

Sunrise Free Radical School
Society for Free Radical Biology and Medicine

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How to Measure ROS and RNS in Biology

Some generality and a singlet molecular oxygen case

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Reactive species have, in generally, a very short half-life, are produced in low amounts. Multiple methods of measurement are available today, each with their own benefits and limits.

Reactive Species measurement methods must be:

- Very, very sensitive**
 - Highly selective**
 - Fast**
 - In situ**
-

How to Measure Reactive Species?

Direct

Electron paramagnetic resonance, EPR (free radical detection)
+Trapping (Spin-traps, “chemical”)

Indirect (but specific)

Mass spectrometry, MS/MS (ESI, MALDI)

+Scavengers

Probes (*spectroscopic investigation*)

Luminescence (direct and indirect)

Combination of Different Techniques !

Electron Paramagnetic Resonance (EPR)

We can detect & measure free radicals and paramagnetic species

- “*High*” sensitivity (nanomolar concentrations)

Direct detection

e.g.: semiquinones, nitroxides, thiyl, ROO[·]...

Indirect detection

Spin-trapping

Species: superoxide, hydroxyl, alkyl, NO

Spin-traps: DMPO, PBN, DEPMPO, Fe-DTCs

We can use EPR to measure free radicals from biological systems
(*in vivo* or *ex vivo*)

Intact tissues, organs ... can be measured.

Other Trapping methods

Hydroxyl radical OH[·] [Trapping methods (without EPR)]

-Reaction with aromatic compounds (Phenylalanine 'Tyrosine', Salicylate, ...)

-Attack of OH[·] on 2-deoxyribose produces a range of products...malondialdehyde

Superoxide O₂^{·-}

- [Fluorescent probes] Dihydroethidium (DHE) conversion to 2-hydroxyethidium and lucigenin =

- Ability to reduce cytochrome c or nitroblue tetrazolium
- Superoxide electrodes
- Histochemical detection: Conversion of diaminobenzidine (DAB) to an insoluble product

Peroxynitrite and other nitrogen speciesNitric oxide NO[·]

-NO[·]: -Light emission in the presence of O₃ (excited NO₂[·])

- NO electrodes (porphyrinic sensors)
- Haemoglobin trapping (ΔA is measured)
- Spin trapping (Haemoglobin, other haem proteins, ...)
- 4,5-Diaminofluorescein diacetate (DAF-2-diacetate), fluorescent product
- Diaminoanthroquinone, red fluorescent product
- “indirect methods”: NO₂[·] measurement, use NOS inhibitors, ...

Nitration assays

ONOO⁻ : ONOO⁻ nitrates many aromatic compounds: tyrosine (**3-nitrotyrosine**), tryptophan, phenylalanine

Reactive halogen species

HOCl / HOBr

- [taurine assay] Conversion of taurine to a chloramine
- bromo- and chlorotyrosine ?

Hydrogen peroxide H₂O₂

- [fluorescent products] Amplex red and DCF-DA
- [non fluorescent products] Fluorescent compound scopoletin
- Inactivation of catalase by aminotriazole....in cells...

Singlet oxygen ${}^1\text{O}_2$ O_2 (${}^1\Delta_g$) in biological system

- Monomol and dimol Light emission
- Use of scavengers (azide), traps (histidine, anthracene derivatives) and D₂O effect

Studies using low-level / ultraweak / dark chemiluminescence

Many methods are available to identify Reactive Species in cultured cells

- 1- Cell culture process itself causes oxidative stress
- 2-Trypsinization increases ROS
- 3- Artifacts are produced

-Dichlorofluorescein diacetate (DCF-DA) is deacetylated by esterases to dichlorofluorescein (DCFH) which can be visualized by fluorescence at 525 nm

More specific for H₂O₂?

