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An oxidatively releasable caging group that senses lipid peroxidation

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Abstract—A new radical-sensitive caging technique releases a caged molecule under lipid peroxidation conditions. In a competitive oxidation study with a model lipid, methyl linoleate, the oxidatively releasable xanthenyl caging groups is found to be 1.93 ± 0.55 times more reactive than the lipid model compound.

Caging biological molecules with a photolabile protecting group is a common technique used in cell biology and biochemistry.[1] Photochemical cleavage of the covalent bond between the caging group and biomolecule releases the biomolecule in its active form.[2] We have been developing a new type of caging concept and technique, oxidatively releasable caging, which releases a covalently bound biomolecule in response to oxidative activity. This caging system should only release the protected biological molecule in the presence of radical species, such as radical reactive oxygen species (ROS) or radical reactive nitrogen species (RNS).

Reactive oxygen and nitrogen species are produced as a result of oxidative metabolism. Important ROS are superoxide radical anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), alkoxyl radicals ($RO^{\bullet}$), peroxyl radical ($ROO^{\bullet}$), and hydroxyl radical ($HO^{\bullet}$).[3] Important RNS are peroxynitrite ($ONOO^-$), nitrosoperoxocarbonate anion ($ONOOCO_2^-$), and nitric oxide ($NO^{\bullet}$).[4] The hydroxyl radical is the most reactive, capable of reacting with any C–H bond on biological molecules to abstract the hydrogen atom and form a radical on the molecule. The other reactive radical species are not as reactive as the hydroxyl radical, but some are capable of reacting selectively with weak C–H bonds. Unsaturated fatty acids, as free acids or as esters in lipids, are especially reactive in radical lipid peroxidation reactions. A lipid C–H bond in a CH_2 group between two alkenes, –CH=CH–CH_2–CH=CH–, is the weakest and most reactive of all because cleavage of that bond produces a resonance-delocalized stabilized radical.[5]

The caging system described here is designed to intercept peroxyl radical intermediates in lipid peroxidation (autoxidation) reactions. The oxidatively releasable caging group must have a radically reactive C–H bond that can divert radicals away from the oxidation of biomolecules. The caging group chosen because of this key feature is a xanthenyl group, shown in Figure 1. Xanthene

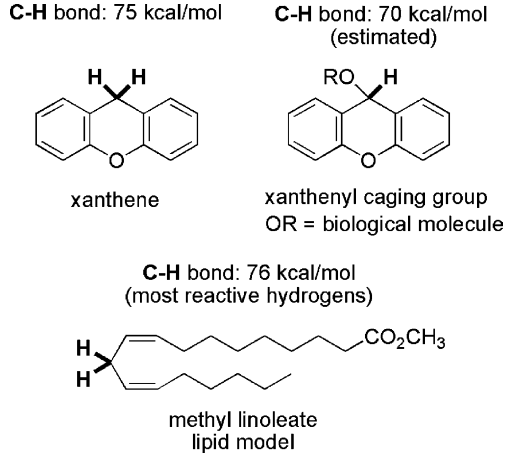

Figure 1. Carbon–hydrogen bond dissociation energies in kcal/mole of xanthene, proposed oxidatively releasable xanthenyl caging group, and reactive hydrogens on the lipid, methyl linoleate.

Keywords: Lipid peroxidation; Reactive oxygen species; Radical reaction mechanism; Caging group.
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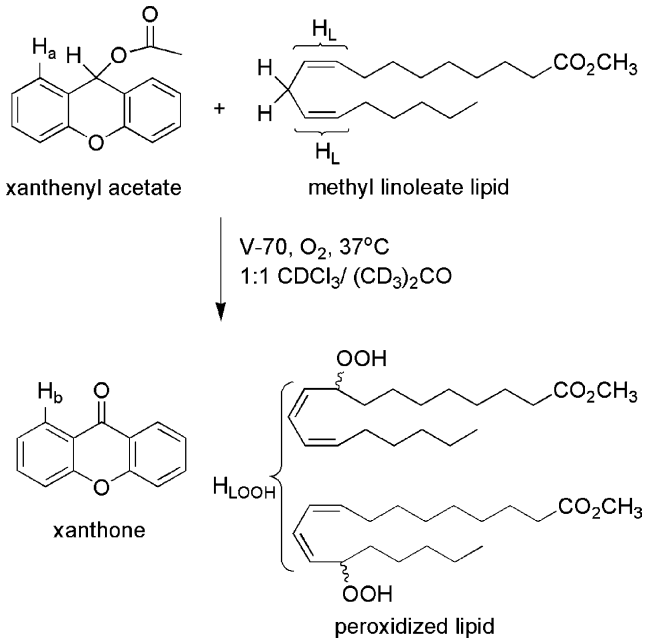
has a relatively weak C–H bond, 75 kcal/mol.[6] In general, an oxygen substituent present on carbon weakens the C–H by about 5 kcal/mol.[8] Therefore, the proposed oxidatively releasable caging group has an even weaker C–H bond, estimated at about 70 kcal/mol. Such a C–H bond is weaker than almost all other bonds commonly found in biomolecules, particularly lipid molecules.[7] Methyl linoleate is a standard model lipid used in lipid peroxidation studies (Fig. 1).[8] The bond energy for the most reactive C–H bond in methyl linoleate is 76 kcal/mol.[9] The C–H bond energy in the proposed xanthenyl caging group is less than the bond energy of the reactive model lipid C–H bond. Thus, the xanthenyl caging group should be more reactive than the model lipid under radical lipid peroxidation conditions.

