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Optimization of labile esters for esterase–assisted accumulation of nitroxides into cells: A model for *in vivo* EPR imaging

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Abstract

Nitroxide–based electron paramagnetic resonance (EPR) imaging agents are useful quantitative probes of O₂ concentration *in vivo* in real time. Lipophilic, labile alkanoyloxymethyl esters of nitroxides can cross the blood–brain barrier, and after hydrolysis, the corresponding anionic nitroxide is intracellularly entrapped at levels sufficient to permit O₂ measurements. The utility of nitroxides as EPR imaging agents depends critically on their ability to accumulate in the brain to high levels. In this study, we systematically investigated the relationship between the structure of the alkanoyl moiety and the ability of the corresponding labile ester to deliver nitroxide intracellularly. We demonstrate, in a cultured cell model, that for nitroxide labile esters with unbranched alkanoyl chains, increasing the chain length improves intracellular loading. Moreover, by studying an isomeric series of labile esters, we conclude that branching of the alkanoyl chain drastically reduces intracellular loading. These structural insights improve our general ability to use labile esters to deliver carboxylates intracellularly, and suggest a strategy for how to enhance delivery of nitroxide imaging agents across the blood–brain barrier in a living animal.

INTRODUCTION

Molecular oxygen is fundamental to many aspects of brain physiology. For example, in neurons, it is essential for the synthesis and catabolism of neurotransmitters such as dopamine, norepinephrine and serotonin. Importantly, diverse pathophysiologies, including stroke, drug abuse, and neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases, are associated with acute or chronic alterations in brain O₂ concentration. *In vivo* measurement of O₂ in the brain in real time is not a trivial task. Blood oxygen level-dependent (BOLD) MR imaging is only capable of qualitative O₂ measurements *in vivo* (1–3). Therefore, an alternative, minimally invasive imaging method for *quantitation* of O₂ in the living brain is highly desirable for pathophysiological studies.

Molecular oxygen, being paramagnetic, broadens the electron paramagnetic resonance (EPR) spectral lines of other paramagnetic species, such as nitroxides or trityl radicals. Changes in EPR spectral linewidth for these compounds have been used to measure O₂ concentrations in homogenous solutions (2,4). The development of very–low–frequency EPR spectroscopy and

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imaging (2), along with suitable imaging probes, make it feasible to quantitate local O₂ concentrations in animals in real time. The concentration of O₂ in the vasculature of tumors has been successfully measured by EPR imaging using trityl radicals (2). Because of their large size and ionic charge, trityls cannot cross the blood–brain barrier or be intracellularly localized. Therefore, trityl radicals are unsuitable for O₂ measurements in the brain. Like trityls, suitably designed nitroxides are relatively resistant to bioreduction, but have the advantages of chemical flexibility and ease of chemical synthesis. Moreover, nitroxides are attractive probes for O₂ imaging in brain because they can be prepared as small labile esters that can cross the blood–brain barrier to enter brain tissue.

Previously, we demonstrated that the lipophilic labile ester, 3-acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [2] (Figure 1), can cross the cell membrane and enter the cytosol, where it is hydrolyzed by intracellular esterases to yield membrane-impermeant 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [1], which, being anionic at physiologic pH, becomes trapped intracellularly (5,6) (Scheme 1). *In vivo*, [2] is also able cross the blood-brain barrier to enter brain tissue, where it is hydrolyzed to [1], making local O₂ measurements possible (4).

Although we have shown that nitroxide [2] is a usable pro-imaging agent (4–6), the potential utility of nitroxides as EPR imaging agents can be maximized by entrapping the highest concentrations of nitroxide [1] in the brain. We reasoned that increasing the lipophilicity of the labile-ester pro-imaging agents would enhance their ability to cross biological membranes, and thus result in higher levels of trapped nitroxide [1]. In the present study, we synthesized a family of labile 3-alkanoyloxymethoxycarbonyl nitroxide esters that can be hydrolyzed to nitroxide [1] (Figure 1). Using our previous *in vitro* cell model (5,6), we examined the ability of cells to accumulate nitroxide [1] when incubated with the different labile-ester pro-imaging agents.