- Dihydrorhodamine 123 (DHR) is used to detect several reactive species
Conversion to rhodamine 123, highly fluorescent 536 nm
- Luminol and lucigenin

“Biomarkers”

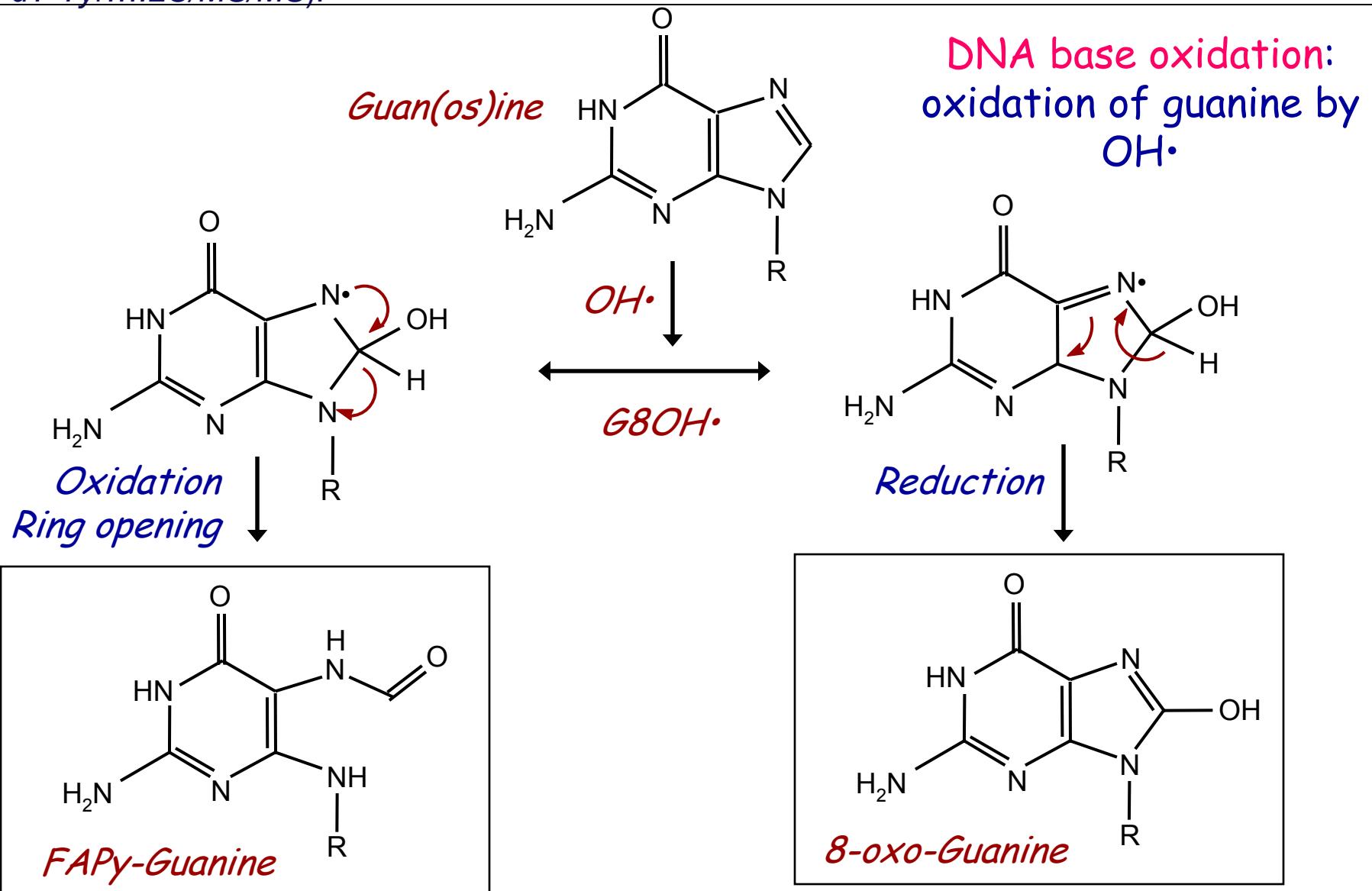
- Of oxidative DNA damage
- Of lipid peroxidation
- Of protein damage

by reactive species

- Single and double strand breaks (Comet assay)
- Oxidative modification of DNA-bases (e.g. 8-oxo-dGuo... LC/MS/MS) Of oxidative DNA damage
- Formation of DNA adducts with oxidized lipids or proteins (e.g. dGuo-MDA, dT-Tyr...LC/MS/MS).

