Carbon Monoxide Release

DOI: 10.1002/anie.201006598

Acyloxybutadiene Iron Tricarbonyl Complexes as Enzyme-Triggered CO-Releasing Molecules (ET-CORMs)**

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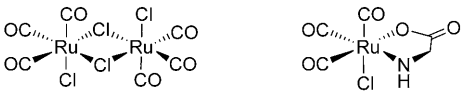
Like nitric oxide (NO), carbon monoxide (CO) is an important yet only recently recognized biological signaling molecule.[1] CO is actually constantly produced in small doses in our body in the course of heme degradation by the heme-oxygenase (HO) enzymes. It exhibits cytoprotective, anti-inflammatory, vasodilatory, and other effects, which are important for instance in our body's response to injuries.[2] Despite these beneficial biological properties, the application of CO as a therapeutic agent is still in its infancy.[3] Not surprisingly, the use of gaseous CO is risky and strongly limited by the high affinity of CO towards hemoglobin and the resulting systemic effects on oxygen transport and low bioavailability.[4]

A promising strategy to circumvent these problems and to deliver controlled amounts of CO directly to a tissue is the use of CO-releasing molecules (CORMs). As pioneers in this field, Motterlini and co-workers have identified a series of transition-metal carbonyl complexes that fulfill this function.[5] While his first CORMs, such as $[Mn_2(CO)_{10}]$, needed UV activation, the dinuclear ruthenium complex **1** (CORM-2) liberates CO upon ligand exchange with DMSO (Scheme 1).[6] The related mononuclear glycinato complex **2** (CORM-3) is better soluble in water and releases CO under physiological conditions.[7]



1 (CORM 2) **2** (CORM 3)

Scheme 1. Selected carbonyl complexes frequently used as CO-releasing molecules in biological studies.

These and several other CORMs[8] were tested in various biological assays, and promising activities (for example vasodilatory, anti-inflammatory, renoprotective, anti-ischemic, and anti-apoptotic effects) were documented, and preclinical studies are in progress.[5] Nevertheless, the search for new CORMs still remains a challenging task as the existing compounds suffer from serious limitations. For example, CO release from CORM-3 is very fast ($t_{1/2} \approx 1$ min) and unspecific,[9] which hampers the delivery of controlled amounts of CO to a target tissue. An approach to overcome this problem could be the use of stable molecules as precursors that are converted into CORMs by means of a trigger.[10] One possibility to achieve this is the pH-dependent CO liberation from a boranocarbonate (CORM-A1)[11] or amino derivatives thereof.[12] Another approach is the photo-induced CO release of transition-metal carbonyl complexes with UV-absorbing organic ligands.[13]

As a new concept, we introduce acyloxybutadiene–iron tricarbonyl complexes as enzyme-triggered CO-releasing molecules (ET-CORMs). The idea resulted from an earlier observation that dienol–iron tricarbonyl complexes like **4** are very labile and readily decompose already under slightly oxidative conditions (presumably via a 16-VE intermediate of type **5**). We now envisioned that such complexes could potentially act as CORMs, provided that they can be generated under physiological conditions from stable precursors. An appealing possibility would be the use of dienylester complexes of type **3**, which are expected to be sufficiently stable. However, once such complexes have entered a cell they may be cleaved by intracellular esterases. The oxidative decomposition of the resulting dienol–iron tricarbonyl complexes **4** would then be linked to the release of three molecules of CO (Scheme 2).

To probe this concept, we first had to synthesize some potentially suitable acyloxydiene–iron tricarbonyl complexes. As such we selected complexes *rac*-**8**, *rac*-**10**, and *rac*-**12**, which were prepared from cyclohexenone **6** (Scheme 3).[14] The synthesis of *rac*-**8** and *rac*-**10** started with the kinetic deprotonation of **6** (LDA, THF, −78 °C) and trapping the intermediate dienolate with either acetic anhydride (to give **7**) or pivalic chloride (to give **9**). Thermal treatment of the obtained dienes with $[Fe_2(CO)_9]$ in diethyl ether gave rise to the complexes *rac*-**8** and *rac*-**10** in good yield. The synthesis of complex *rac*-**12** (the isomer of *rac*-**8**) was initiated by thermodynamically controlled deprotonation of **6** with LiHMDS in the presence of 1.5 equiv of TPPA followed by acetylation of the resulting dienolate. Complexation of **11**

