Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Controlled Oxygen Release from Pyridone Endoperoxides Promotes Cell Survival under Anoxic Conditions

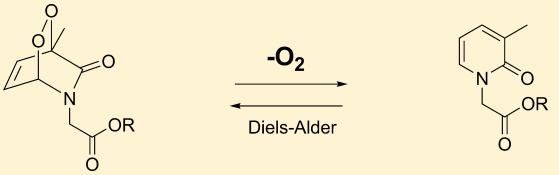
Sebastian Benz,[†] Sarah Nötzli,[‡] Jay S. Siegel,[§,†] Daniel Eberli,*[,‡] and Henning J. Jessen*[,†]

[†]Institute of Organic Chemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

[‡]Department of Urology, University of Zürich, University Hospital, Frauenklinikstrasse 10, 8091 Zürich, Switzerland

Supporting Information

ABSTRACT: In tissue engineering, survival of larger constructs remains challenging due to limited supply of oxygen caused by a lack of early vascularization. Controlled release of oxygen from small organic molecules represents a possible strategy to prevent cell death under anoxic conditions. A comprehensive study of methylated pyridone-derived endoperoxides has led to the development of water-soluble molecules that undergo retro-Diels–Alder reactions in aqueous environment releasing oxygen in high yield and with half-lives of up to 13 h. These molecules in combination with vitamin C as singlet oxygen quencher significantly improved survival of 3T3 fibroblasts and rat smooth muscle cells challenged with oxygen-depleted conditions.



INTRODUCTION

The support of anoxic tissue with oxygen to enhance cell survival holds promise in different areas of medical research and applications. In regenerative medicine, necrosis of engineered tissue as a result of oxygen depletion has remained a challenging problem, especially in larger grafts.[1−7] The efficiency of oxygen supply in engineered grafts is reduced, due to the poor vascularization[8−10] of these implants and the diffusion limit of molecular oxygen.[11] Necrosis and apoptosis of cells in engineered grafts and a shift to anaerobic metabolism controlled by hypoxia-inducible factor 1 (HIF-1)[12,13] under oxygen-deprived conditions can impair the healing process significantly. As a result of coagulative necrosis, cell debris and denatured proteins accumulate and further reduce oxygen supply causing failure in vital functions of the tissue. Thus, effort has been dedicated to the development of techniques that can supply oxygen to hypoxic or anoxic tissue.[5,14−16] (Normoxic conditions are generally referred to as 21% oxygen, hypoxic conditions 1−5% oxygen, and anoxic conditions <1% oxygen. It should be noted that most cells behave differently under hypoxic conditions found in their respective tissue compared with normoxia, which is hardly the oxygen partial pressures to which most cells are exposed. One needs to be aware of the fact that normoxia (21% O_2) is in fact for most cells a hyperoxic condition and hypoxic conditions are actually representative of an *in situ* normoxia for many cells.[17]) An intriguing strategy is the use of (biodegradable) polymers as scaffolds that slowly release oxygen in the graft.[18−21] The release must occur on a time-scale enabling the growth of a vascular network that can eventually support engineered tissue autonomously. From this perspective, the release of oxygen should occur over several days.[22] The molecules that have been studied in tissue engineering of bones and cardiosphere derived stem-cell therapy are hydrogen peroxide, sometimes combined with catalase,[19] and in the context of pancreatic islet regeneration[21] inorganic peroxides such as calcium peroxide that eventually produce hydrogen peroxide.[23] Soaking of biocompatible polymers such as poly(lactic-*co*-glycolic acid) (PLGA)[24] has led to promising results, showcasing the possibility to rescue hypoxic tissue and, as a consequence, decrease necrosis of tissue over multiple days in a naked mouse skin flap model.[18] However, an issue can be the cytotoxicity of the H_2O_2/catalase mixture or, more generally, uncontrolled release of oxygen and ions such as Ca^{2+}.[25,26] On this account, endoperoxides incorporated into small organic molecules are interesting alternatives owing to their ability to release oxygen in a retro-Diels–Alder reaction following first order kinetics.[27] The reaction rate can likely be controlled by the substitution pattern on the scaffold and does not rely on the presence of enzymes or other triggers. Moreover, these scaffolds could be attached covalently to a solid support, opening up the possibility to synthesize oxygen-releasing polymers with defined rates of oxygen release and without soluble byproducts, such as calcium ions. Systems that have been shown to reversibly incorporate oxygen are derived from polyaromatic hydrocarbons, for example, naphthalene and anthracene or different 2-pyridones.[28−32] The latter provide a scaffold that allows straightforward generation of interesting substitution patterns, while their polar nature should enhance solubility in water compared with, for example, anthracene-derived endoperoxides. Thus, the controlled release of oxygen from substituted 2-pyridone-derived endoperoxides in an aqueous environment followed by subsequent quenching[33] of initially formed $^1O_2$ to $^3O_2$, for example, by solvent deactivation[34] potentially provides a new strategy to rescue anoxic tissue from necrosis.

Received: October 18, 2013
Published: December 3, 2013

CHEMISTRY

To study the rate and efficiency of oxygen release from pyridone-derived endoperoxides, a series of substituted 2-pyridones had to be obtained and then subjected to type two photooxygenations.[35] It has been shown in naphthalene-derived endoperoxides, that substituting the bridgehead position significantly decreases the rate of the retro-Diels–Alder reaction.[31,36] Because one goal was the attenuated release of oxygen to prolong tissue supply and reduce oxygen-related toxicity, a series of methylated 2-pyridones was targeted to study the effect of simple substituents and combinations thereof in the system. In this context, the yield of oxygen release in water is clearly another important parameter. To increase the overall stability of the pyridone endoperoxides[35] and their solubility in water, a short tri(ethylene glycol) ether moiety was attached to the pyridone via N-alkylation that strongly reduced lipophilicity (see Table 1, ClogP values). As starting materials for the synthesis, some (methylated) 2-pyridones, such as **9** and **10**, are commercially available; others had to be obtained by chemical transformations (see Scheme 1).

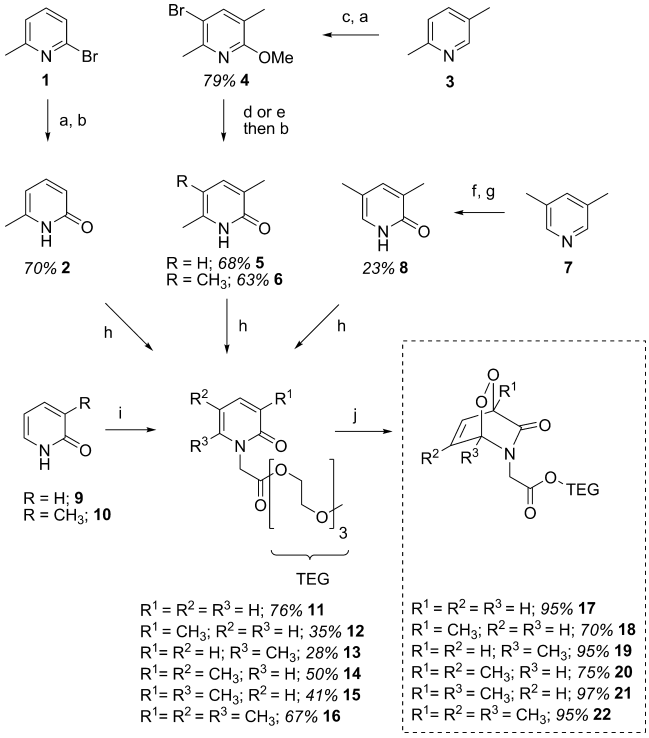
Briefly, 2-bromo-6-methylpyridine **1** was treated with sodium methoxide in methanol under microwave irradiation for 1 h, and the intermediate methoxypyridine was directly converted into the 6-methyl-2-pyridone **2** with hydrochloric acid in 1,4-dioxane at 100 °C in 70% yield over both steps.[37]

2,5-Lutidine **3** was selectively brominated with 1 equiv of tribromoisocyanuric acid (TBCA)[38] in 20% oleum giving an intermediate dibromide[39] in 89% yield after stirring for 3 h at room temperature. Nucleophilic substitution of bromide with sodium methoxide in methanol under microwave irradiation delivered 2-methoxypyridine derivative **4**. Lithium bromide exchange yielded the 3,6-dimethyl-2-pyridone **5** after protonation of the lithiated intermediate with water and cleavage of the methyl ether. Alternatively, reaction of the lithiated intermediate with methyl iodide delivered the 3,5,6-trimethyl-2-pyridone[40] **6** after cleavage of the methyl ether.

3,5-Lutidine **7** was converted with *meta*-chloroperbenzoic acid into its corresponding *N*-oxide, which was first treated with acetic acid anhydride and then with water at elevated temperature giving 3,5-dimethyl-2-pyridone **8**.[41]

The series of 2-pyridones was N-alkylated with 2-[2-(2-methoxyethoxy)ethoxy]ethyl 2-bromoacetate.[42] Careful adjustment of the reaction conditions for each 2-pyridone was necessary to control N- versus O-alkylation.[43] The ester moiety was important, because otherwise the endoperoxide could not be stably incorporated into the scaffold.[35] It should be noticed that this is also a suitable attachment point for covalent linkage to different polymers. The synthetic and commercially available 2-pyridones **11**–**16** were converted into their endoperoxides **17**–**22** by type two photooxygenations using triplet oxygen and tetraphenylporphyrin (TPP) as a sensitizer under irradiation with a high-pressure sodium lamp.[44,45] The Diels–Alder reactions occurred smoothly and with high yield within 1 h in DCM at 0 °C. The products were purified by flash column chromatography over SiO_2. All endoperoxides were stable over a period of many months if stored appropriately at −20 °C.