Biomarkers

Of oxidative DNA damage

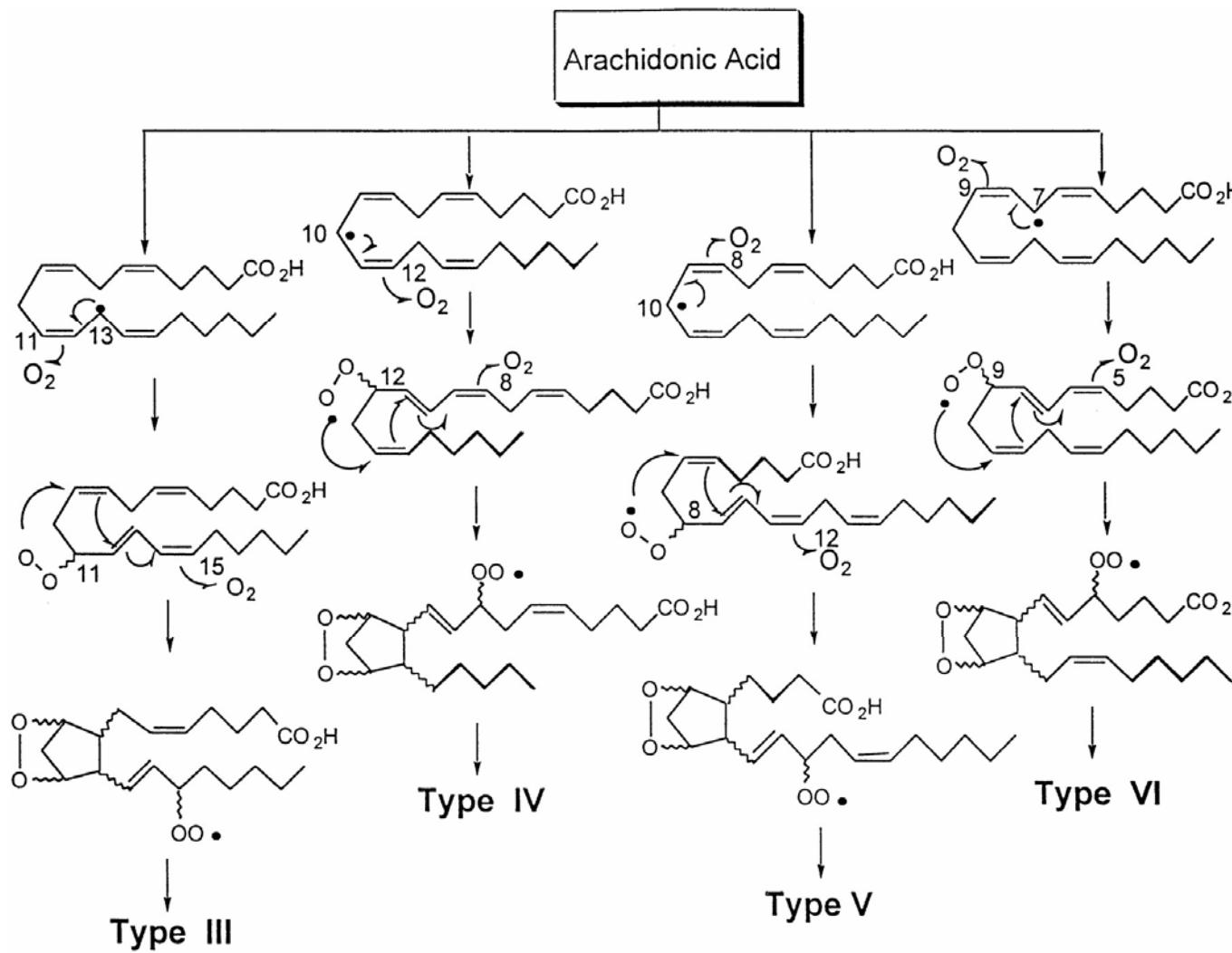


Arachidonic acid oxidation products:

"Biomarkers"

Of lipid peroxidation

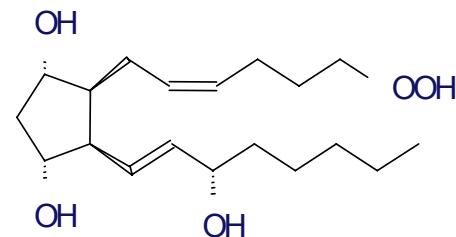
Isoprostanes: Relatively stable endproducts that are currently viewed as most reliable marker of lipid oxidation *in vivo*



Isoprostanes comprise four regioisomers with eight stereoisomers.
in vivo (Lawson et al., J. Biol. Chem. 1999: 274, 2444-24444).