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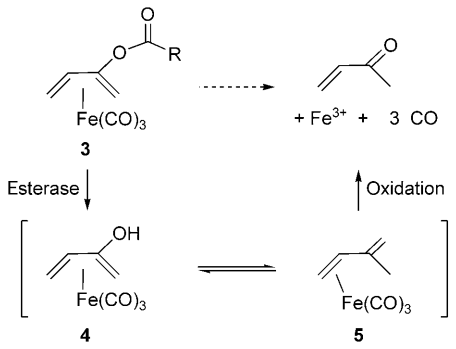
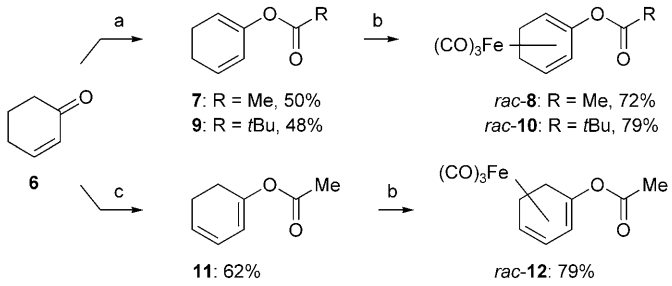
[**] This work was supported by the Deutsche Forschungsgemeinschaft (FOR 630) and the Fonds der Chemischen Industrie (doctorate stipend to S.R. and a Liebig fellowship to S.A.)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201006598.

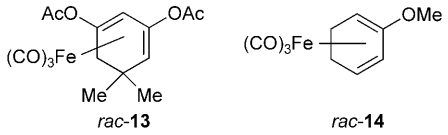


Scheme 2. Proposed mode of action of enzyme-triggered CO-releasing molecules (ET-CORMs) of type **3**.



Scheme 3. Synthesis of complexes *rac*-**8**, *rac*-**10**, and *rac*-**12**. a) LDA, THF, −78 °C, then Ac_2O or PivCl; b) $[Fe_2(CO)_9]$, Et_2O, 40 °C, 20 h; c) LiHMDS, TPPA, THF, −78 °C, then Ac_2O. LDA = lithium diisopropylamide, LiHMDS = lithium hexamethyldisilazide, PivCl = pivaloyl chloride, TPPA = trispyrrolidinophosphoric acid triamide.

then afforded *rac*-**12**. Furthermore, the diacetoxydiene complex *rac*-**13**,[15] derived from dimedone as a nontoxic precursor, and the methoxy-substituted complex *rac*-**14**,[16] as an esterase-insensitive reference sample, were synthesized by known procedures (Scheme 4).



Scheme 4. Structure of complexes *rac*-**13** and *rac*-**14**.

All of the complexes thus prepared proved to be reasonably air-stable and were fully characterized by common spectroscopic methods. The structural assignments were further confirmed by an X-ray crystal structure analysis of *rac*-**12** (Figure 1), which also revealed the conformational preferences of this compound in the solid state.

While we expected the acetates *rac*-**8**, *rac*-**12**, and *rac*-**13** to be readily cleaved by esterases, such as pig-liver esterase (PLE), we considered the pivalate *rac*-**10** to be less reactive towards hydrolysis (also under non-enzymatic conditions), thus making this complex a particularly meaningful probe for esterase activation.

In preparation for testing the ET-CORM properties of the complexes, we first had to identify suitable esterases for the individual complexes. The enzymatic ester cleavage of compounds *rac*-**8**, *rac*-**10**, *rac*-**12**, and *rac*-**13** was investigated under the conditions of the myoglobin assay later used to detect the CO release (see below). Typically, a solution of a complex in a small amount of DMSO was mixed with a solution of an esterase in phosphate buffer, and the conversion was monitored by means of HPLC on a chiral stationary phase (or GC in the case of *rac*-**8**). This also allowed us to detect kinetic resolution as an unambiguous proof of an enzymatic process.[17] After testing a variety of commercially available esterases, we found suitable esterases for all ester substrates (Table 1). The reaction of complexes

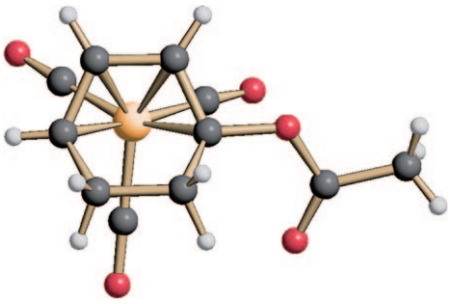


Figure 1. Structure of complex *rac*-**12** in the crystalline state. C gray, H white, Fe orange, O red.