EXPERIMENTAL PROCEDURES

General Materials and Methods

Reagents and solvents from commercial vendors were used without further purification. Reagents were obtained from Aldrich Chemical Company (Milwaukee, WI), and solvents were from VWR (West Chester, PA). Silica gel (230 – 400 mesh) and TLC plates (Silica Gel 60 F254) were from EMD Chemicals Inc. (Gibbstown, NJ). Cell culture media and biochemicals were from Invitrogen Corp. (Carlsbad, CA). 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [1] was prepared as described by Rozantsev (7). 3-Acetoxymethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [2] was prepared as previously described (6). IR spectra were recorded on an FT-IR spectrometer (model 1600, Perkin-Elmer, Norwalk, CT) in CHCl₃. Mass spectrometric analysis was performed by the Mass Spectrometry Facility in the Department of Chemistry and Biochemistry at the University of Maryland, College Park. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA). Origin 8.0 software (OriginLab Corp., Northampton, MA) was used for data analysis. Unless otherwise indicated, standard deviations are reported for measurements.

Synthesis of 3-(2,2-Dimethylpropanoyl)oxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [6]

To a solution of nitroxide [1] (0.25 g, 1.3 mmol) in DMSO (2 mL) was added K₂CO₃ (0.37 g, 2.7 mmol). After the reaction mixture was stirred at room temperature for 5 min, chloromethyl pivalate (0.2 g, 0.19 mL, 1.4 mmol) was added. Stirring was continued at room temperature for 3 h before the mixture was diluted with CH₂Cl₂ (50 mL) and brine (100 mL). The organic layer was dried over anhydrous MgSO₄ and evaporated under high vacuum to remove traces of DMSO. The resulting crude product was purified by chromatography on silica gel; elution

with 1% (v/v) acetone in CHCl_3 afforded compound [6] (0.29 g, 75% yield). IR (CHCl_3): 1753 cm^{-1} (broad ester peak). Anal. calculated for $\text{C}_{15}\text{H}_{26}\text{NO}_5$: C, 59.98; H, 8.73; N, 4.66; found: C, 59.90; H, 8.84; N, 4.53.

Synthesis of 3-Chloromethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [8]

This compound was prepared following the general procedure of Harada *et al.* (8). A mixture of CH_2Cl_2 (20 mL) and 20 mL of aqueous solution containing 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [1] (0.5 g, 2.7 mmol), NaHCO_3 (0.9 g, 10.8 mmol), and tetrabutylammonium bisulfate (91 mg, 0.27 mmol) was stirred for 10 min at room temperature before addition of chloromethyl chlorosulfate (0.54 g, 0.33 mL, 3.3 mmol). The reaction mixture was vigorously stirred at room temperature for 2 h, during which the yellow color of the nitroxide moved from the aqueous phase into the organic phase. The CH_2Cl_2 phase was separated, washed with brine (100 mL), dried over anhydrous Na_2SO_4 , filtered, and evaporated under reduced pressure to yield compound [8] (0.54 g, 85% yield). TLC (silica gel, 2:1 (v/v) hexane/EtOAc) showed only one spot. The product was used in subsequent reactions without further purification.

Synthesis of 3-pentanoylmethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [4]

A mixture of chloromethyl ester [8] (0.75 g, 3.2 mmol), K_2CO_3 (0.88 g, 6.4 mmol) and DMSO (2 mL) was stirred at room temperature for 5 min before addition of pentanoic acid (0.33 g, 0.34 mL, 3.2 mmol) and a few crystals of NaI. The reaction mixture was stirred at room temperature overnight and then diluted with CH_2Cl_2 (50 mL) and brine (100 mL). The organic phase was dried over anhydrous MgSO_4 , filtered, and evaporated under high vacuum to remove traces of DMSO. The resulting crude product was purified on silica gel (7:3 (v/v) hexane:EtOAc) to give compound [4] (0.76 g, 80% yield). IR (CHCl_3): 1753 cm^{-1} (broad ester peak). Anal. calculated for $\text{C}_{15}\text{H}_{26}\text{NO}_5$: C, 59.98; H, 8.73; N, 4.66; found: C, 59.94; H, 8.83; N, 4.72.