A proposed mechanism for the release of the oxidatively triggered caging group is shown in Scheme 1.[10] Under lipid peroxidation conditions the reactive hydrogen on the caged biomolecule (**1**) should be abstracted. Oxygen will react with the alkyl radical (**2**) to form the caged peroxyl radical (**3**) compound. This radical then would abstract the reactive hydrogen on another caged biomolecule which propagates the autoxidation of the caged biomolecule. The peroxidized caged biomolecule (**4**) undergoes hydrolysis to release the active biomolecule (**8**). To test if this caging group can release a molecule under radical oxidation conditions, a model compound was synthesized and tested.

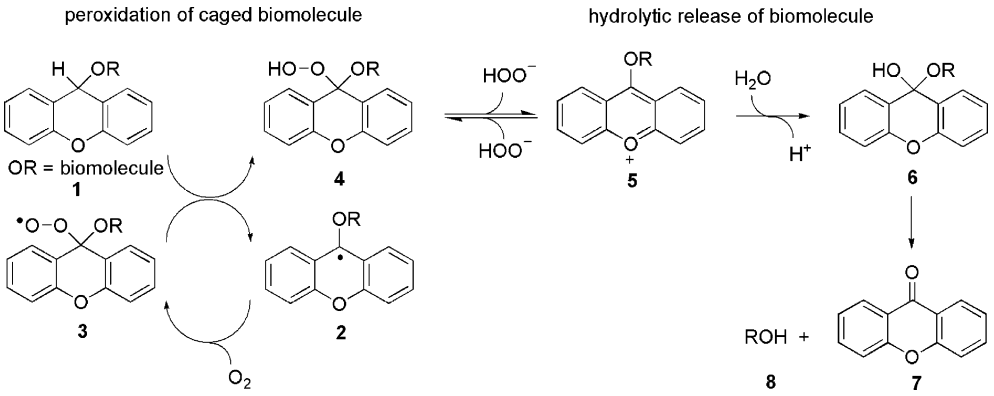
Xanthenyl acetate (Scheme 2) was chosen as a model compound due to the simplicity of the acetyl group compared to a larger, more complex biological molecule. Xanthenyl acetate was prepared by acetylation of xanthenol.[11] A xanthenyl acetate autoxidation study, monitored by ^{1}H NMR, was carried out to investigate its autoxidation reactivity. Xanthenyl acetate has a doublet at 7.6 ppm for the hydrogen *ortho* to the acetyl-substituted methylene bridge (H_a). The equivalent proton on xanthone is shifted downfield to 8.3 ppm (H_b). The appearance of the H_b doublet at 8.3 ppm monitors the production of xanthone. Autoxidation of xanthenyl acetate was carried out over a 4 h period in an NMR tube,[12] with 1:1 deuterated acetone/deuterated chloroform as the solvent[13]



Scheme 2. Competitive autoxidation reaction between xanthenyl acetate and methyl linoleate in an NMR tube carried out for 4 h.

with V-70 radical initiator.[14] The NMR tube reaction was incubated at 37 °C (body temperature), while oxygen was bubbled through a syringe needle into the solution. The characteristic H_b doublet at 8.3 ppm for xanthone appeared early into the reaction. The H_a doublet at 7.6 ppm for xanthenyl acetate disappeared proportionate with the increase of the xanthone H_b doublet at 8.3 ppm. Such experiments clearly established the production of xanthone, demonstrating the release of the caged moiety.

A competitive oxidation study was carried out between xanthenyl acetate and methyl linoleate to determine if the xanthenyl caging group could compete in diverting radicals from the lipid peroxidation reaction of methyl linoleate. Reaction conditions for the competitive study, shown in Scheme 2, were the same as for the autoxidation of xanthenyl acetate. Figure 2 shows the ^{1}H NMR results of one of the competitive oxidation



Scheme 1. Proposed mechanism for oxidatively releasable caging group to release a biomolecule (OR). The caged biomolecule **1** undergoes autoxidation to form the peroxidized caging group **4**, which should undergo hydrolysis to release the active biomolecule **8** (ROH).