Scheme 1. Synthesis of Substituted 2-Pyridones and Their Corresponding Endoperoxides[a]



[a]Reagents and conditions: (a) 4 equiv of NaOMe, MeOH, microwave 100 °C, 1 h; (b) HCl concn/1,4-dioxane, microwave 100 °C, 2 h; (c) 1 equiv of TBCA, oleum 20% SO_3, 2.5 h; (d) (i) 1.1 equiv of *n*-BuLi, THF, −78 °C, (ii) H_2O, 0 °C to RT; (e) (i) 1.1 equiv of *n*-BuLi, THF, −78 °C, (ii) 3 equiv of MeI, −78 °C to RT; (f) 1.1 equiv of *m*CPBA, DCM, RT, 5 h; (g) (i) Ac_2O, reflux, 20 h, (ii) H_2O, reflux, 2 h; (h) (i) 1.1 equiv of NaH, DME/DMF, 0 °C, 15 min, (ii) 2.0 equiv of LiBr, RT, 15 min, (iii) 2.0 equiv of 2-[2-(2-methoxyethoxy)ethoxy]ethyl 2-bromoacetate, 65 °C, 4–12 h; (i) 3.0 equiv of K_2CO_3, then 1.5 equiv of 2-[2-(2-methoxyethoxy)ethoxy]ethyl 2-bromoacetate, DMF, overnight, RT; (j) TPP cat., *hν*, DCM, 0 °C, 1 h.

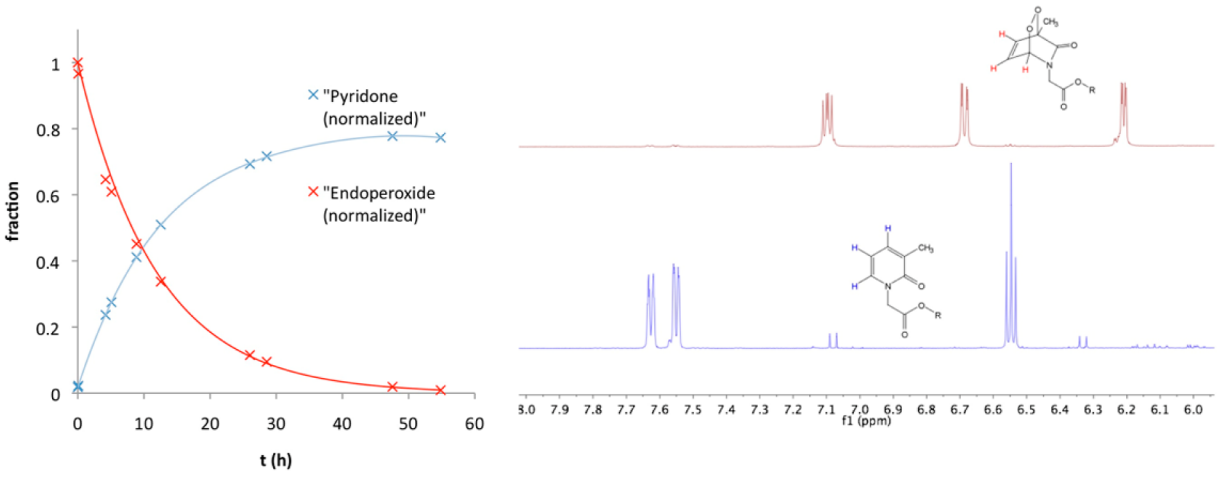
BIOLOGY

For the evaluation of cell expansion, the 3T3 fibroblast cell line and primary smooth muscle cells (SMCs) freshly isolated from rat bladder tissue[46] were cultured at a density of 4000 cells/cm^2. Endoperoxide **18** or pyridone **12** was dissolved in a 1:50 H_2O/DMSO (DMSO Hybri-Max, Sigma, Switzerland) mix, and different amounts (10, 50, 100, and 200 μg/mL) were directly added to cells in culture medium (CM) consisting of Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (DMEM/F-12, GlutaMAX and Pen/Strep, Gibco; HyClone FBS Defined, ThermoScientific). Vitamin C (Sigma, Switzerland) dissolved in H_2O was used in different concentrations as antioxidant (100 and 500 μg/mL to fibroblasts; 100 and 200 μg/mL to SMCs) and added to the endoperoxide **18** under the different assay conditions. All experiments were performed in triplicate. Cells with or without additives served as controls. Further, the parent pyridone **12** (the other product of the retro-Diels–Alder process of **18** besides oxygen) was tested with SMCs only.

All tests were performed in normoxic (aerobic, 37 °C, 5% CO_2) and anoxic environments (N_2). For the anoxic condition, CM was degassed prior to use with N_2 for 2–3 h (continuous bubbling of N_2 through a sterile tube into the medium). The experiments were conducted in an O_2-free environment by adding nitrogen gas to an airtight bag system (Sekuroka-Glove-Bags, Carl Roth GmbH, Switzerland). Additionally, the 24-well plates were individually packed in airtight minigrip bags and kept in the incubator (37 °C, 5% CO_2) without following

dx.doi.org/10.1021/jm4016137 | J. Med. Chem. 2013, 56, 10171–10182

Scheme 2. Exponential Decay of Endoperoxide 18 and Evolution of Parent Pyridone 12[a] in Water



[a]Values in the graph (left) are normalized on starting endoperoxide **18**. The values were obtained by integration of explicit proton resonances (right) in the ^{1}H NMR spectrum (H_2O/D_2O 9:1) and normalized to resonances of a defined amount of added DMSO. The partial ^{1}H NMR spectrum shows the distinct signals before (red, 0 h) and after (blue, >60 h) retro-Diels–Alder reaction.

medium change for up to 4 days. For the aerobic condition, cells were processed in a laminar flow hood and kept in the same incubator without airtight sealing.

Cell viability was measured on days 1–4 using a colorimetric assay with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt reagent (WST-1, Roche, Switzerland) according to the supplier. Briefly, CM was substituted with 0.5 mL/well fresh CM containing 50 μL/well WST-1 reagent (1:10). After an incubation time of 3 h, absorbance of the supernatant was measured at 450 nm in a scanning multiwell spectrophotometer (KC4, Software BioTek Instruments, Inc. Luzern, Switzerland).

RESULTS AND DISCUSSION

Chemical Behavior in Water. The half-lives of the retro-Diels–Alder process at 37 °C in H_2O/D_2O 9:1 v/v were measured by ^{1}H NMR spectroscopy with water suppression assuming first order kinetics. Since the chemical shift of the released 2-pyridones was known, quantification of oxygen release was possible. The half-lives were estimated by fitting of an exponential regression curve and using the equation $t_{1/2} = \ln(2)/k$ (see Scheme 2). The obtained values are summarized in Table 1.

For endoperoxides **17**, **18**, and **20**, more precise values were obtained by internal standardization with DMSO. Additionally, the NMR studies were used for an evaluation of the selectivity of the process, because the chemical shifts of the starting 2-pyridone were known. Thus, peaks arising from the retro-Diels–Alder process were readily assigned. The 2-pyridone and the endoperoxide that had the most favorable properties, that is, a long half-life combined with a good yield of oxygen and little byproduct formation, were then examined in cell assays (*vide supra*). Non-methylated endoperoxide **17** was the least stable in water and decomposed quickly and unspecifically within 30 min at 37 °C. This behavior is in contrast to an earlier publication, where oxygen release from a related non-methylated pyridone was measured in chloroform and found to occur slowly and selectively.[30] Endoperoxide **18** with a methyl group in the 3-position slowly released oxygen in water with a half-life of 8 h with only little byproduct formation (ca. 20%, see Table 1). Because of its favorable properties, this compound was eventually chosen for the cell-based assays. Methylation in the 6-position as in **19**, **21**, and **22** opened up new decomposition pathways other than retro-Diels–Alder thus increasing byproduct formation (>50%) and decreasing oxygen yield and half-lives (ca. 4 h). Compound **20** with methyl groups in the 3- and 5-position displayed an increased half-life of about 13 h and acceptable oxygen yield (50%). The main byproduct in most of the cases is the rearranged pyridone resulting from the Kornblum–Delamare reaction,[32,47,48] which is triggered both under basic and acidic conditions (results not shown). From these studies, it became obvious that modification of the pyridone in the 3-position resulted in favorable properties, whereas methylation, for example, in the 6 position strongly reduced the propensity of the system to undergo retro-Diels–Alder reaction.

Table 1. ClogP, Half-Lives, and Amount of Oxygen Release from Endoperoxides in Water at 37 °C

compd	ClogP[a]	$t_{1/2}$ [h]	% retro-Diels–Alder
17	−0.8	0.5	18[b]
18	−0.4	8.5	78[b]
19	−0.4	4	ca.50[c]
20	0.0	13	47[b]
21	0.0	4	ca.50[c]
22	0.1	15	ca.10[c]

[a]ChemDraw Pro (version 12). [b]Measurement normalized on DMSO standard. [c]Not normalized.