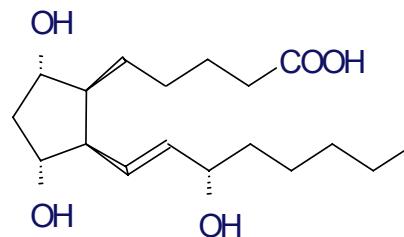
"Biomarkers"
Of lipid peroxidation

Metabolic Fate of 15-F_{2t}-IsoP (8-Iso-PGF_{2α}) in Humans



15-F_{2t}-IsoP

↓
 β -oxidation Δ^5 -reduction



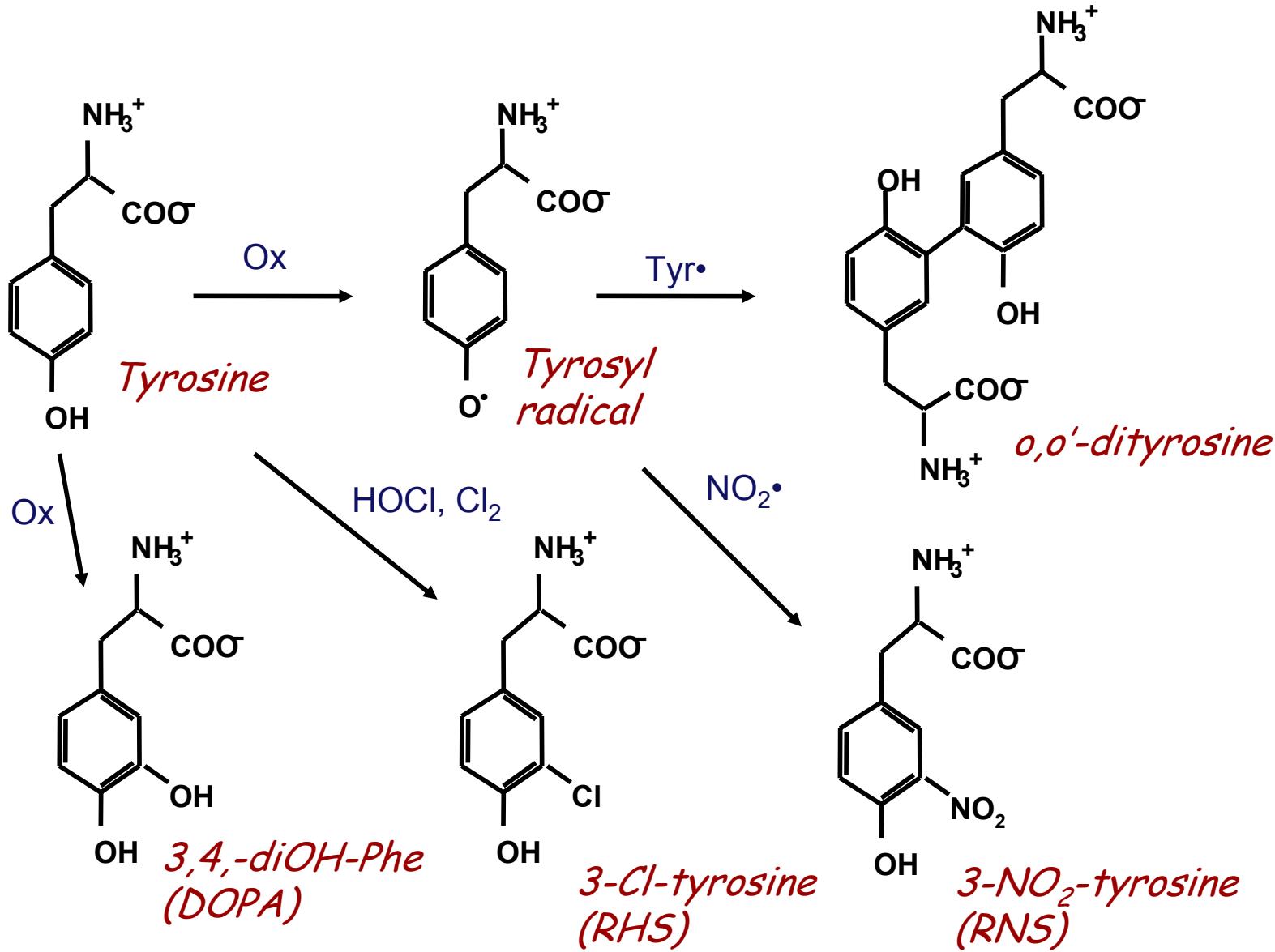
Major urinary metabolite

A GC/MS assay for F₂-IsoP-M has been developed

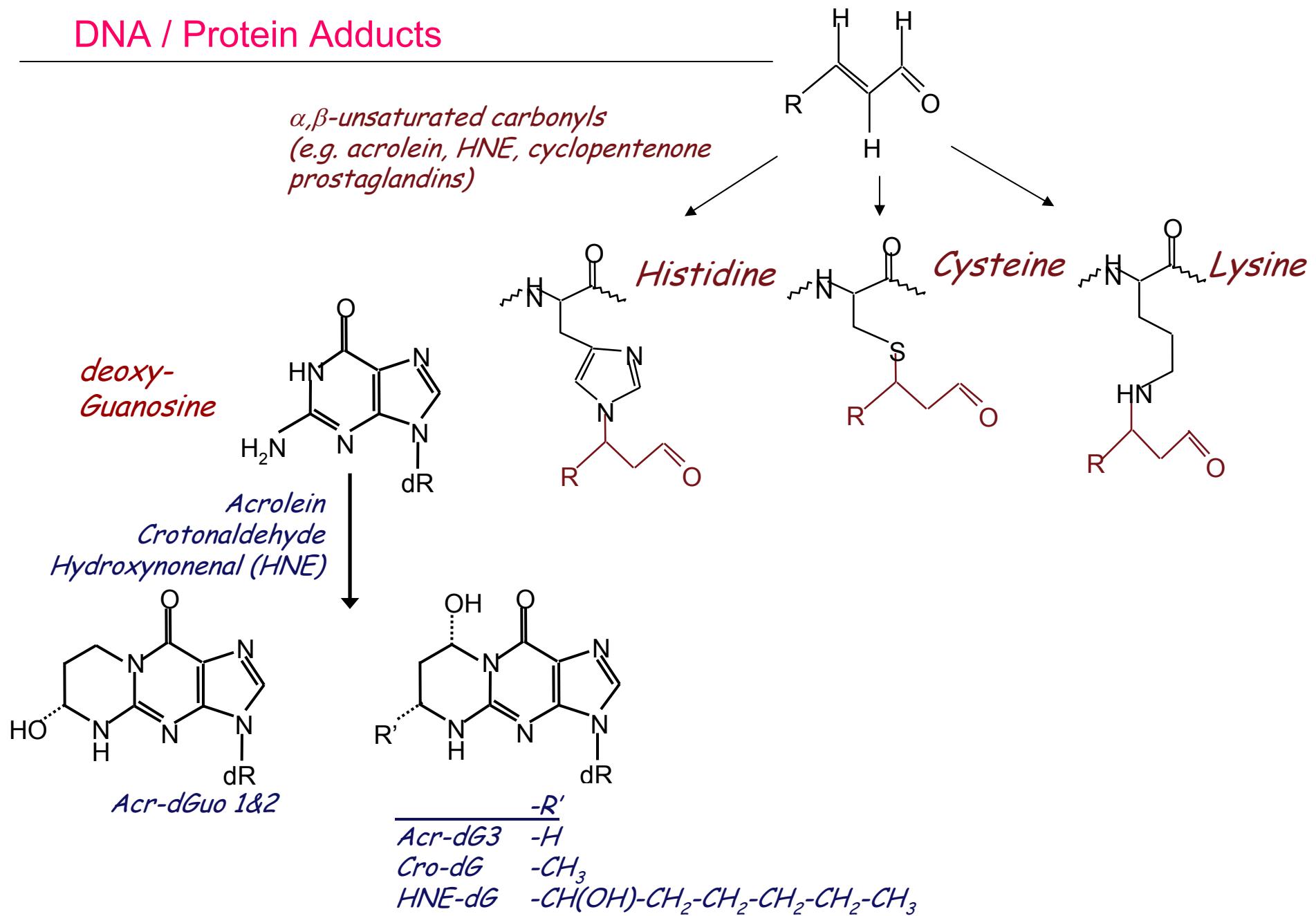
2,3-Dinor-5,6-Dihydro-15-F_{2t}-IsoP
(F₂-IsoP-M)