Table 1: Identification of suitable esterases for the enzymatic hydrolysis of acyloxydiene–iron tricarbonyl complexes as detected by kinetic resolution.

Enzyme[a]	*rac*-**8**	*rac*-**10**	*rac*-**12**	*rac*-**13**	*rac*-**14**
PLE	−	−	+	+	−
LCR	+	+	+	−	−

[a] PLE: pig-liver esterase, LCR: lipase from *Candida rugosa*.

rac-**12** and *rac*-**13** with PLE was clearly associated with kinetic resolution (70 % to over 90 % *ee* of the remaining complex at 80 % conversion). For complexes *rac*-**8** and *rac*-**10**, a lipase from *Candida rugosa* (LCR) proved to be superior to PLE. Not unexpectedly, the pivalate *rac*-**10** reacted significantly slower than the acetoxydiene complexes *rac*-**8**, *rac*-**12**, and *rac*-**13**. The methoxy complex *rac*-**14** did not react at all under these conditions, as expected.

The esterase-triggered CO release from the complexes (triggered by enzymatic hydrolysis) was then assessed by means of the myoglobin (Mb)-based assay established by Motterlini et al.[6] In this assay, the conversion of deoxymyoglobin (deoxy-Mb; generated in situ by reduction of Mb with dithionite) to carbonmonoxy myoglobin (MbCO) is determined spectrophotometrically (500–750 nm). In accordance with the proposed mechanism (Scheme 2), a clean CO release was detected from *rac*-**12** (Figure 2) and *rac*-**13** in the presence of PLE, and from *rac*-**8**, *rac*-**10**, and *rac*-**12** in the presence of LCR. Under the same conditions, no CO release (or very slow CO release in case of *rac*-**13**) was detected in the absence of an esterase.[18] Thus, the postulated enzyme-triggered CO release was unambiguously demonstrated.[19]

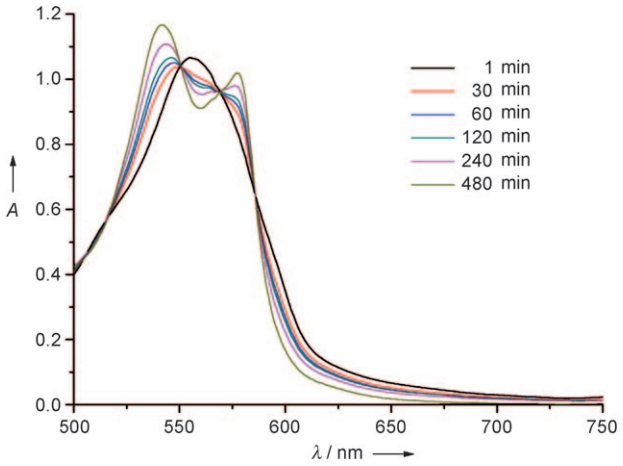


Figure 2. Detection of enzyme-triggered CO release by monitoring the changes of the Q-band region of the UV/Vis spectra of reduced horse skeletal muscle myoglobin (76 μM) in the presence of complex *rac*-**12** (1.3 equiv) and PLE (ca. 0.01 equiv) in phosphate buffer solution (0.1 M, pH 7.4).