Cell Viability and Cytotoxicity. The properties of endoperoxide **18** were studied in combination with rat smooth muscle cells (SMCs) and 3T3 fibroblasts (FBs). These cell lines play important roles in tissue regeneration, because they are constituents of blood vessels and connective tissue, respectively.[49−51] It is important to note that their natural environments generally feature hypoxic conditions (sometimes referred to as *in situ* normoxia),[17] which might explain the slow growth under normoxic, ambient conditions (actually hyperoxia) that was generally observed in the assays.[52] Interestingly, fibroblasts contain significantly increased VEGF mRNA if grown under hypoxic conditions,[53] initiating angiogenesis in

dx.doi.org/10.1021/jm4016137 | J. Med. Chem. 2013, 56, 10171−10182

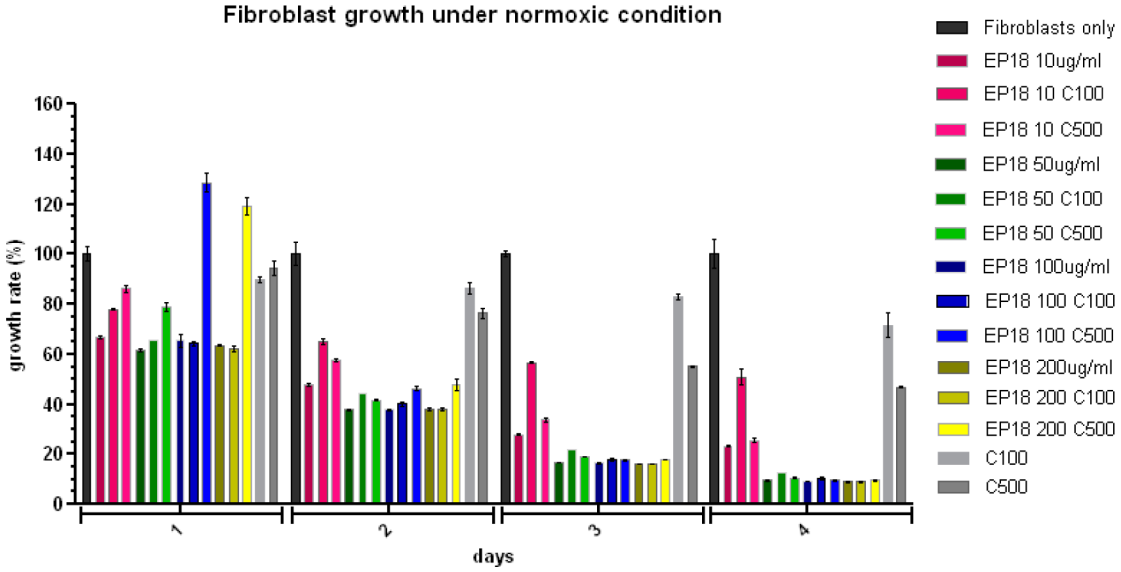


Figure 1. Normalized growth rate of fibroblasts under normoxic conditions. Fibroblasts were grown under normoxic conditions over 4 days. The growth rate without additives (black bars) was normalized to 100%. Addition of different concentrations of endoperoxide **18** (EP18) without or with vitamin C (C). The colors indicate one set of experiments at one fixed concentration of **18** (EP18). Vitamin C alone (gray bars) served as control. In general, all additives were detrimental for cell growth except high concentrations of vitamin C and **18** (blue bars, only on the first day). Read legend as follows: EP18 10 C500, endoperoxide **18** at 10 μg/mL + 500 μg/mL vitamin C.

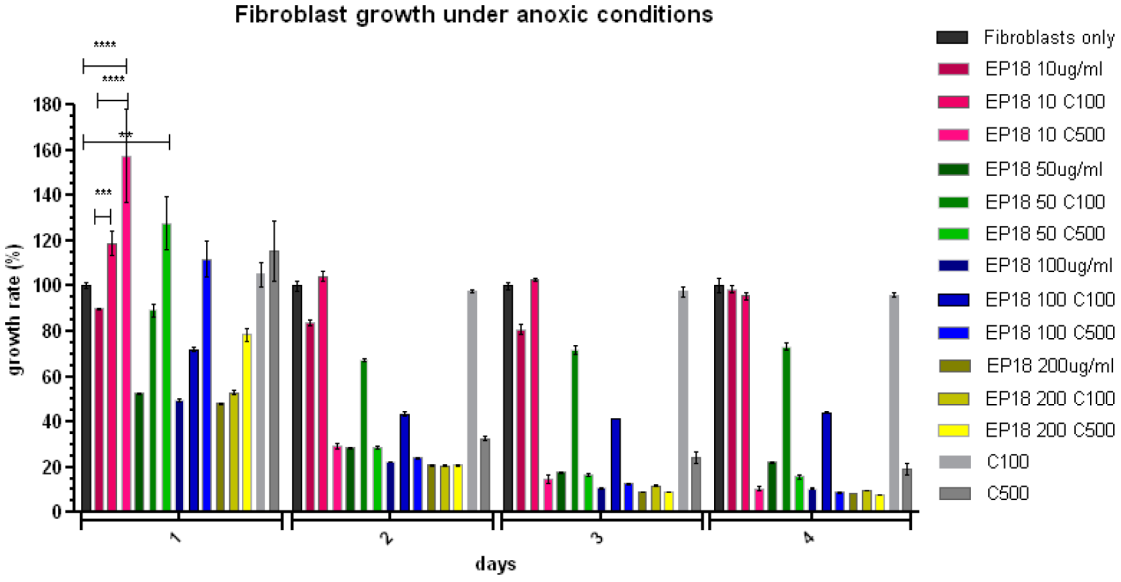


Figure 2. Normalized growth rate of fibroblasts under anoxic conditions. Fibroblasts were grown under anoxic conditions over 4 days. The growth rate without additives was normalized to 100%. Addition of different concentrations of endoperoxide **18** (EP18) without or with vitamin C (C). The colors indicate one set of experiments at one fixed concentration of **18** (EP18). Vitamin C alone (gray bars) served as control. Addition of vitamin C (100 μg/mL) reduced cytotoxicity of **18**, and a significant effect on cell growth was observed on day 1 (red bars). For the following days 2–4, cell growth was reduced to normal levels again. Read legend as follows: EP18 10 C500, endoperoxide **18** at 10 μg/mL + 500 μg/mL vitamin C.

hypoxic tissue along with other factors.[54,55] Initially, growth of FB and SMC under normoxic and anoxic conditions was followed and quantified over 4 days. The untreated control was normalized to 100% growth rate and compared with cells grown in the presence of additives. Increased growth rate was attributed to improved survival and avoidance of cell-cycle arrest under the respective conditions.[20,56] From the experimental setup, one would expect the following observations: (1) Under normoxic conditions, the endoperoxide should not lead to growth enhancement. (2) High concentrations of the endoperoxide should reduce cell viability under normoxic conditions, as singlet oxygen is released.[57] (3) The addition of an antioxidant like vitamin C (physical and chemical quenching of $^1O_2$)[58] should reduce the cytotoxicity exerted by the endoperoxide. (4) Under anoxic conditions, the endoperoxide should enhance the normalized growth rate of the cells, if used at nontoxic concentrations. (5) Small amounts of endoperoxide could already have an effect on the growth rate, because both cell types usually grow under hypoxic conditions. (6) The byproduct of the retro-Diels–Alder process, the corresponding pyridone, should not have an effect on cell growth both under normoxic and under anoxic conditions.

dx.doi.org/10.1021/jm4016137 | J. Med. Chem. 2013, 56, 10171–10182

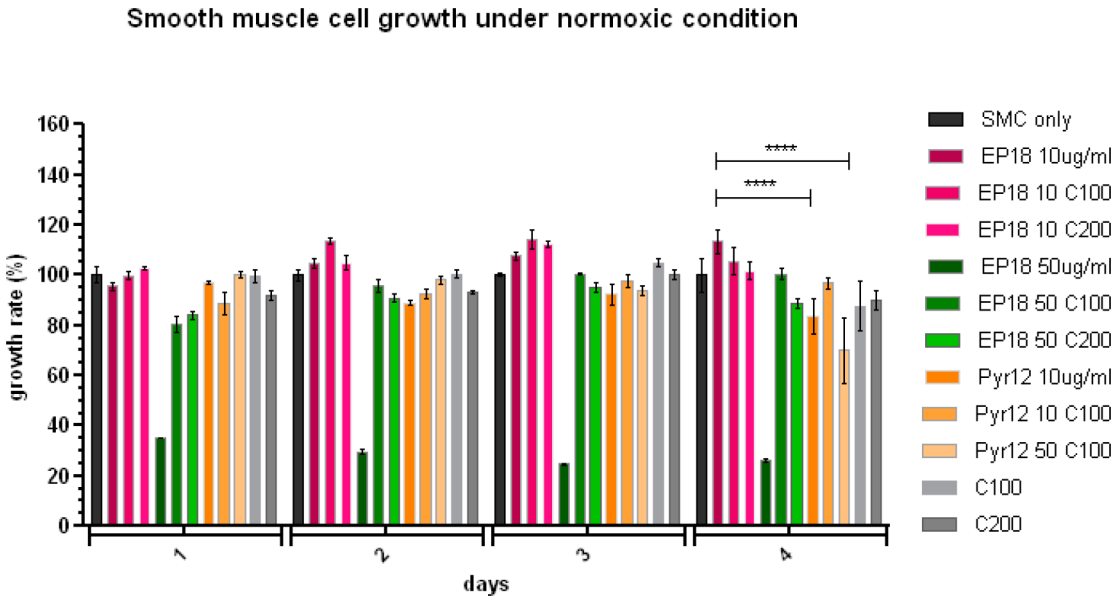


Figure 3. Normalized growth rate of smooth muscle cells under normoxic conditions. Smooth muscle cells were grown under normoxic conditions over 4 days. The growth rate without additives was normalized to 100%. Addition of two different concentrations of endoperoxide **18** (EP18) without or with vitamin C. The colors indicate one set of experiments at one fixed concentration of **18** (EP18). Vitamin C (C) alone (gray bars) served as control. Pyridone **12** at 10 μg/mL (Pyr12) served as a negative control (orange bars) and had no beneficial effect on cell growth in the presence or absence of vitamin C (C). Read legend as follows: EP18 10 C100, endoperoxide **18** at 10 μg/mL + 100 μg/mL vitamin C.