Synthesis of 3-propanoylmethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [3]

This nitroxide was synthesized using propionic acid in the procedure for nitroxide [4]; the yield was 85%. IR (CHCl_3): 1752 cm^{-1} (broad ester peak). HR FAB MS (m/z): calculated for $\text{C}_{13}\text{H}_{22}\text{NO}_5$ (M^+) 272.1498; found, 272.1498. Anal. calculated for $\text{C}_{13}\text{H}_{22}\text{NO}_5$: C, 57.33; H, 8.14; N, 5.14; found: C, 57.46; H, 8.00; N, 5.10.

Synthesis of 3-(3-methylbutanoyl)methoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [5]

This nitroxide was synthesized using isovaleric acid in the procedure for nitroxide [4]; the yield was 74%. IR (CHCl_3): 1753 cm^{-1} (broad ester peak). Anal. calculated for $\text{C}_{15}\text{H}_{26}\text{NO}_5$: C, 59.98; H, 8.73; N, 4.66; found: C, 59.86; H, 8.81; N, 4.71. Synthesis of 3-heptanoylmethoxycarbonyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy [7]. This nitroxide was synthesized using heptanoic acid in the procedure for nitroxide [4]; the yield was 84%. IR (CHCl_3): 1753 cm^{-1} (broad ester peak). Anal. calculated for $\text{C}_{15}\text{H}_{26}\text{NO}_5$: C, 62.17; H, 9.21; N, 4.26; found: C, 62.33; H, 9.40; N, 4.22.

Determination of logP Values of Labile Esters

For each of the labile esters, the $\log_{10}$ of the octanol-water partition coefficient ($\log P$) was determined. The $\log P$ values of ester 2 – 7 were evaluated from their capacity factors (k') as determined by HPLC on a reverse-phase column (Kromasil 100 RP-C8; Higgins Analytical, Mountain View, CA), with a mobile phase consisting of methanol and 0.02 M sodium phosphate buffer (pH 6.0) in a volume ratio of 7:3 (9,10). The measurements were performed using isocratic elution (1 mL/min) on a Waters HPLC equipped with a diode-array detector

(Model 600; Milford, MA); the absorbance at 222 nm was used to construct the chromatograms. The capacity factor is defined as $k' = (t_R - t_0)/t_0$, where t_R and t_0 are, respectively, the elution times of the compound of interest and the void marker (thiourea). To generate a $\log P$ vs. $\log k'$ calibration line, we used 9 compounds with known $\log P$ values (in parentheses): caffeine (−0.07), 2-butanone (0.29), cycloheximide (0.55), benzyl alcohol (1.10), hydrocortisone (1.53), acetophenone (1.58), nitrobenzene (1.85), anisole (2.11) and naphthalene (3.37) (11, 12). The calibration line (Supporting Information Fig. S1) was used to convert the $\log k'$ values of esters **2** – **7** into the corresponding $\log P$ values. Two to four replicates of each measurement were made.