Having successfully characterized our ET-CORMs in vitro, we next turned our attention to probe the biological potential of these compounds in a cellular assay. An important biological target that can be addressed by CO is the pro-inflammatory acting enzyme inducible nitric oxide synthase (iNOS). Nitric oxide synthases (NOS) produce NO and L-citrulline from L-arginine and molecular oxygen using NADPH as a co-substrate. NOS possess a tightly bound heme cofactor that is needed for their oxygenase activity (Figure 3).[20] iNOS itself is the inducible form of NOS and plays an important role in cellular defense mechanisms against bacteria, viruses, parasites, and tumors.[21] With respect to iNOS, CO is able to act in two different ways. It can prevent the dimerization of the inactive monomeric iNOS protein to the enzymatically active dimer. Secondly, iNOS activity can be suppressed by binding of CO to the iron center of heme (Fe protoporphyrin IX) to generate the inactive complex carbonmonoxy-iNOS (Figure 3).[23]

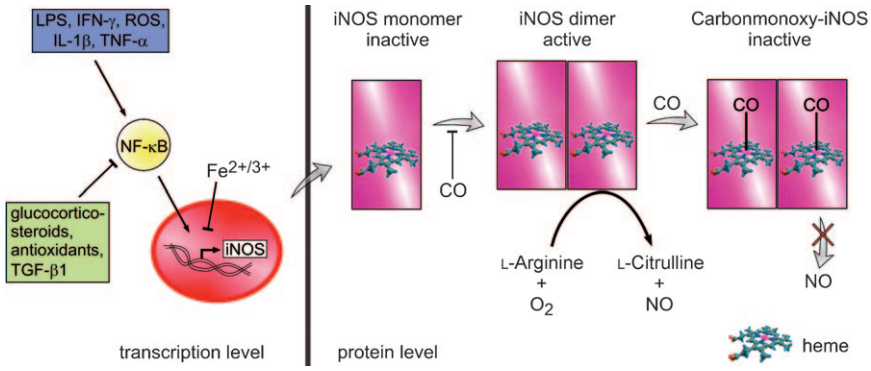


Figure 3. Regulation of transcription and protein activity of inducible nitric oxide synthase (iNOS) by iron species and CO.[22] The gene transcription of iNOS can be induced for example, by lipopolysaccharide (LPS), interferon γ (IFN-γ), oxidative stress/reactive oxygen species (ROS), interleukin 1β (IL-1β), or tumor nekrosis factor α (TNF-α). This occurs by activation of nuclear factor κB (NF-κB). The activation of NF-κB can be prohibited for example by glucocorticosteroids, antioxidants, or transforming growth factor 1β (TGF-β1).

Prior to performing the iNOS inhibition assays, we assessed the potential cytotoxicity of the ET-CORMs in two different ways using the murine macrophage cell line RAW267.4 in which iNOS can be easily induced by lipopolysaccharide (LPS).[24] The so-called MTT test was used to measure the viability of cells when exposed to the complexes.[25] Furthermore, the number of intact cells was determined photometrically by staining the cell nuclei with crystal violet dye. Table 2 shows the concentrations of ET-CORMs that caused inhibition of 50% (IC_{50}) and 20% (IC_{20}) in the two assays.

Table 2: Cytotoxicity data of ET-CORMs.[a]

	MTT		Crystal violet	
Compound	IC_{50} [μM]	IC_{20} [μM]	IC_{50} [μM]	IC_{20} [μM]
rac-**8**	67.1 ± 3.6	28.1 ± 0.6	73.6 ± 6.9	28.6 ± 7.2
rac-**10**	[a]	[a]	> 100	> 100
rac-**12**	39.6 ± 0.9	11.2 ± 2.2	47.0 ± 2.7	14.4 ± 3.5
rac-**13**	54.3 ± 1.0	21.8 ± 0.6	95.2 ± 3.8	37.7 ± 1.0
rac-**14**	> 100	> 100	> 100	> 100

[a] Determined by MTT and crystal violet assay using RAW264.7 cells stimulated with LPS (10 ng mL^{-1}). [b] An unusual color was observed with *rac*-**10** in the MTT assay that precluded the correct assessment of the cell viability.

For the pivalate *rac*-**10**, no significant toxicity was found in the crystal violet assay at the concentrations tested. The methoxy-substituted complex *rac*-**14** also did not display any toxicity up to 100 μM. In contrast, the acetates *rac*-**8**, *rac*-**12**, and *rac*-**13** gave IC_{20} values in a range 11–28 μM in the MTT test and a slightly wider range 14–38 μM in the crystal violet staining.