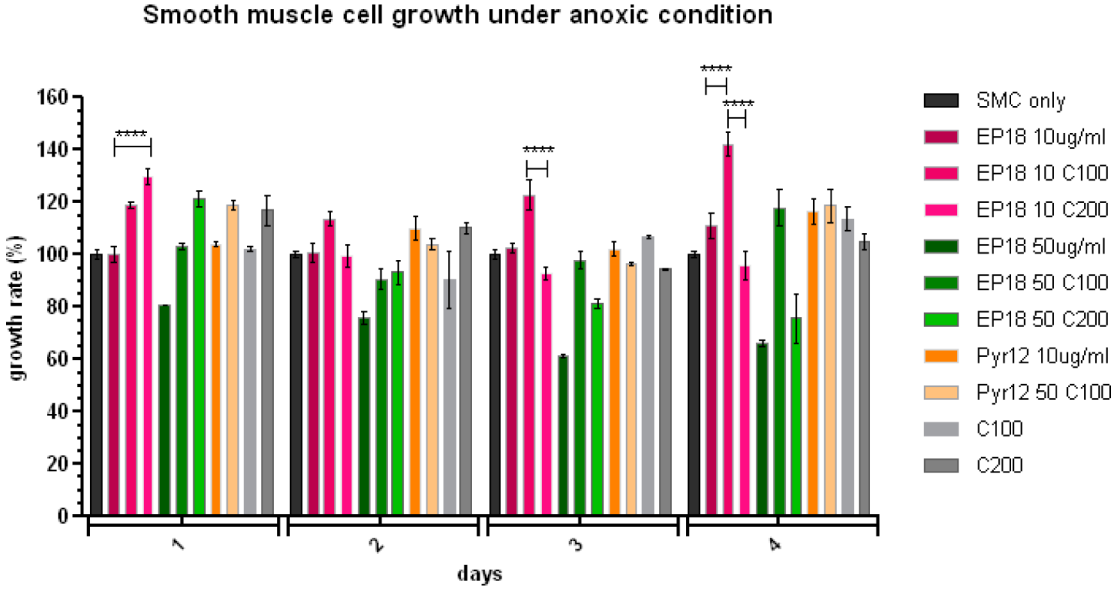


Figure 4. Normalized growth rate of smooth muscle cells under anoxic conditions. Smooth muscle cells were grown under anoxic conditions over 4 days. The growth rate without additives was normalized to 100%. Addition of different concentrations of endoperoxide **18** (EP18) without or with vitamin C (C). The colors indicate one set of experiments at one fixed concentration of **18** (EP18). Vitamin C alone (gray bars) served as control. Addition of vitamin C (100 μg/mL) and **18** resulted in a significant effect on cell growth over 4 days (dark red bars). Pyridone 12 (Pyr12) served as a negative control. Read legend as follows: EP18 10 C100, endoperoxide **18** at 10 μg/mL + 100 μg/mL vitamin C.

Based on the above considerations, the cytotoxicity of the compounds had to be determined first. The growth rate was quantified after the indicated periods with a colorimetric assay (WST-1, Roche). As expected, the growth rate of both SMCs and FBs was inhibited upon addition of endoperoxide **18** in a concentration-dependent manner. Concentrations of 29 μM (10 μg/mL) to 580 μM (200 μg/mL) were analyzed, and already at the lowest concentration, an effect on cell growth was observed under normoxic conditions in both FBs and SMCs (see Figure 1). However, this effect was significantly reduced under anoxic conditions in fibroblasts (10 μg/mL **18**). Intriguingly, addition of vitamin C as singlet oxygen quencher (100 μg/mL) led to significant reduction of the cytotoxic effect of endoperoxide **18** under normoxic conditions and completely abolished the negative effect of **18** on cell viability under anoxic conditions (see Figure 2). High amounts of vitamin C (500 μg/mL) were usually found to be detrimental in the assays (see Figure 1, gray bars), at least after 2 days of incubation, whereas on the first day this concentration pronouncedly increased the rate of cell growth under anoxic conditions (see Figure 2, red,

dx.doi.org/10.1021/jm4016137 | J. Med. Chem. 2013, 56, 10171−10182

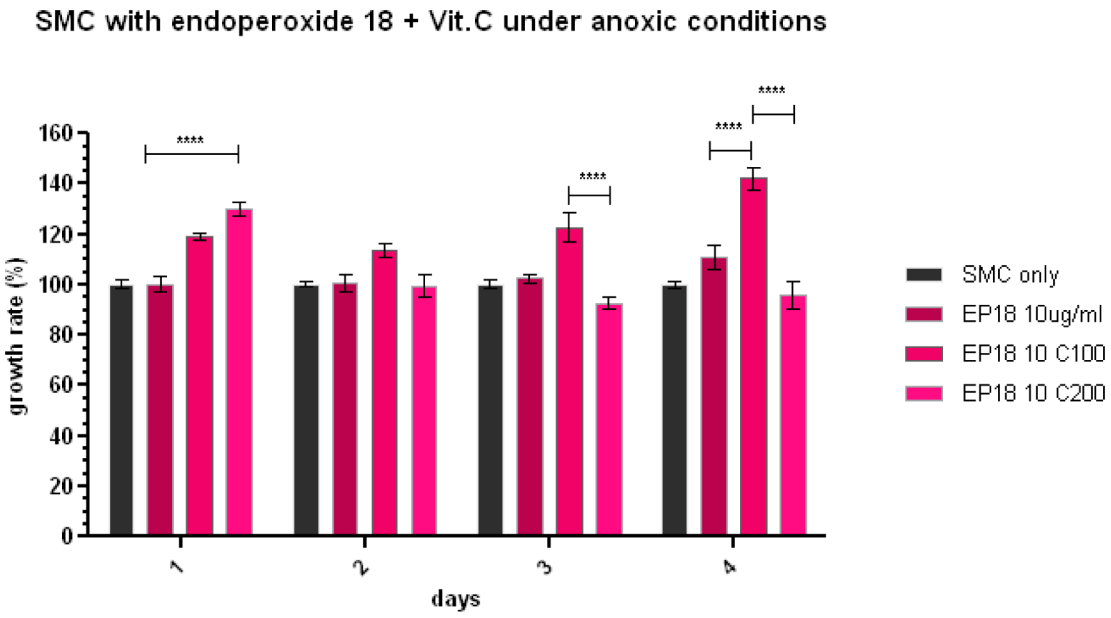


Figure 5. Normalized growth rate of smooth muscle cells under anoxic conditions. Smooth muscle cells were grown under anoxic conditions over 4 days. A clear beneficial effect of endoperoxide **18** (EP18) on growth rate in the presence of vitamin C (100 μg/mL) was observed over 4 days. Read legend as follows: EP18 10 C100, endoperoxide **18** at 10 μg/mL + 100 μg/mL vitamin C.

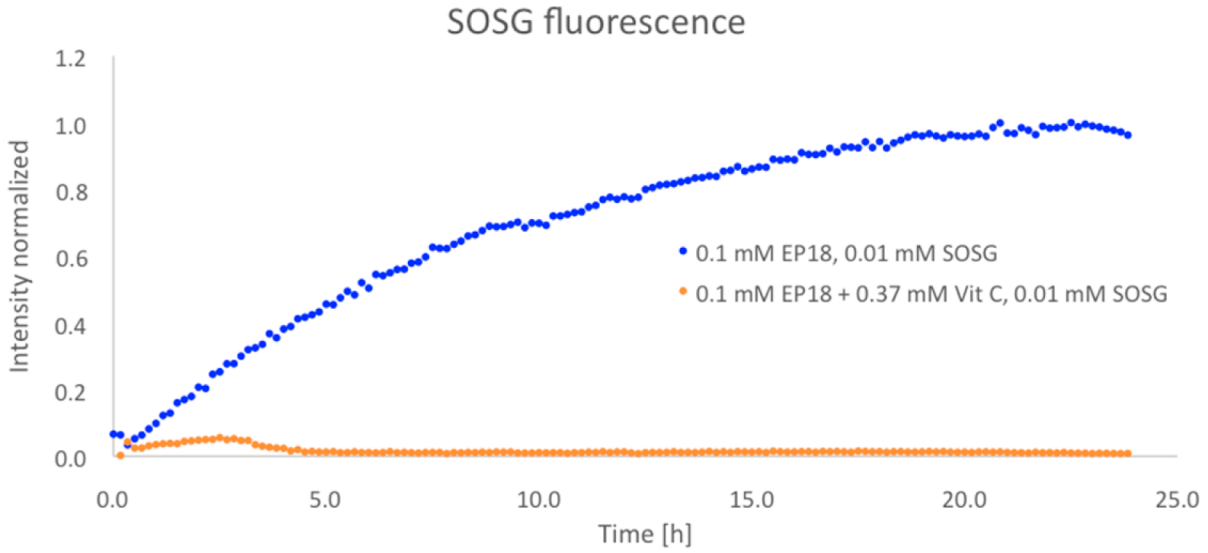


Figure 6. SOSG assay to detect singlet oxygen released from endoperoxide **18**. Singlet oxygen sensor green (SOSG) detects singlet oxygen released from pyridone endoperoxide **18** (EP18) in water at 37 °C (blue dots). Fluorescence was monitored with the exitation/emission pair 488/525 nm. In the presence of vitamin C, no singlet oxygen is detected anymore (orange dots).