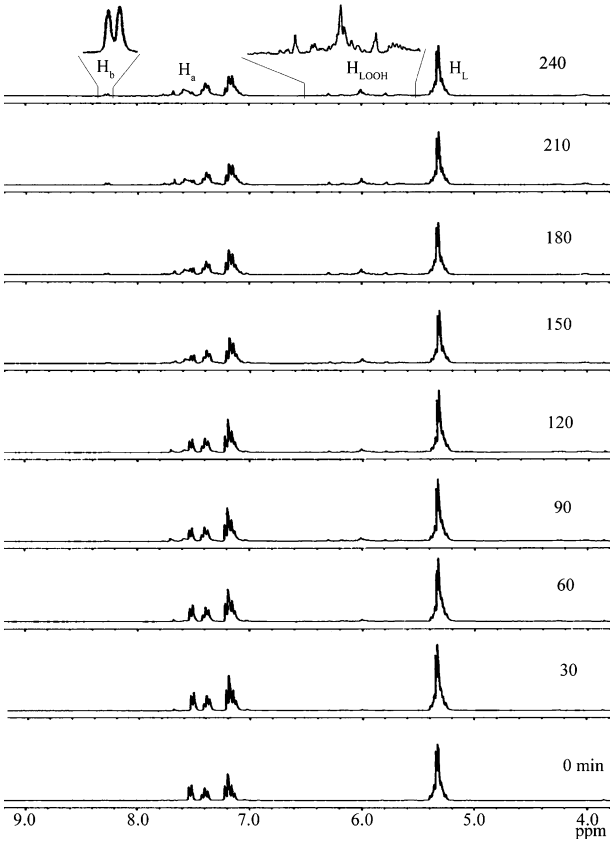


Figure 2. Competitive autoxidation study of xanthenyl acetate and methyl linoleate monitored by ^{1}H NMR. H_a is the proton on xanthenyl acetate, H_b is the proton on xanthone, H_L are the alkene protons on methyl linoleate, and H_{LOOH} are the conjugated alkene protons on peroxidized methyl linoleate.

studies. The alkene protons on the lipid (H_L) appear around 5.3 ppm. As autoxidation time progresses, new peaks appear downfield in the region of 5.5–6.7 ppm, for the conjugated alkene protons on the peroxidized lipid (H_{LOOH}). The doublet at 8.3 ppm (H_b) also appears as time progresses, signifying the oxidation of xanthenyl acetate (H_a) to produce xanthone (H_b). The reaction was only carried out to about 10% completion to focus on the formation of the primary reaction products. If the reaction was carried out for a longer time period, secondary reaction products from the decomposition of lipid peroxides might begin to be formed, such as well known fragmentation, radical cyclization, or epoxide rearrangement reactions, complicating the analysis of the reaction.[15]

To determine which molecule is more reactive, the relative reactivity was calculated comparing the reactive C–H bond in xanthenyl acetate with the reactive C–H bond in the methyl linoleate lipid model (based on NMR integrals and adjusted for such factors as the number of hydrogen on each molecule and the moles of each reactant). It was found that the reactive C–H bond of xanthenyl acetate was 1.93 ± 0.55 times more reactive[16] than the most reactive C–H bond of the methyl linoleate lipid model.

The method described here cages molecules with OH functional groups. The oxygen attachment to the xanthenyl group weakens the adjacent C–H bond in the xanthenyl group to enhance its reactivity in radical peroxidation reactions. Many interesting biomolecules that could be caged have OH groups and have their activity blocked by caging of the OH functional group, for example, the antioxidants vitamin C and vitamin E. A common fluorescent probe, fluorescein, also contains the OH group.

Setsukinai et al.[17] recently developed a novel fluorescent probe for reactive oxygen species (ROS). Their caged fluorescein has an aryl group attached to the OH on fluorescein to block its fluorescence. Reaction of the aryl group with ROS leads to release of free fluorescein in its fluorescent state. The release of their probe is through oxidative O-dearylation, a different mechanism than our proposed mechanism. To the best of our knowledge, the work of Setsukinai and co-workers is the only approach other than ours to ROS-sensitive caging.

In summary, we have demonstrated successful release of a simple model biomolecule, an acetate group, using an oxidatively releasable caging group. This type of caging/uncaging system could be used in numerous ways such as the release of a drug in sites of high oxidative activity or to replenish essential molecules in cells, such as antioxidants, that are consumed in radical reactions. This system could also be used to release a fluorophore to monitor sites of oxidative stress.

Acknowledgments

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