Kinetics of Nitroxide Reduction by Ascorbate

The background buffer used for all kinetic measurements was a 9:1 (v/v) mixture of Dulbecco's Phosphate Buffered Saline (Ca^{2+} - and Mg^{2+} -free) and absolute ethanol (final pH 7.3). Diethylenetriamine-*N,N,N',N',N''*-penta-acetic acid (DTPA; 0.5 mM) was added to the background buffer to sequester trace heavy metal ions. Nitroxide esters [**2**] – [**7**] were each dissolved in absolute ethanol to make a 20 mM stock solution. A 200 mM stock solution of crystalline L-ascorbic acid (SigmaUltra grade) was prepared in deionized water (18.3 MΩ·cm resistivity) that had been sparged with argon gas for 30 min; the solution was maintained at 0° C under N_2 atmosphere. Each experimental sample contained 50 μM nitroxide in background buffer. At time zero, 2 mM ascorbate was added to the sample, which was immediately transferred into a quartz flat cell and placed in the EPR spectrometer for spectroscopic measurement (details below). The amplitude of the downfield peak in the nitroxide EPR spectrum was monitored over 20 – 25 min. In all cases, peak amplitude as a function of time was well fit by a single-exponential decay characterized by an apparent first-order rate constant, k_{red} (see Fig. S2 in Supporting Information for typical data set and nonlinear curve fit). All experiments were conducted at 23°C. Duplicate measurements were performed for each nitroxide labile ester; the sequence of measurements was randomized.

Cellular Loading of Nitroxides

Jurkat lymphocytes were cultured and loaded with nitroxides as previously described (5,6). Briefly, a suspension of 1.8×10^7 cells, at a density of $2 \times 10^7 \text{ mL}^{-1}$, were incubated for 70 min at room temperature in serum-free RPMI 1640 medium containing the indicated concentration of the appropriate nitroxide labile ester and 0.0015% (w/v) of the surfactant Pluronic F-127 (BASF Corp, Florham Park, NJ). After incubation, cells were washed 3 times in Hanks' Balanced Salt Solution (HBSS), and resuspended in 400 μL HBSS. To lyse cells, 120 μM digitonin was added to each cell suspension, which was then sonicated for 1 min in a bath sonicator (model G112SPIG, Laboratory Supplies Company, Inc., Hicksville, NY). The lysate was assayed for nitroxide content by EPR spectroscopy. Each loading experiment was repeated two to four times.

EPR Spectroscopy

EPR spectra were recorded on an X-band spectrometer (model E-109, Varian Inc, Palo Alto, CA) at the following settings: microwave power, 20 mW; microwave frequency, 9.55 GHz; field set, 3335 G; modulation frequency, 1 kHz; modulation amplitude, 0.5 G; field sweep, 80 G at 26.7 G min^{-1} . Where the amplitude of only the center spectral line was required, the sweep width was 8 G. EWIN software (Scientific Software Solutions, Northville, MI) was used for spectrometer control and data acquisition. Nitroxide signal was measured as the peak-to-trough amplitude of the center line of the three-line spectrum.

RESULTS AND DISCUSSION

To adjust the lipophilicity of EPR pro-imaging agents systematically, we synthesized a series of alkanoyloxymethyl esters of [1] in which the hydrophobicity and steric bulk of the alkanoyl moiety was varied (Figure 1). Compound [6] was synthesized through the procedure developed for [2] (6), with commercially available chloromethyl pivalate used instead of bromomethyl acetate as the alkylating agent to esterify nitroxide [1] (Scheme 2A). However, for compounds [3], [4], [5] and [7], it was simpler to prepare the chloromethyl ester [8], which could undergo reaction with a series of inexpensive carboxylic acids to yield the corresponding labile esters (Scheme 2B).

Previously, we had shown that compound [2] is relatively resistant to bioreduction (5,6). The new compounds [3] – [7], bearing different alkanoyloxymethyl ester functions but the identical nitroxide moiety as [2], are expected to be similar to compound [2] in their resistance to reduction. We assessed the susceptibility of all seven compounds used in this study to reduction by 2 mM ascorbate. The apparent first-order rate constants for reduction (k_{red}) of compounds [2] – [7] are given in Table 1. The differences in the values are not statistically significant. When all measurements were pooled, the mean value for compounds [2] – [7] is $k_{\text{red}} = 7.7 (\pm 0.7) \times 10^{-2} \text{ min}^{-1}$ ($n = 12$).