Stable oxidation markers:
Tyrosine oxidation, chlorination, and nitration

"Biomarkers"
Of protein damage



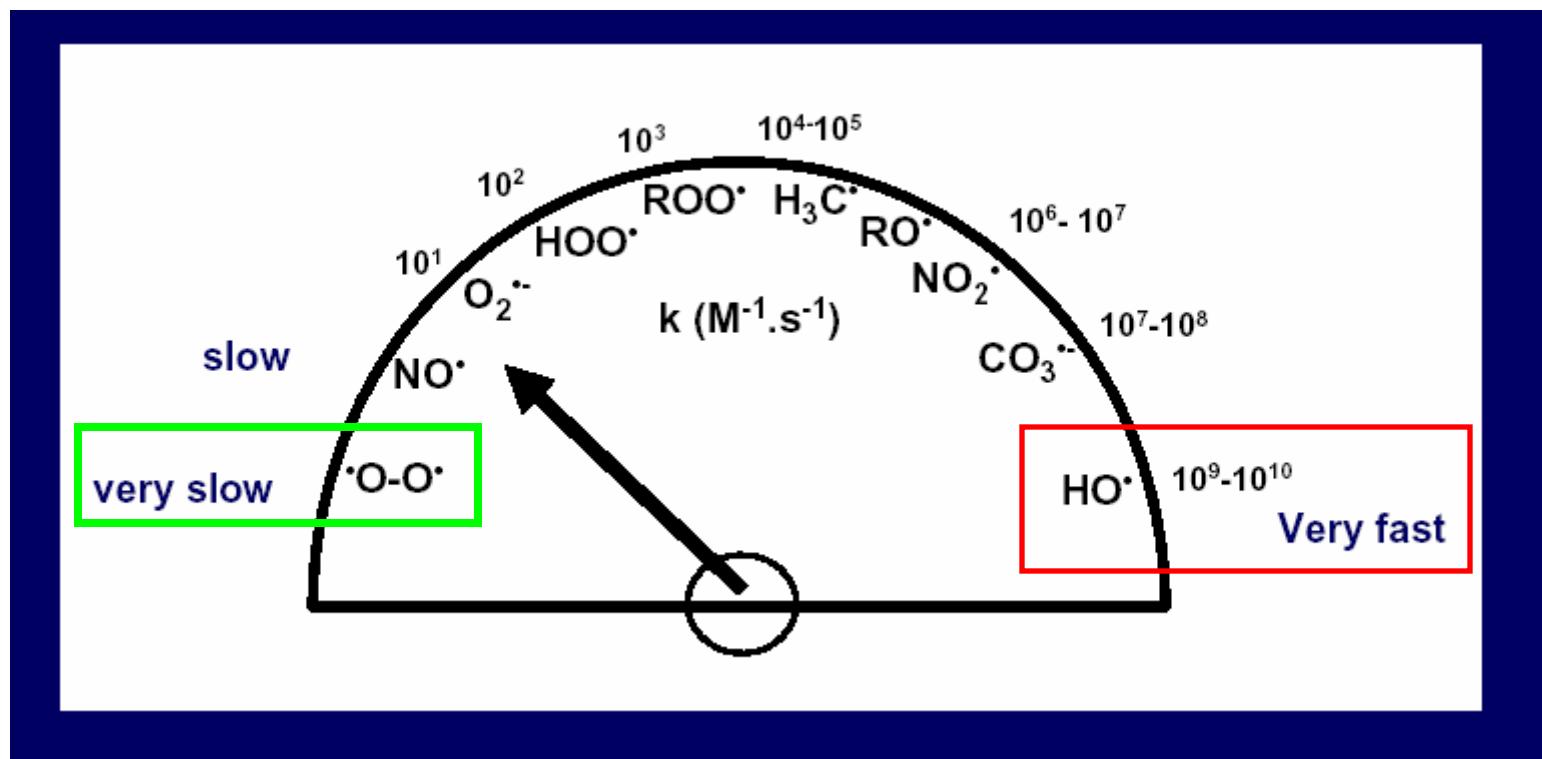
