THF, TMS). δ = 198.8 (*C*=O)$_{ketone}$, 172.4, 171.6, 169.7, 169.4 (*C*=O)$_{ester}$, 149.4*, 149.3 (C-2′), 138.7*, 138.5, 138.2, 138.0*, 135.5, 135.2*, 134.6*, 134.3, 132.6, 132.5*, 129.9, 129.7, 122.5 (C-1′a, C-4′a, C-5′a, C-8′a, C-9′, C-10′, *C-ipso*-phenyl), 132.8, 132.7*, 132.5*, 132.4, 130.0*, 130.0*, 129.9, 129.7, 129.2, 129.1*, 129.0*, 129.0, 128.6, 128.0, 126.9, 126.0, 125.0, 124.8, 123.1, 115.0, 114.9, 106.9 ($CH_{arom.}$), 80.61, 80.54*, 80.48*, 80.42 (OCH_2O), 64.29, 64.14, 63.91, 63.77 (C-3,C-5), 51.54, 50.93, 48.05, 47.83*, 45.19, 45.14*, 44.31*, 44.12, 43.10 (C-1, C-2, C-6, C-7, C-8, C-9), 39.94*, 39.89 (NCH_3), 34.73 (C-10), 20.40 (CH_3); IR (KBr): $\tilde{\nu}$ = 3068, 3030 (=CH), 2976, 2922, 2801 (CH), 2793 (N−CH_3), 1779 (C=O_{ketone}), 1764 (C=O_{ester}), 1614 (C=C), 1370 (O−CO−CH_3), 1367 (C−N), 1007 cm^{-1} (C−O); UV/Vis (THF): λ_{max} (lg ε) = 398 (3.53), 369 (3.54), 334 (4.16), 310 nm (4.32); Fluorescence (THF): λ_{exc} = 380 nm, λ_{em} = 460 nm; MS (70 eV, EI): *m*/*z* (%): = 751 (27) [M^+], 723 (100) [M^+ − CO]; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{46}H_{41}N_1O_9$: 751.277341; found 751.278132; difference 1.1 ppm.

Photolysis of ketones 10a–d: Ketones **10a--d** were photolysed at λ = 300 nm in [D_8]THF or THF in a Rayonet Photochemical Reactor at −30 °C. For ^{1}H NMR spectral investigations suprasil NMR tubes were used, and for preparative purposes quartz tubes of 7 mm diameter and a volume of 10 mL were used. The concentration of **10a–d** was 0.015 M (ca. 10 mg mL^{-1}) for ^{1}H NMR spectroscopical studies and 3 mg mL^{-1} for preparations. The solutions of the ketones were purged with nitrogen for 30 min and photolysed at −30 °C for up to 100 h. The necessary duration of the photolysis was determined by running a ^{1}H NMR spectroscopic and a preparative sample simultaneously. After termination of the photolysis the solvent was removed, the *o*-quinodimethanes characterized by spectroscopic techniques, and the samples stored at −78 °C.

3,6-Diphenyl-4,5-(2,2′-biphenylene)tricyclo[6.2.1.0^{2,7}]undeca-3,5-diene-9,10-(*E*)-dicarboxylic acid (4a): M.p. 265–267 °C; ^{1}H NMR (300 MHz, [D_8]THF, TMS, HH-COSY): δ = 11.02 (br s, 2H; COO*H*), 7.74 (d, ^3J = 8.1 Hz, 2H; 6′-C, 6″-H), 7.39–7.46 (m, 4H; $CH_{arom.}$), 7.15–7.26 (m, 6H; $CH_{arom.}$), 7.06–7.13 (m, 4H; $CH_{arom.}$), 6.72–6.78 (m, 2H; 5′-C, 5″-H), [3.34–3.39 (m, 1H), 3.24–3.29 (m, 2H), 3.07–3.10 (m, 1H), 2.90 (br s, 1H), 2.80–2.83 (m, 1H), 1-H, 2-H, 7-H, 8-H, 9-H, 10-H], 2.11 (d, ^2J (11a-H, 11b-H) = 10.3 Hz, 1H; 11a-H), 1.57 (d, ^2J (11a-H, 11b-H) = 10.3 Hz, 1H; 11b-H); ^{13}C NMR (75 MHz, [D_8]THF, TMS, inverse-CH-COSY): δ = 175.63, 174.77 (*C*=O)$_{acid}$, 135.74, 135.55, 135.46, 134.70, 134.60, 132.74 (C-3, C-4, C-5, C-6, C-1′, C-1″, C-2′, C-2″), 132.93, 130.90, 130.87, 129.67, 129.53, 128.67, 128.56 (C-3′, C-3″,*C*H-phenyl), 128.29, 128.22, 127.56, 127.47 (C-4′, C-4″, C-5′, C-5″), 124.59 (C-6′, C-6″), 52.93, 52.16 (C-9, C-10), 50.85, 50.14, 49.53, 48.22 (C-1, C-2, C-7, C-8), 35.03 (C-11); IR (KBr): $\tilde{\nu}$ = 3340–3600 (−OH), 3060, 3021 (=CH), 2930–2970 (−CH), 1708 cm^{-1} (C=O); UV/Vis (THF): λ_{max} (lg ε) = 446 nm (3.91); Fluorescence (phosphate buffer pH 7.4): λ_{exc} = 395–400 nm, $\lambda_{em(max)}$ = 565 nm; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{37}H_{28}O_4$: 536.1988; found 536.1971; difference 3.2 ppm.