Knowing the cytotoxicity profiles of the different complexes (Figure 4, left side), the stage was set for the investigation of the influence of the ET-CORMs on nitric oxide production by iNOS. For this purpose, the generation of NO was determined indirectly by measuring the accumulation of nitrite in the cell-culture medium using a microplate assay based on the Griess test.[26] We only used concentrations of ET-CORMs below the IC_{20} values (Table 2) to avoid pseudo positive results caused by cytotoxic effects.

Amongst the five ET-CORMs investigated, the diacetate *rac*-**13** was the most active inhibitor of NO production in LPS-stimulated RAW267.4 cells. Treatment of cells with *rac*-**13** at a concentration of 15 μM resulted in up to (68 ± 6)% suppression of LPS-induced NO formation relative to control cells that were only treated with LPS. At 20 μM (a value that is close to the cytotoxic range), no further increase of activity was observed. Overall, *rac*-**13** caused a significant NO inhibition already at 5 μM ((33 ± 6)% inhibition). Compared to *rac*-**13**, complex *rac*-**12** exhibits very similar activity,

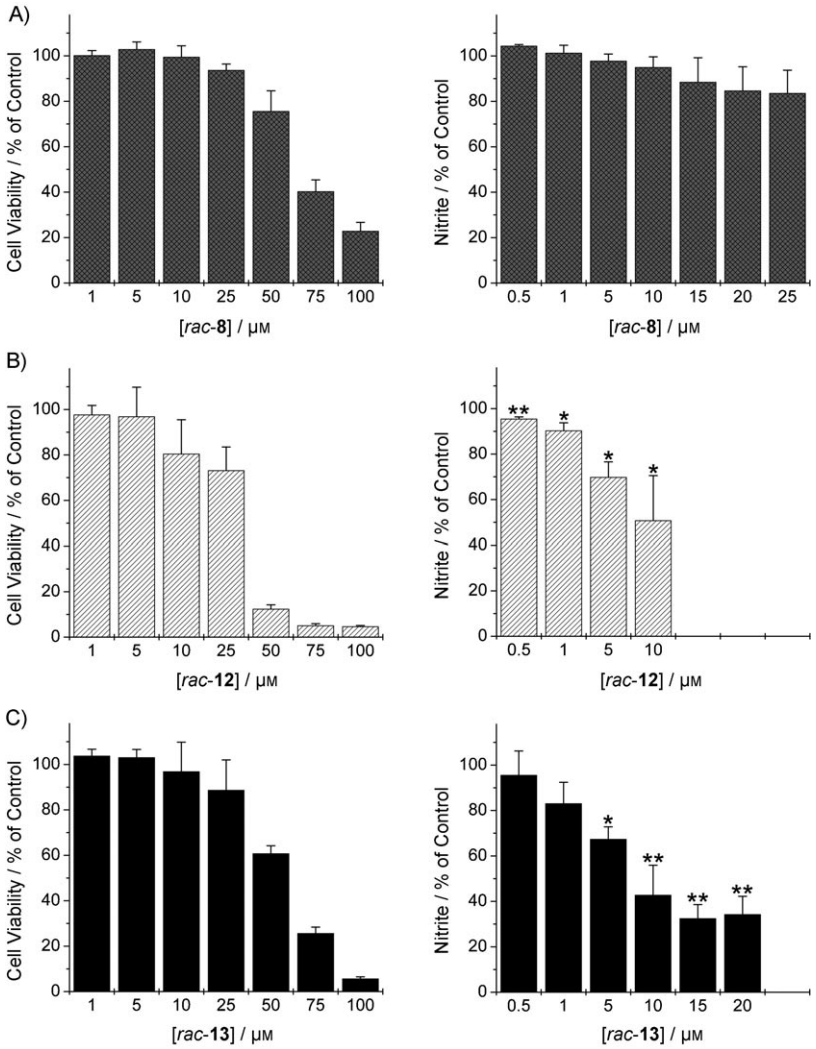


Figure 4. Results of the in vitro assays performed with RAW264.7 cells stimulated with 10 ng mL^{-1} of LPS. Left: MTT tests after an incubation time of 24 h at different concentrations. Right: the influence of ET-CORMs on NO production (Griess assay). Data represent three independent experiments performed in quadruplicates. Levels of significance: * $p \leq 0.05$, ** $p \leq 0.01$.