To investigate the effectiveness of the various labile esters to deliver nitroxide [1] intracellularly, we incubated Jurkat lymphocytes with the esters over a range of concentrations and assayed the intracellular content of nitroxide [1]. We first compared the intracellular loading after incubation with esters [2], [3], [4], and [7]—a family in which the length of the alkanoyl chain was systematically increased. The results are presented in Figure 2. It can be seen that while increasing the alkanoyl chain length from 2 to 3 had negligible effect on intracellular loading (compare results for esters [2] and [3]), increasing the chain length to 5 (ester [4]) resulted in a substantial enhancement of intracellular loading. A further increase of the chain length to 7 (ester [7]), however, brought no additional improvement in loading.

We next investigated the effect of branching in the alkanoyl chain in the labile ester on intracellular loading of nitroxide [1] by incubating the cells with the isomeric esters [4], [5], and [6]. The results in Figure 2 show that while ester [4], with an *n*-pentanoyl moiety, gave high intracellular levels of nitroxide [1], esters [5] and [6], with 3-methylbutanoyl (isovaleryl) and 2,2-dimethylpropanoyl (pivaloyl) moieties, respectively, gave very poor intracellular loading of nitroxide [1]. These findings indicate that increased branching in the alkanoyl chain drastically diminished the ability of the labile ester to deliver nitroxide [1] intracellularly.

Besides changing the steric bulk of the labile ester, increased branching in the alkanoyl chain is also expected to decrease the lipophilicity of the molecule. A commonly-used measure of lipophilicity is the $\log_{10}$ of the octanol-water partition coefficient, $\log P$. The experimentally determined $\log P$ values for esters 2 – 7 (Table 1) show that lipophilicity does decrease with increased alkanoyl chain branching, but the effect is very slight. Thus, the isomeric esters [4], [5] and [6], with primary, secondary and tertiary alkanoyl chains, respectively, have $\log P$ values that decrease systematically but slightly, from 2.67 down to 2.60. Moreover, intracellular loading of the branched-chain esters [5] and [6] is much worse than that of straight-chain esters that are either less lipophilic ([2] and [3]) or more lipophilic ([7]). Therefore, the differences in lipophilicity cannot be invoked to explain differential intracellular loading. A reasonable inference is that intracellular esterases that hydrolyze alkanoyloxymethyl esters have a strong preference for straight over branched alkanoyl chains.

Having investigated the relationship between labile ester structure and the levels of intracellularly entrapped nitroxide [1], we must investigate this relationship *in vivo*. Our cellular incubation model provided extremely promising data regarding the ability to load cells

with nitroxides, but ultimate application of these compounds will depend on the ability of the lipophilic nitroxide esters to cross the blood-brain barrier. In anticipation of using the labile esters for EPR imaging of O₂ in living brain, studies are under way to determine the pharmacokinetics and pharmacodynamics of esters [3], [4], [5], and [7].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. 3-Carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl [1] and its labile ester derivatives characterized in this study.

Figure 2.

Intracellular loading of nitroxide [1] after Jurkat lymphocytes were incubated with various concentrations of the labile esters [2] – [7]. Error bars represent standard deviations; where not shown, the error bar is smaller than the symbol. For visual clarity, only down error bars are shown.

Scheme 1.

Esterase-assisted intracellular accumulation of nitroxide [1]

Scheme 2.
Synthesis of nitroxide labile esters

Table 1Log P values and reduction kinetics of nitroxide labile esters [2] – [7]

Ester	R	log P	k_{red}^a ($\times 10^{-2} \text{ min}^{-1}$)
2	$-\text{CH}_3$	1.12	7.2
3	$-\text{CH}_2\text{CH}_3$	1.33	7.4
4	$-(\text{CH}_2)_3\text{CH}_3$	2.67	8.1
5	$-\text{CH}_2\text{CH}(\text{CH}_3)_2$	2.62	8.0
6	$-\text{C}(\text{CH}_3)_3$	2.60	8.0
7	$-(\text{CH}_2)_5\text{CH}_3$	3.54	7.6

^a Apparent first-order rate constant for reduction by 2 mM ascorbate at pH 7.3, 23°C. Each value is the average from two experiments.