3,6-Diphenyl-4,5-(2,2′-biphenylene)-tricyclo[6.2.1.0^{2,7}]undeca-3,5-diene-9,10-(*E*)-dicarboxylic acid diacetoxymethylester (4b): M.p. 98–100 °C; ^{1}H NMR (300 MHz, [D_8]THF, TMS): δ = 7.74 (d, ^3J = 7.5 Hz, 2H; 6′-H, 6″-H), 7.44 (dd, ^3J = 7.8 Hz, ^4J = 1.4 Hz, 2H; *o*-H or *o*′-H), 7.35 (dd, ^3J = 7.5 Hz, ^4J = 1.4 Hz, 2H; *o*-H or *o*′-H), 7.30–7.10 (m, 8H; 2′, 9′, *m*, *m*′, *m*″, *p*-H), 7.10–7.05 (m, 2H; 4′-H, 4″-H), 6.80–6.68 (m, 2H; 5′-H, 5″-H), 5.80 (d, ^2J = 5.6 Hz, 1H; OCH_2O), 5.70 (d, ^2J = 9.0 Hz, 1H; OCH_2O), 5.68 (d, ^2J = 8.8 Hz, 1H; OCH_2O), 5.66 (d, ^2J = 5.6 Hz, 1H; OCH_2O), 3.35–3.30 (m, 2H; 2-H, 7-H), 3.30 (br s, 1H; 1-H or 8-H), 3.20 (br d, ^3J = 4.8 Hz, 1H; 9-H or 10-H), 2.90 (br s, 1H; 1-H or 8-H), 2.85 (br d, ^3J = 3.3 Hz,1H; 9-H or 10-H), 2.15 (br d, ^2J(11a-H, 11b-H) = 10.5 Hz, 1H; 11a-H), 2.02 (s, 3H; CH_3), 2.00 (s, 3H; CH_3), 1.60 (br d, ^2J(11a-H, 11b-H) = 10.0 Hz, 1H; 11b-H); ^{13}C NMR (75 MHz, [D_8]THF, TMS): δ = 172.8, 172.1, 169.6, 169.5 (*C*=O), 143.4, 143.1 (C-3,C-6), 135.2, 132.6, 130.6, 130.5, 129.6, 129.4, 128.1, 128.1, 124.3 ($CH_{arom.}$), 134.9, 134.8, 134.2, 133.9, 128.5, 128.4, 127.3, 127.2 (C-4, C-5, C-1′, C-1″, C-2′, C-2″, *C-ipso*-phenyl), 80.90, 81.27 (OCH_2O), 52.36, 51.68, 50.26, 49.72, 48.92, 47.75 (C-1, C-2, C-7, C-8, C-9, C-10), 34.90 (C-11), 20.58, 20.35 (CH_3); IR (KBr): $\tilde{\nu}$ = 3068, 3030 (=CH), 2993, 2913, 2880 (CH), 1757 (C=O_{ester}), 1369 (O−CO−CH_3), 1233 , 1153, 1004 cm^{-1} (C−O); UV/Vis (THF): λ_{max} (lg ε) = 445 nm (3.69); Fluorescence (THF): negligible; MS (70 eV, EI): *m*/*z* (%): = 680 (100) [M^+]; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{43}H_{36}O_8$: 680.2411; found 680.2449; difference −5.8 ppm.