green, and gray bars) and for very high concentrations of **18** and vitamin C also under normoxic conditions (see Figure 1, blue and yellow bars). From these data, it becomes obvious that the cells react very differently to the application of endoperoxide **18** under normoxic and anoxic conditions. Given the fact that 100 μg/mL of vitamin C did not significantly alter the growth rate of cells alone under anoxic conditions (Figure 2, light gray bars), this amount was used in the following experiments to distinguish the effect exerted by oxygen release from the endoperoxide.

Next, it was important to find out the optimal concentration range of the endoperoxide **18** in the presence of vitamin C. Compound **18** at 100 μg/mL was still found to reduce cell viability by a factor of ca. 10 in FBs in the presence of vitamin C under normoxic conditions (Figure 1, blue bars, day 4), whereas under anoxic conditions, the viability was only impaired by a factor of 2 (Figure 2, blue bars, day 4). If 200 μg/mL **18** was used, no beneficial effect of vitamin C could be observed anymore (Figures 1 and 2, yellow bars). The cytotoxicity of **18** at 50 μg/mL was still considerable even in the presence of vitamin C (Figure 1 and 2, green bars). The best results were obtained with 10 μg/mL of endoperoxide **18** in the presence of 100 μg/mL vitamin C (Figures 1 and 2, red bars).

Under anoxic conditions, the combination of endoperoxide **18** (10 μg/mL) with vitamin C (100 μg/mL) displayed no cytotoxicity in FBs but a significant beneficial effect on day 1 (Figure 2, red bars).

For SMCs, a similar picture was obtained. Under normoxic conditions, SMC growth rate was impaired starting from a

dx.doi.org/10.1021/jm4016137 | J. Med. Chem. 2013, 56, 10171−10182

concentration of 50 μg/mL endoperoxide **18**. This cytotoxicity of **18** was much less pronounced in SMCs compared with FBs. Vitamin C alone (100 and 200 μg/mL) had neither a beneficial nor detrimental effect on cell growth (Figure 3, gray bars), but it enhanced cell survival in the presence of endoperoxide **18** at 50 μg/mL (Figure 3, green bars). As a control, the parent pyridone **12**, which is also released in the retro-Diels–Alder reaction, did not show a beneficial effect on cell growth (Figure 3, orange bars).

Strikingly, SMCs showed increased cell growth under anoxic conditions even over 4 days in the presence of endoperoxide **18** (10 μg/mL) and vitamin C (100 μg/mL) compared with the control experiment and normoxic conditions (Figure 4, red bars, shown in more detail in Figure 5). Endoperoxide **18** alone did not have this significant effect, likely counterbalancing the cytotoxicity of singlet oxygen. Also at 50 μg/mL **18**, the beneficial effect of O_2 release was counterbalanced by the cytotoxicity at this concentration, which is also valid for high amounts of vitamin C (200 μg/mL). Pyridone **12** (50 μg/mL) in the presence of vitamin C did not increase cell survival over 4 days significantly (Figure 4, orange bars) and was nontoxic up to concentrations of 100 μg/mL if tested alone (data not shown).

In summary, pyridone endoperoxide **18** in the presence of vitamin C can supply cells under anoxic conditions with O_2 and increase survival over extended periods of time.

$^1O_2$ Release. Given a molar volume of 24 L/mol at 25 °C of O_2, in principle an 80% retro-Diels–Alder reaction of 10 μg of endoperoxide **18** can release 600 nL of oxygen. To further support the idea of initial singlet oxygen release from the pyridone endoperoxide **18**, the singlet oxygen sensor green assay from Invitrogen was applied. Briefly, a singlet oxygen responsive moiety attached to a fluorophore and quenching its fluorescence is the basis of this assay. After reaction of the responsive moiety with singlet oxygen, the emission of the fluorophore is restored, allowing fluorimetric readout.[59] Figure 6 shows the results of the experiments conducted in the presence and absence of vitamin C. From the data, it becomes obvious, that pyridone endoperoxide **18** releases singlet oxygen and that addition of vitamin C efficiently quenches the released singlet oxygen.

CONCLUSION

While in many studies involving oxygen generation cytotoxicity of a compound is the ultimate goal, as, for example, in cancer research,[60,61] the support of damaged tissue with small organic molecules is a largely unexplored area. In tissue engineering, survival of the grafts is critical and failure in sufficient oxygen supply can lead to necrosis. Usually, poor vascularization is one severe problem in larger grafts. Hypoxic conditions are generally well tolerated by cells as reduced oxygen pressure can be found in most tissues. However, anoxic conditions can have devastating effects on cell survival. Thus, it is likely that minute amounts of oxygen can rescue anoxic tissue from necrosis. Moreover, fibroblasts entering hypoxic tissue have increased amounts of mRNA encoding vascular endothelial growth factor (VEGF) an important signaling protein in vasculogenesis and angiogenesis.

In this study, fibroblasts and smooth muscle cells were grown under normoxic (21% O_2) and anoxic (0% O_2) conditions in the presence of water-soluble pyridone endoperoxides. The endoperoxide used in the assays was optimized toward long half-life in water (8–13 h) and efficient retro-Diels–Alder reaction (ca. 80%), releasing (singlet) oxygen. The cytotoxicity of the released singlet oxygen was controlled by addition of vitamin C. Efficient quenching of singlet oxygen by vitamin C was demonstrated by applying the singlet oxygen sensor green assay. The cell studies clearly show a reduction of the cytotoxicity of endoperoxides in the presence of vitamin C. Moreover, FBs showed increased growth rate under anoxic conditions on the first day of the study, and SMCs had increased growth rates over 4 days compared with the controls. Thus, pyridone endoperoxides efficiently release oxygen under cell culture conditions and can rescue cell growth under strictly anoxic conditions in two important cell lines responsible for early vascularization. It should be possible to covalently attach these molecules to polymeric scaffolds, thus enabling the generation of oxygen releasing polymers that can be used in tissue engineering. The endoperoxides, either in free form or attached to bandages, could in principle be used to support necrotic wounds with oxygen. The efficiency of the latter process will be studied in a mouse skin flap model in due course.

EXPERIMENTAL SECTION

Synthesis. Chemicals were purchased from commercial suppliers (Acros, Aldrich, TCI) and used without further purification, unless noted otherwise. Deuterated solvents were obtained from Armar Chemicals, Switzerland, in the indicated purity grade. ^{1}H NMR spectra were recorded on Bruker 300 and 400 MHz spectrometers or Bruker 500 MHz spectrometers (equipped with a cryo platform) at 298 K in the indicated deuterated solvent. Data are reported as follows: chemical shift (δ, ppm), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; or not resolved signal; br, broad signal), coupling constant(s) (J, Hz), integration. All signals were referenced to the internal solvent signal as standard ($CDCl_3$, δ 7.26; D_2O, δ 4.79; CD_3OD, δ 3.31; DMSO-d_6, δ 2.50). ^{13}C NMR spectra were recorded with ^{1}H-decoupling on Bruker 75, 101, or 125 MHz spectrometers (equipped with a cryo platform) at 298 K in the indicated deuterated solvent. All signals were referenced to the internal solvent signal as standard ($CDCl_3$, δ 77.16; CD_3OD, δ 49.0; DMSO-d_6, δ 39.5). IR spectra were recorded on a JASCO FT-IR-4100 spectrometer, and data are reported in terms of frequency of absorption (cm^{-1}).

Microwave experiments were conducted on a CEM Discover microwave (300 W, 10 mL tube volume).

Mass spectra were recorded by the Mass Spectroscopy Service of UZH on Finnigan MAT95 MS, Bruker EsquireLC MS, Bruker maXis QTof HR MS, and Finnigan TSQ700 MS machines. For thin layer chromatography (TLC), Merck precoated 60 F_{254} plates with a 0.2 mm layer of silica gel were used. For column chromatography, Merck silica gel 60, 230–400 mesh, was used. The purity of the compounds was additionally checked by HPLC analysis (CN modified silica, elution with hexane/*i*PrOH gradient) and found to be >95% unless indicated otherwise.

Fluorescence Measurements. One vial of singlet oxygen sensor green (Invitrogen) was dissolved in 33 μL of MeOH. The endoperoxide was dissolved in 10 μL of MeOH and diluted with water to the desired concentrations. Vitamin C was dissolved only in water. Total volume of 200 μL was introduced into a black 96-well plate (Greiner Bio One) and covered with a transparent plastic foil. The plate was placed into a plate reader (Spectra Max M5, Molecular Devices), and every 10 min, a value was recorded at 37 °C (excitation/emission 488/525 nm). All measurements were conducted in triplicate. Blank measurements containing SOSG and SOSG/vitamin C were subtracted from the results.