with (30 ± 7)% inhibition of NO release at 5 μM. Its higher toxicity, however, did not allow measurements above 10 μM. Interestingly, its isomer *rac*-**8** was found to be less active ((16 ± 10)% reduction of NO generation at 25 μM). The esterase-insensitive reference sample *rac*-**14** and the pivalate *rac*-**10**, which was also expected to be less prone to hydrolysis, did not significantly suppress NO production at concentrations of up to 50 μM (see the Supporting Information, Figure S18D and E).[27]

In conclusion, we have shown that acyloxycyclohexadiene–iron tricarbonyl complexes are a novel class of powerful CO-releasing molecules (ET-CORMs), which are activated by enzymatic cleavage of the ester functionality. The proposed trigger mechanism was substantiated by CO-release experiments (myoglobin assay) and by a cellular assay based on iNOS inhibition as a meaningful biological response to CO. The best of the compounds prepared to date (*rac*-**13**) exhibited a stronger activity, as for example cyclopentadienyl-based iron carbonyls ([(γ-$C_5H_4CO_2Me$)$Fe(CO)_2Br$])[28] or pyrone-based iron tricarbonyl complexes.[29] Although this compound displays a certain cytotoxicity, it is to the best of our knowledge the most potent CORM ever studied in this type of assay (30% NO inhibition at 5 μM).[30]

The concept introduced herein offers promising new options for the development of a new generation of CORMs, thus allowing a controlled and possibly even tissue-selective CO delivery. The short and flexible synthesis will at least allow the variation of the dienylester ligand thus enabling us to tune the pharmacological and biological properties (for example water solubility) in the future.

Received: October 20, 2010
Published online: February 14, 2011

Keywords: carbonyl ligands · CO release · drug delivery · enzyme catalysis · inhibitors

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[18] In a control experiment, it was confirmed that compound *rac*-**14** did not show CO release under the conditions of the myoglobin assay in the presence of PLE or LCR.

[19] The presence of small amounts of oxygen is required for the CO release, and the rate of CO release depends on the concentration of esterase (enzymatic hydrolysis) and of dithionite added (redox potential). While some oxygen is essential for the oxidative decomposition (Scheme 2), the assay does not tolerate higher O_2 concentrations (re-oxidation of the CO-binding Fe^{II} form of Mb). Under the assay conditions, typical times for the release of 0.5 mol of CO per mol of ET-CORM are as follows: 56 min for *rac*-**8** (0.09 equiv of LCR), 183 min for *rac*-**12** (0.01 equiv of PLE) and 86 min for *rac*-**13** (0.01 equiv of PLE). Owing to the complexity of the parameter space, such values do not allow a quantitative comparison of the ET-CORMS or any predictions of their biological performance.

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[22] Exceptionally high NO quantities account for cell damage, which is a crucial factor in the pathophysiology of human diseases. The gene transcription of iNOS can be induced for example, by lipopolysaccharide (LPS), interferon-γ (IFN-γ), oxidative stress/reactive oxygen species (ROS), interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α). This occurs via an activation of nuclear factor-κB (NF-κB). The activation of NF-κB can be prohibited for example by glucocorticosteroids, antioxidants or transforming growth factor-1β (TGF-β1).

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[24] Cytotoxicity is an important issue when dealing with CO-releasing molecules. The toxicity of CO, which leads to its deadly effect in mammals, is due to its high affinity to the prosthetic heme groups of hemoglobin and myoglobin, resulting in hypoxia, together with binding of CO to cytochrome c oxidase in mitochondria. Thus, the mitochondrial electron transport chain is inhibited and a breakdown of the energy supply of the cells is caused.

[25] This assay is based upon the conversion of the yellow MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenases into a violet formazan dye, which can be quantified photometrically.

[26] Supernatants of cells were mixed 1:1 (v/v) with Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride in 2% phosphoric acid) and the absorbance was measured at 560 nm. Nitrite content was quantified by using sodium nitrite as standard. See: E. Park, M. R. Quinn, *J. Leukocyte Biol.* **1993**, *54*, 119 – 124.

[27] The ether *rac*-**14** showed a significant iNOS inhibition of (11 ± 3)% at a concentration of 100 μM. This effect most likely results from some esterase-independent decomposition reaction.

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