3,6-Diphenyl-4,5-(4-dimethylamino-2,2′-biphenylene)tricyclo[6.2.1.0^{2,7}]undeca-3,5-diene-9,10-(*E*)-dicarboxylic acid (4c): M.p. 230–235 °C; ^{1}H NMR (300 MHz, [D_8]THF, TMS): δ = 7.60 (d, ^3J = 8.2 Hz, 1H; 6′-H or 6″-H), 7.58 (d, ^3J = 7.8 Hz, 1H; 6′-H or 6″-H), 7.46 (dd, ^3J = 7.8 Hz, ^4J = 1.5 Hz, 1H; *o*′-H), 7.40 (dd, ^3J = 7.8 Hz, ^3J = 1.4 Hz, 2H; *o*-H), 7.33–7.27 (m, ^3J = 7.8 Hz, ^3J = 1.4 Hz, 2H; *o*″-H, 3′-H), 7.27–7.14 (m, 5H; 3″-H, *m*-H, *m*′-H, *m*″-H), 7.08–6.98 (m, 2H; C$H_{arom.}$), 6.67–6.48 (m, 3H; 4″-H, 5′-H, 5″-H), [3.32–3.12 (m, 3H), 3.03–2.95 (m, 1H), 2.82–2.78 (m, 1H), 2.72–2.67 (br d, ^3J = 4.2 Hz, 1H), 1-H, 2-H, 7-H, 8-H, 9-H, 10-H], 2.50, 2.48* (s, 6H; CH_3), 2.13 (br d, ^2J(11a-H, 11b-H) = 10.5 Hz, 1H; 11a-H), 1.60 (br dd, ^2J(11a-H, 11b-H) = 10.2 Hz, *J*(7-H, 11b-H) = 9.3 Hz, 1H; 11b-H); ^{13}C NMR (75 MHz, [D_8]THF, TMS): δ = 175.2, 175.1*, 174.2 (*C*=O), 149.6, 149.5* (C-4′), 144.8, 144.5*, 143.2, 143.1* (C-3,C-6), 135.6*, 135.6, 135.0, 134.7*, 134.3*, 134.1, 133.7*, 133.5, 133.4*, 133.3, 132.9*, 132.8, 128.0*, 127.9, 127.5, 127.3* (C-4, C-5, C-1′, C-1″, C-2′, C-2″, *C-ipso*-phenyl), 130.6, 130.5*, 130.2, 130.1*, 129.4, 129.2*, 128.8*, 128.7, 125.2*, 125.2, 124.6, 124.0, 123.9*, 127.8, 116.9*, 116.9, 113.3, 113.2* (C-3′, C-3″, C-4″, C-5′, C-5″, C-6′, C-6″, C$H_{arom.}$), 52.92, 52.32*, 51.99, 50.44, 50.33*, 49.68*, 49.62, 48.25, 48.06*, 47.15 (C-1, C-2, C-7, C-8, C-9, C-10), 40.00, 39.93* (NCH_3), 34.57, 34.40* (C-11); IR (KBr): $\tilde{\nu}$ = 3066, 3029 (=CH), 2977, 2921, 2802 (CH), 2787 (N−CH_3), 1708 (C=O_{acid}), 1612 (C=C), 1362 (C−N), 1006 cm^{-1} (C−O); UV/Vis (THF): λ_{max} (lg ε) = 466 (3.30), 295 nm (4.36); Fluorescence (phosphate buffer pH 7.2): λ_{exc} = 460 nm, λ_{em} = 600 nm; MS (70 eV, EI): *m*/*z* (%): = 579 (100) [M^+]; MS (70 eV, EI, high resolution): *m*/*z*: [M^+] calcd for $C_{39}H_{33}N_1O_4$: 579.132730; found 579.135874; difference 6.3 ppm.