6-Methyl-2-pyridone, 2. 2-Bromo-6-methylpyridine **1** (500 mg, 2.90 mmol) and freshly prepared sodium methoxide (313 mg, 5.80 mmol) were dissolved in MeOH (4 mL) and heated in the microwave for 1 h at 105 °C. The mixture was concentrated under reduced pressure, extracted with diethyl ether, and washed with water. The

organic layer was concentrated *in vacuo*. HCl, 32% (1.5 mL), and 1,4-dioxane (2 mL) were added, and the solution was heated in the microwave for 2 h at 100 °C. The reaction mixture was concentrated *in vacuo*, dissolved in DCM, and washed with water. The organic layer was dried with $MgSO_4$ and concentrated to yield the title compound as a colorless solid (220 mg, 2.02 mmol, 70%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 13.57−12.19 (bs, 1H), 7.38 (dd, J = 9.1, 6.9 Hz, 1H), 6.43 (d, J = 9.1 Hz, 1H), 6.09 (d, J = 6.9 Hz, 1H), 2.37 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.2, 146.1, 142.1, 116.5, 106.2, 19.1; HRMS (ESI) calcd for C_6H_7NNaO $[M]^+$ 132.04198, found 132.04192.

2,5-Dibromo-3,6-dimethylpyridine. Oleum (20% in water, 20 mL) was cooled to 0 °C and 2,5-lutidine, **3** (1.50 g, 14.0 mmol), was added slowly. After warming to room temperature, TBCA (5.12 g, 14.0 mmol) was added in 1 g portions over a period of 2.5 h to the stirred solution. The reaction mixture was poured on ice water (80 mL), neutralized with sodium carbonate, and extracted with diethyl ether. The organic layer was dried over magnesium sulfate and evaporated to give the title compound as a colorless solid (3.30 g, 12.5 mmol, 89%). This material was used in the next step without further purification.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.62 (s, 1H), 2.60 (s, 3H), 2.33 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 155.6, 142.1, 142.0, 134.1, 120.2, 24.1, 21.3; HRMS (ESI) calcd for $C_7H_7Br_2N$ $[M]^+$ 263.90180, found 263.90167.

3-Bromo-2-methoxy-3,6-dimethylpyridine, 4. 2,5-Dibromo-3,6-dimethylpyridine (3.00 g, 11.3 mmol) was added to freshly prepared sodium methoxide (2.45 g, 45.3 mmol) in MeOH (12 mL), divided in to three equal portions, and irradiated in the microwave at 100 °C for 1 h. The mixtures were combined, concentrated *in vacuo*, and redissolved in EtOAc and water. The organic layer was washed with brine, dried over magnesium sulfate, and concentrated *in vacuo*. The residue was diluted with hexane, and a white solid was filtered off. The filtrate was concentrated to give the product as a clear liquid (2.10 g, 9.72 mmol, 89%). The product was used in the next step without further purification.

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (s, 1H), 3.91 (s, 3H), 2.50 (s, 3H), 2.12 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 160.7, 151.1, 141.7, 119.9, 111.4, 53.6, 24.0, 15.3; IR ν_{max}/cm^{-1} 2948, 1569, 1459, 1356, 1313, 1246, 1174, 1036, 962; HRMS (ESI) calcd for $C_8H_{11}BrNO$ $[M]^+$ 216.00185, found 216.00168.

3,6-Dimethyl-2-pyridone, 5. Pyridine **4** (700 mg, 3.23 mmol) was dissolved in 10 mL of dry THF and cooled to −78 °C. To this solution was slowly added *n*-BuLi (2.5 M in hexane, 1.43 mL, 3.56 mmol), and after stirring for 2.5 h, the mixture was warmed to 0 °C and H_2O (1.5 mL) was added carefully. The solution was concentrated *in vacuo*. HCl, 32% (2.5 mL), and 1,4-dioxane (2 mL) were added, and the solution was heated in the microwave at 100 °C for 2 h. The mixture was neutralized with sodium carbonate and extracted with ethyl acetate, washed with brine, and concentrated *in vacuo*. Purification by flash chromatography (9:1 DCM/MeOH v/v) yielded the title compound as a colorless solid (270 mg, 2.19 mmol, 68%).

Melting point 173−175 °C; ^{1}H NMR (500 MHz, $CDCl_3$) δ 12.90−12.26 (bs, 1H), 7.19 (d, J = 6.9 Hz, 1H), 5.95 (d, J = 6.9 Hz, 1H), 2.32 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.7, 142.7, 139.4, 125.4, 105.7, 18.9, 16.3; IR ν_{max}/cm^{-1} 2924, 1642, 1580, 1480, 593; HRMS (ESI) calcd for C_7H_9NNaO $[M + Na]^+$ 146.05764, found 146.05776.

3,5,6-Trimethyl-2-pyridone 6. Pyridine **4** (700 mg, 3.23 mmol) was dissolved in 10 mL of dry THF and cooled to −78 °C. To this solution was slowly added *n*-BuLi (2.5 M in hexane, 1.43 mL, 3.56 mmol). After the solution was stirred for 2.5 h, methyl iodide (1.38 g, 9.73 mmol) was added, and the mixture was warmed to room temperature. The solution was stirred for 50 min, concentrated *in vacuo*, and dissolved in HCl, 32% (1.5 mL), and 1,4-dioxane (2 mL). The mixture was heated in the microwave for 1.5 h at 100 °C, concentrated *in vacuo*, and extracted with DCM. The organic layer was washed with sat. sodium carbonate, sat. ammonium chloride, 1 M sodium thiosulfate, and brine. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (9:1 DCM/MeOH v/v) yielded the title compound as an off-white solid (310 mg, 2.26 mmol, 70%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 12.7−11.28 (bs, 1H), 7.12 (s, 1H), 2.26 (s, 3H), 2.11 (s, 3H), 2.02 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.9, 142.7, 139.1, 125.0, 112.5, 16.7, 16.2, 16.1; HRMS (ESI) calcd for $C_8H_{12}NO$ $[M]^+$ 138.09134, found 138.09143.

3,5-Dimethylpyridine-*N*-oxide. 3,5-Dimethylpyridine **7** (3.00 g, 28.0 mmol) was dissolved in DCM (50 mL) and cooled to 0 °C. *m*CPBA (75% stabilized with water, 7.00 g, 30.8 mmol) was added in three portions over a period of 10 min. The solution was warmed to room temperature and stirred for 5 h. The organic layer was mixed with sat. $NaHCO_3$ and extracted four times with DCM. The combined organic layers were dried over $MgSO_4$ and concentrated *in vacuo* to yield the product as a colorless solid (2.10 g, 17.0 mmol, 61%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.95 (s, 2H), 6.95 (s, 1H), 2.27 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 136.5, 136.0, 129.0, 18.1.; HRMS (ESI) calcd for $C_7H_{10}NO$ $[M]^+$ 124.07569, found 124.07562.

3,5-Dimethyl-2-pyridone, 8. 3,5-Dimethylpyridine-*N*-oxide (2.10 g, 17.0 mmol) was dissolved in acetic acid anhydride (30 mL) and heated under reflux for 20 h. The solution was concentrated *in vacuo*. Water (25 mL) was added, and the resulting mixture was heated under reflux for 2 h. The mixture was concentrated *in vacuo* and dried by repeated azeotropic distilliation with toluene. Purification by flash chromatography (9:1, DCM/MeOH, v/v) yielded the title compound as an off-white solid (0.80 g, 6.5 mmol, 38%).

Melting point 86−89 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.67−11.95 (bs, 1H), 7.19 (d, J = 1.1 Hz, 1H), 7.04 (s, 1H), 2.15 (s, 3H), 2.06 (d, J = 0.8 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 164.6, 142.0, 129.6, 128.5, 115.9, 17.1, 16.7; IR ν_{max}/cm^{-1} 3390, 1656, 1630, 1563,1472, 1375, 964, 881, 701; HRMS (ESI) calcd for $C_7H_{10}NO$ $[M]^+$ 124.07569, found 124.07569.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-Bromoacetate.[42] Bromoacetic acid (5.00 g, 36.0 mmol), tri(ethylene glycol) monomethyl ether (5.61 g, 34.2 mmol), and a catalytic amount dimethylaminopyridine (DMAP, 50 mg) were dissolved in DCM (200 mL) and cooled to 0 °C. Dicyclohexylcarbodiimide (DCC, 8.17 g, 39.6 mmol) was slowly added. The mixture was warmed to room temperature and stirred overnight. The white precipitate was filtered off, and the filtrate was concentrated *in vacuo*. The precipitation/filtration process was repeated once more. Purification by flash chromatography (20:1 DCM/MeOH) gave the product as a colorless liquid (5.10 g, 52%).

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.31−4.23 (m, 2H), 3.83 (s, 2H), 3.71−3.65 (m, 2H), 3.64−3.54 (m, 6H), 3.53−3.45 (m, 2H), 3.32 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.2, 71.9, 70.6, 70.5, 70.5, 68.7, 65.3, 59.0, 25.8; HRMS (ESI) calcd for $C_9H_{17}BrNaO_5$ $[M + Na]^+$ 307.0152, found 307.0154.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(2-Oxo-1,2-dihydropyridin-1-yl)acetate, 11. 2-Pyridone (100 mg, 1.05 mmol) was alkylated according to the same procedure described for product **13** (see below). Yield: 239 mg (0.798 mmol, 76%, colorless oil). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.26−7.20 (m, 1H), 7.19−7.13 (m, 1H), 6.41 (d, J = 9.1 Hz, 1H), 6.06 (td, J = 6.7, 1.3 Hz, 1H), 4.55 (s, 2H), 4.23−4.12 (m, 2H), 3.63−3.53 (m, 2H), 3.53−3.44 (m, 6H), 3.44−3.34 (m, 2H), 3.22 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 167.5, 162.1, 140.1, 138.1, 120.4, 105.9, 71.6, 70.3, 70.3, 70.2, 68.5, 64.5, 58.7, 50.1; IR ν_{max}/cm^{-1} 1748, 1660, 1540,1197, 1097; HRMS (ESI) calculated for $C_{14}H_{21}NNaO_6$ $[M + Na]^+$ 322.12611, found 322.12655.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(3-Methyl-2-oxo-1,2-dihydropyridin-1-yl)acetate, 12. 3-Methyl-2-pyridone **10** (200 mg, 1.83 mmol), 2-[2-(2-methoxyethoxy)ethoxy]ethyl 2-bromoacetate (784 mg, 2.75 mmol), and potassium carbonate (760 mg, 5.50 mmol) were dissolved in DMF (20 mL) at room temperature, and the solution was stirred overnight. The mixture was concentrated *in vacuo*. The residue was dissolved in ethyl acetate and washed with water. The organic layer was dried over $MgSO_4$ and concentrated *in vacuo*. Purification by flash chromatography (9:1 DCM/MeOH v/v) yielded the title compound as a colorless oil (200 mg, 0.64 mmol, 35%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.21−7.16 (m, 1H), 7.11−7.06 (m, 1H), 6.08 (t, J = 6.8 Hz, 1H), 4.63 (s, 2H), 4.34−4.19 (m, 2H), 3.72−3.45 (m, 10H), 3.33 (s, 3H), 2.10 (s, 3H); ^{13}C NMR (75 MHz,

$CDCl_3$) δ 168.0, 163.0, 137.5, 135.4, 130.0, 106.0, 72.0, 70.6, 70.6, 70.6, 68.9, 64.8, 59.1, 50.8, 17.2; IR ν_{max}/cm^{-1} 1748, 1652, 1592, 1561, 1196, 1099, 768; HRMS (ESI) calculated for $C_{15}H_{23}NNaO_6$ $[M + Na]^+$ 336.14176, found 336.14193.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(6-Methyl-2-oxo-1,2-dihydropyridin-1-yl)acetate, 13. 6-Methyl-2-pyridone, **2** (50 mg, 0.46 mmol), dissolved in 10 mL of DME, was cooled to 0 °C, and NaH (60% suspension in oil, 19 mg, 0.48 mmol) was added. After the mixture was stirred for 10 min, LiBr (80 mg, 0.92 mmol) was added, and the mixture was stirred for 20 min. 2-[2-(2-Methoxyethoxy)-ethoxy]ethyl 2-bromoacetate (157 mg, 0.55 mmol) was added. The mixture was heated to 60 °C for 4 h, cooled to room temperature, and filtered. The filtrate was concentrated *in vacuo*. Purification by flash chromatography yielded the desired product as a colorless oil (40 mg, 0.13 mmol, 28%).

^1H NMR (300 MHz, $CDCl_3$) δ 7.18 (dd, J = 9.2, 6.8 Hz, 1H), 6.38 (d, J = 9.2 Hz, 1H), 5.98 (d, J = 6.7 Hz, 1H), 4.77 (s, 2H), 4.33−4.17 (m, 2H), 3.75−3.41 (m, 10H), 3.30 (s, 3H), 2.22 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 167.9, 163.3, 145.8, 139.5, 117.4, 106.7, 71.8, 70.5, 70.5, 70.4, 68.7, 64.6, 58.9, 45.3, 20.4; IR ν_{max}/cm^{-1} 1746, 1660, 1550, 1197, 1099, 794; HRMS (ESI) calcd for $C_{15}H_{23}NNaO_6$ $[M + Na]^+$ 336.1418, found 336.1421.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(3,5-Dimethyl-2-oxo-1,2-dihydropyridin-1-yl)acetate, 14. 3,5-Dimethyl-2-pyridone, **8** (100 mg, 0.810 mmol), was alkylated according to the same procedure described for product **13**. Yield: 132 mg (0.404, 50%, colorless oil).

^1H NMR (400 MHz, CDCl3) δ 7.08 (d, J = 1.1 Hz, 1H), 6.85 (s, 1H), 4.61 (s, 2H), 4.36−4.24 (m, 2H), 3.73−3.67 (m, 2H), 3.66−3.59 (m, 6H), 3.57−3.49 (m, 2H), 3.35 (s, 3H), 2.10 (s, 3H), 2.02 (d, J = 0.8 Hz, 3H); ^{13}C NMR (101 MHz, CDCl3) δ 168.2, 162.2, 140.4, 132.6, 129.4, 114.8, 72.0, 70.7, 70.7, 70.6, 68.9, 64.79, 59.1, 50.7, 17.2, 17.1; IR ν_{max}/cm^{-1} 1747, 1664, 1593, 1195, 1100, 1036; HRMS (ESI) calcd for $C_{16}H_{25}NNaO_6$ $[M + Na]^+$ 350.1574, found 350.1579

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(3,6-Dimethyl-2-oxo-1,2-dihydropyridin-1-yl)acetate, 15. 3,6-Dimethyl-2-pyridone **5** (100 mg, 0.81 mmol) was dissolved in DME (9 mL) and cooled to −20 °C. *n*-BuLi (2.5 M in hexane, 0.36 mL, 0.89 mmol) was slowly added under an argon atmosphere, and the solution was stirred for 20 min. 2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-bromoacetate (347 mg, 1.22 mmol) in DME (0.5 mL) was added; the mixture was warmed to room temperature and stirred overnight. Concentration under reduced pressure and purification by flash chromatography (9:1 DCM/MeOH v/v) yielded the product as a colorless oil (108 mg, 0.33 mmol, 41%).

^1H NMR (300 MHz, $CDCl_3$) δ 7.12 (dd, J = 6.9, 0.9 Hz, 1H), 5.98 (d, J = 6.9 Hz, 1H), 4.84 (s, 2H), 4.40−4.29 (m, 2H), 3.80−3.49 (m, 10H), 3.38 (s, 3H), 2.26 (s, 3H), 2.11 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.3, 163.8, 142.7, 137.0, 126.3, 106.4, 72.0, 70.7, 70.6, 70.6, 69.0, 64.7, 59.1, 45.8, 20.4, 17.1; IR ν_{max}/cm^{-1} 1747, 1651, 1568, 1194, 1101; HRMS (ESI) calcd for $C_{16}H_{25}NNaO_6$ $[M + Na]^+$ 364.1731, found 364.1733.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-(3,5,6-Trimethyl-2-oxo-1,2-dihydropyridin-1-yl)acetate, 16. 3,5,6-Trimethyl-2-pyridone, **6** (90 mg, 0.66 mmol), was dissolved in a mixture of dry DME (6 mL) and dry DMF (1.5 mL). Under a nitrogen atmosphere, the solution was cooled to 0 °C, sodium hydride (60% in oil, 28 mg, 0.69 mmol) was added, and the solution was stirred for 15 min. To this mixture, LiBr (114 mg, 1.31 mmol) was added. The solution was warmed to room temperature and stirred for 15 min. 2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-bromoacetate (374 mg, 1.31 mmol) was added, and the reaction mixture was heated to 65 °C overnight. Concentration under reduced pressure followed by purification by flash chromatography (9:1 DCM/MeOH v/v) yielded the product as a colorless oil (150 mg, 0.44 mmol, 67%), purity >85%.

^1H NMR (400 MHz, $CDCl_3$) δ 7.04 (s, 1H), 4.87 (s, 2H), 4.36−4.23 (m, 2H), 3.71−3.65 (m, 2H), 3.65−3.57 (m, 6H), 3.55−3.47 (m, 2H), 3.34 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 168.6, 163.1, 141.1, 138.9, 125.6, 112.4, 72.0, 70.6, 70.6, 70.6, 68.9, 64.6, 59.1, 46.0, 18.0, 16.9, 16.4; ν_{max}/cm^{-1} 1746, 1639, 1196, 1098; HRMS (ESI) calcd for $C_{17}H_{27}NNaO_6$ $[M + Na]^+$ 350.1574, found 350.1577.