3,6-Diphenyl-4,5-(4-dimethylamino-2,2″-biphenylene)tricyclo[6.2.1.0^{2,7}]-undeca-3,5-diene-9,10-(*E*)-dicarboxylic acid diacetoxymethylester (4d): M.p. 82–83 °C; ^{1}H NMR (300 MHz, [D_8]THF, TMS): δ = 7.59 (dd, ^3J = 8.4 Hz, ^3J = 8.7 Hz, 2H; 6′-H, 6″-H), 7.52–7.46 (m, 1H; *o*′-H), 7.43 (m, 2H; *o*-H), 7.32–7.24 (m, 3H; *o*″-H, 3′-H, 3″-H), 7.22–7.14 (m, 4H; *m*-H, *m*′-H), 7.05–6.97 (m, 2H; *p*-H), 6.67–6.48 (m, 3H; 4″-H, 5′-H, 5″-H), 5.82–5.56 (m, 4H; OCH_2O), [3.35–3.10 (m, 3H), 3.12–3.10 (m, 1H), 2.84–2.82 (m, 1H), 2.74 (br d, ^3J = 4.2 Hz, 1H), 1-H, 2-H, 7-H, 8-H, 9-H, 10-H], 2.51, 2.49* (s, 6H; NCH_3), 2.18 (br d, ^2J(11a-H, 11b-H) = 9.0 Hz, 1H; 11a-H), 2.03, 2.01* (s, 3H; CH_3), 1.98, 1.97* (s, 3H; CH_3), 1.60 (br dd, ^2J(11a-H, 11b-H) = 9.0 Hz, *J*(7-H, 11b-H) = 8.4 Hz, 1H; 11b-H); ^{13}C NMR (75 MHz, [D_8]THF, TMS): δ = 172.5, 172.5*, 171.8, 171.8*, 169.3*, 169.2 (*C*=O), 149.7, 149.7* (C-4′), 144.50*, 144.4, 143.3, 142.9* (C-3, C-6), 135.6*, 135.6, 134.3*, 134.1, 133.8*, 133.7, 133.4*, 133.2, 133.0*, 132.9, 132.5, 132.5*, 128.2*, 128.0, 127.6*, 127.4, (C-4, C-5, C-1′, C-1″, C-2′, C-2″, *C-ipso*-phenyl), 130.5*, 130.5, 130.2, 130.0*, 129.9*, 129.4, 129.0, 128.9*, 125.2, 125.1*, 124.7*, 124.7, 123.9*, 123.8, 122.8, 116.9*, 116.8, 113.4, 113.3* ($CH_{arom.}$), 80.18, 80.06 (OCH_2O), 52.52, 51.89*, 51.82, 50.28, 50.06*, 49.51*, 49.26, 48.14*, 47.87, 46.97 (C-1, C-2, C-7, C-8, C-9, C-10), 39.99, 39.90 (NCH_3), 34.57, 34.39

(C-11), 20.13, 20.05 (CH_3); IR (KBr): $\tilde{\nu}$ = 3070, 3033 (CH=), 2980, 2924, 2803 (CH), 2795 (N−CH_3), 1765 (C=O_{ester}), 1615 (C=C), 1372 (O−CO−CH_3), 1369 (C−N), 1009 cm^{-1} (C−O); UV/Vis (THF): λ_{max} (lg ε) = 465 (3.40), 298 nm (4.44); Fluorescence (THF): negligible; MS (70 eV, EI): *m/z* (%): = 723 (100) [M^+]; MS (70 eV, EI, high resolution): *m/z*: [M^+] calcd for $C_{45}H_{41}N_1O_8$: 723.177341; found 723.178132; difference 1.3 ppm.

Formation of hydroxylamines 6b and 6d: An equimolar amount of acetaldehyde (0.42 μL) was added to a solution of **4b** (15 mM, 5.1 mg in 0.5 mL) or **4d** (5.4 mg in 0.5 mL) in [D_8]THF. This solution was mixed with an equimolar amount of NO dissolved in [D_8]THF (0.5 mL). Immediate analysis by ^{1}H NMR spectroscopy revealed about 40% conversion of **4b** and **4d** to **6b** and **6d**, respectively. The ^{1}H NMR spectra of **4b** and **4d** are very similar to those of **10b** and **10d**, respectively; MS (70 eV, EI) *m/z* (%): **6b**: 711 (4) [M^+], 680 (100) [M^+ − NOH] or [M^+] of **4b**; **6d**: 754 (8) [M^+], 723 (100) [M^+ − NOH] or [M^+] of **4d**.