General Procedure for the Photooxygenation of 2-Pyridones. The respective pyridone was dissolved in ca. 10 mL of DCM in a reaction tube, TPP (1 mg) was added, and oxygen was bubbled through the solution with vigorous stirring for 5 min. The mixture was kept under an oxygen atmosphere with a balloon, cooled to 0 °C, and irradiated for 1 h with a high-pressure sodium lamp. The solution was concentrated *in vacuo*, and the residue was purified by flash column chromatography.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{6-Oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***17.*** Pyridone **11** (85 mg, 0.28 mmol); flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **17** as a clear oil (90 mg, 271 mmol, 95%). ^1H NMR (400 MHz, $CDCl_3$) δ 6.96−6.89 (m, 1H), 6.73−6.66 (m, 1H), 5.81−5.77 (m, 1H), 5.01−4.96 (m, 1H), 4.63 (d, J = 18.1 Hz, 1H), 4.34−4.17 (m, 2H), 3.90 (d, J = 18.1 Hz, 1H), 3.74−3.43 (m, 10H), 3.33 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.7, 168.0, 135.2, 127.7, 84.5, 77.8, 71.9, 70.5,70.5, 70.5, 68.6, 64.6, 59.0, 43.9; IR ν_{max}/cm^{-1} 1744, 1704, 1447, 1202, 1098, 891; HRMS (ESI) calculated for $C_{14}H_{21}NNaO_8$ $[M + Na]^+$ 354.11594, found 354.11597.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{1-Methyl-6-oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***18.*** Pyridone **12** (90 mg, 0.29 mmol); flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **18** as a slightly purple oil (70 mg, 0.20 mmol, 70%).

^1H NMR (300 MHz, $CDCl_3$) δ 6.95 (dd, J = 7.8, 5.3 Hz, 1H), 6.45 (dd, J = 7.8, 1.8 Hz, 1H), 5.75 (dd, J = 5.3, 1.8 Hz, 1H), 4.72 (d, J = 18.1 Hz, 1H), 4.39−4.20 (m, 2H), 3.92 (d, J = 18.1 Hz, 1H), 3.75−3.49 (m, 10H), 3.38 (s, 3H), 1.62 (s, 3H), 1.56 (s, 3H); 4.20 (m, 2H), 3.92 (d, J = 18.1 Hz, 1H), 3.75−3.49 (m, 10H), 3.38 (s, 3H), 1.62 (s, 3H), 1.56 (s, 3H); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.6, 168.8, 134.7, 132.5, 84.7, 81.3, 71.8, 70.5, 70.5, 68.6, 64.5, 59.0, 44.2, 14.5; IR ν_{max}/cm^{-1} 1747, 1705, 1448, 1201, 1104; HRMS (ESI) calcd for $C_{15}H_{23}NNaO_8$ $[M + Na]^+$ 368.1316, found 368.1316; R_t = 4.9 min (CN modified silica, 10% iprOH in hexanes, isocratic, 1 mL/min, then gradient to 40% iprOH at 35 min, purity >95%).

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{4-Methyl-6-oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***19.*** Pyridone **13** (40 mg, 0.13 mmol); flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **19** as a clear oil (42 mg, 0.12 mmol, 95%).

^1H NMR (400 MHz, $CDCl_3$) δ 6.72−6.65 (m, 2H), 4.98 (dd, J = 4.5, 3.3 Hz, 1H), 4.60 (d, J = 18.2 Hz, 1H), 4.33−4.15 (m, 2H), 3.94 (d, J = 18.2 Hz, 1H), 3.73−3.45 (m, 10H), 3.34 (s, 3H), 1.70 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.2, 168.9, 140.0, 126.8, 89.2, 77.2, 72.0, 70.6, 70.6, 70.6, 68.8, 64.6, 59.1, 40.8, 17.0; IR ν_{max}/cm^{-1} 1744, 1703, 1400, 1196, 1100, 782; HRMS (ESI) $C_{15}H_{23}NNaO_8$ $[M + Na]^+$ 368.1316, found 368.1315.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{1,8-Dimethyl-6-oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***20.*** Pyridone **14** (100 mg, 0.305 mmol); flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **20** as a clear oil (82 mg, 0.23 mmol, 75%).

^1H NMR (400 MHz, $CDCl_3$) δ 6.03−5.99 (m, 1H), 5.48 (d, J = 2.1 Hz, 1H), 4.74 (d, J = 18.1 Hz, 1H), 4.30−4.25 (m, 2H), 3.85 (d, J = 18.2 Hz, 1H), 3.68 (t, J = 4.7 Hz, 2H), 3.64−3.58 (m, 6H), 3.56−3.48 (m, J = 5.7, 3.6 Hz, 2H), 3.35 (s, 3H), 2.07 (d, J = 1.8 Hz, 3H), 1.54 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.0, 169.1, 145.7, 125.4, 88.9, 81.5, 72.0, 70.7, 70.7, 70.7, 68.8, 64.7, 59.1, 44.0, 17.6, 14.6; IR ν_{max}/cm^{-1} 1745, 1705, 1446, 1200, 1101; HRMS (ESI) calcd for $C_{16}H_{25}NNaO_8$ $[M + Na]^+$ 382.1472, found 382.1473.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{1,4-Dimethyl-6-oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***21.*** Pyridone **15** (60 mg, 0.18 mmol); flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **21** as a clear oil (64 mg, 0.18 mmol, 97%), purity >90%.

^1H NMR (400 MHz, $CDCl_3$) δ 6.66 (d, J = 7.8 Hz, 1H), 6.38 (d, J = 7.8 Hz, 1H), 4.60 (d, J = 18.2 Hz, 1H), 4.32−4.16 (m, 2H), 3.94 (d, J = 18.2 Hz, 1H), 3.71−3.46 (m, 10H), 3.34 (s, 3H), 1.67 (s, 3H), 1.57 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 169.6, 139.9, 132.1, 89.6, 81.1, 72.4, 71.1, 71.0, 71.0, 69.3, 65.0, 59.5, 41.7, 17.7, 15.2; IR

ν_{max}/cm^{-1} 1744, 1702, 1397, 1197, 1102, 790; HRMS (ESI) calcd for $C_{16}H_{25}NNaO_8$ $[M + Na]^+$ 382.1472, found 382.1473.

2-[2-(2-Methoxyethoxy)ethoxy]ethyl 2-{1,4,8-Trimethyl-6-oxo-2,3-dioxa-5-azabicyclo[2.2.2]oct-7-en-5-yl}acetate, ***22****.* Pyridone **16** (40 mg, 0.13 mmol) flash chromatography (20:1 DCM/MeOH v/v) yielded the endoperoxide **22** as a clear oil (31 mg, 0.08 mmol, 95%), purity >90%.

^{1}H NMR (300 MHz, $CDCl_3$) δ 6.08−6.04 (m, 1H), 4.71 (d, J = 18.3 Hz, 1H), 4.37−4.19 (m, 2H), 3.92 (d, J = 18.3 Hz, 1H), 3.76−3.48 (m, 10H), 3.38 (s, 3H), 1.97 (d, J = 1.8 Hz, 3H), 1.64 (s, 3H), 1.56 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.1, 169.5, 148.1, 125.7, 91.4, 80.8, 72.1, 71.9, 70.7, 68.9, 64.7, 59.2, 59.1, 41.3, 16.2, 15.1, 14.9; IR ν_{max}/cm^{-1} 1745, 1703, 1397, 1194, 1096; HRMS (ESI) calcd for $C_{17}H_{27}NNaO_8$ $[M + Na]^+$ 396.1630, found 396.1631.

Statistics. For data analysis, GraphPad Prism 5.04 software was used, and all data were expressed as mean and standard error of the mean. To determine the differences between the different conditions, one-way and two-way analysis of variance (ANOVA), respectively, with a Bonferroni posthoc test were performed for the numeric data. A p value of <0.05 was considered significant; * denotes $p < 0.05$, ** denotes $p < 0.01$, *** denotes $p < 0.001$, **** denotes $p < 0.0001$.

■ ASSOCIATED CONTENT

Supporting Information

NMR spectra, exponential decay curves, and cell micrographs. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*D.E. E-mail: Daniel.Eberli@usz.ch. Phone: +41 44 255 9619. *H.J.J. E-mail: henningjacob.jessen@uzh.ch. Phone: +41 44 63 5 42 41.

Present Address

§J.S.S.: School of Pharmaceutical Science and Technology; Tianjin University (A210/Bldg 24); 92 Weijin Road, Nankai District; Tianjin, 300072 PRC.

Author Contributions

Dr. Eberli and Dr. Jessen both serve as last authors because this work was only possible through combination of the distinct knowledge of the two research groups (Dr. Eberli, cell work and tissue engineering; Dr. Jessen, chemistry). The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. S.B. and S.N. contributed equally.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was funded by the Fonds der Chemischen Industrie (FCI, Germany), the Swiss National Science Foundation (SNF), and the University of Zürich (UZH).

■ ACKNOWLEDGMENTS

Generous financial support from the FCI (Liebig Stipend to H.J.J.) and the SNF (SNF, Ambizione Grant PZ00P2_136816 to H.J.J.) is acknowledged. We thank the members of the MS and NMR facilities at the University of Zürich (Laurent Bigler and Oliver Zerbe). J.S.S. gratefully acknowledges the Swiss National Science Foundation (SNSF), the Qian Ren Scholar Program of P.R. China, and Synergetic Innovation Center of Chemical Science and Engineering (Tianjin).

■ ABBREVIATIONS

VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; FB, 3T3 fibroblasts; SMC, smooth muscle cell; TLC, thin layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; TBCA, tribromoisocyanuric acid; *m*CPBA, *meta*-chloroperbenzoic acid; DMSO, dimethyl sulfoxide; DMF, dimethylformamide; DME, dimethoxyethane; TBCA, tribromoisocyanuric acid; TPP, tetraphenyl porphyrin; Vit. C, vitamin C; SOSG, singlet oxygen sensor green

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