Stopped-flow kinetic measurements: The reactions of FNOCTs with nitric oxide were carried out at 23 °C in benzene (c = 90 μM, λ = 450 nm) and in phosphate buffer (pH 7.4) for **4a** (c = 50 μM, λ = 450 nm) and at 21 °C in THF (c = 100 μM, λ = 465 nm) and in phosphate buffer (pH 7.3) for **4c** (c = 10 μM, λ = 465 nm) under pseudo-first-order conditions (c_{NO}(benzene) = 12 mM, c_{NO}(THF) = 15 mM, c_{NO}(water) = 2.0 mM).[40]

ESR spectroscopic measurements: Solutions were prepared in septum-capped, argon-flushed 4 mm o.d. quartz tubes (for organic solvents) or a 0.4 mm quartz flat cell (for aqueous solutions). Deoxygenated solutions of the FNOCTs (1 mM, 0.5 mL) and saturated solutions of NO in the same solvent (0.5 mL) were injected into the tubes by means of a gas-tight syringe and briefly mixed by shaking. Recording conditions: microwave frequency, 9.4 GHz; modulation, 0.03 mT; sweep range, 10 mT; sweep time, 15 min. The ESR parameters were refined by computer simulation.

Cell isolation and culture: Alveolar macrophages were isolated from male Wistar rats (250 – 310 g) by bronchoalveolar lavage as described previously.[41] The cells were seeded onto 6.2 cm^2 glass coverslips in 6-well cell-culture plates and cultured in Dulbecco's modified eagles medium (DMEM) supplemented with L-glutamine (2 mM), gentamycin (50 mg mL^{-1}), and 10% heat-inactivated fetal calf serum.

Cell experiments: Experiments with alveolar macrophages were started 15 – 20 hours after isolation of the cells. Cell density in the experiments was $3.25 \pm 0.5 \times 10^4$ cells cm^{-2}. The cells were used either without further treatment, or were primed for NO production by 11 hours preincubation with lipopolysaccharides (LPS; 0.5 μg mL^{-1}), and/or NO production was inhibited by 11 h preincubation with the NO synthase inhibitor NMA (0.1 mM). The media of stimulated cultures and respective controls were supplemented with L-arginine (0.5 mM). The experiments were performed in Krebs-Henseleit buffer (NaCl 115 mM, $NaHCO_3$ 25 mM, KCl 5.9 mM, $MgCl_2$ 1.2 mM, NaH_2PO_4 1.2 mM, Na_2SO_4 1.2 mM, $CaCl_2$ 2.5 mM, HEPES 20 mM, pH 7.4) supplemented with L-glutamine (2 mM) and D-glucose (10 mM; modified Krebs-Henseleit buffer). This buffer was equilibrated with 95% air/5% CO_2 throughout the experiments. Cell viability was determined routinely by trypan blue or propidium iodide exclusion.

Digital fluorescence microscopy: Cells were loaded with FNOCT using the membrane-permeable acetoxymethyl ester **4d**. Retention of the dye in the cells was improved by the addition of probenecid. In order to evaluate the optimal loading conditions, the following variations were performed: the cells were incubated for 10 – 120 minutes at 37 °C in the dark in modified Krebs-Henseleit buffer containing probenecid (1.4 mM), FNOCT **4d** (10 – 100 μM), NMA (0.1 mM) where appropriate, Pluronic® F-127 (0.02 – 0.2%), and DMSO (0.2 – 2%), depending on the FNOCT concentration. After this incubation the glass coverslips were rinsed three times, modified Krebs-Henseleit buffer supplemented with probenecid (1.4 mM), L-arginine (0.5 mM) or NMA (0.1 mM) was added, and fluorescence microscopy was started immediately. Fluorescence microscopy was performed on an inverted microscope (Axiovert 135TV, Zeiss, Oberkochen, Germany) equipped with the Attofluor imaging system (Atto Instuments, Rockville, Maryland, USA).

Nitrite determination: Cells were isolated, cultured, and stimulated as described above. After different incubation periods (following stimulation), nitrite was determined in the supernatant using the Griess[42] and DAN[43] tests.

Statistics: The experiments with alveolar macrophages were performed in duplicate and repeated 4 times with cells from different rats. Data are expressed as means ± standard deviation. Data obtained from two groups were compared by means of the Student's t test. A p value of < 0.05 was considered significant.

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