DNA / Protein Adducts



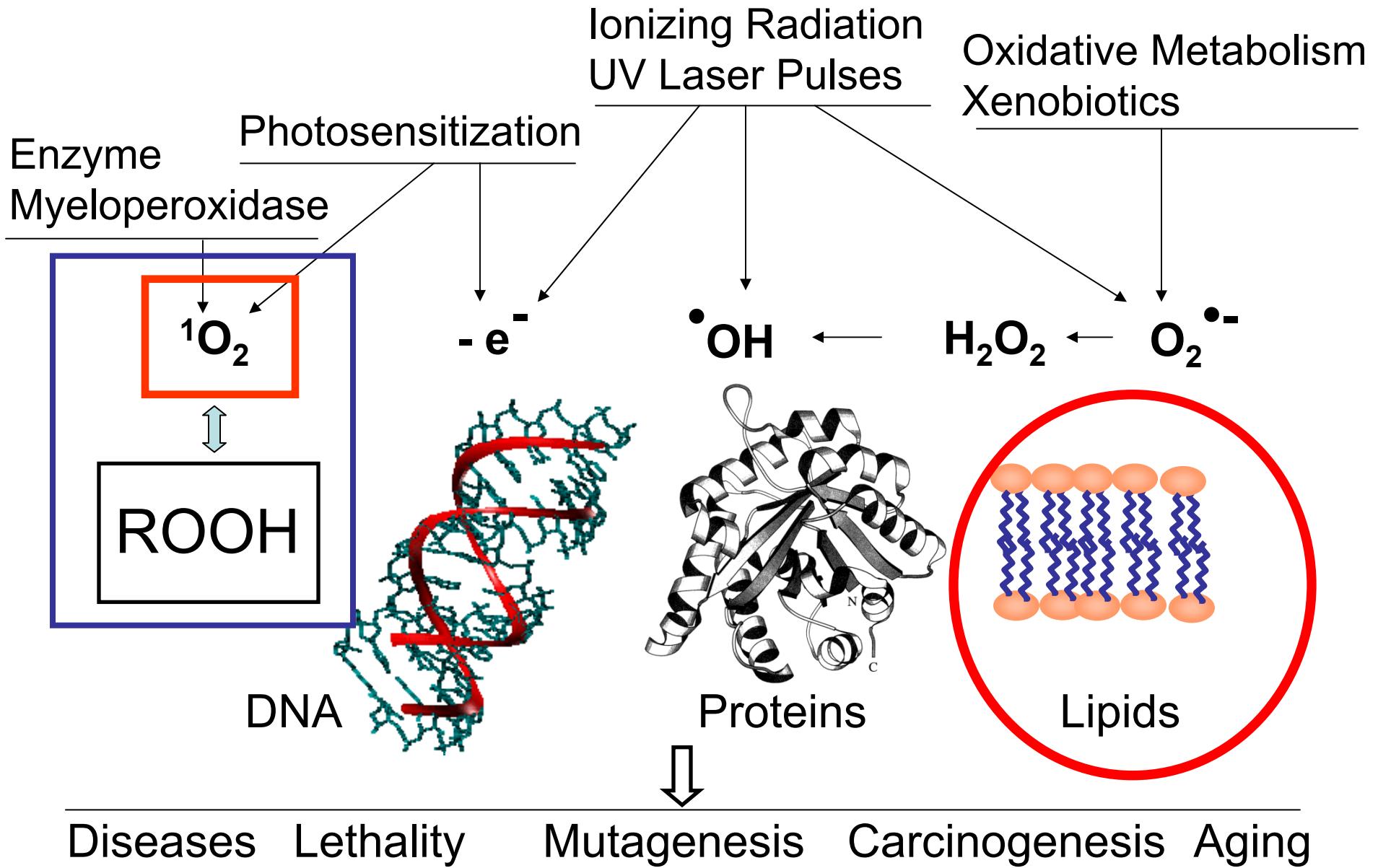
Half-life of some reactive species

Reactive species	Half-life (s)	Physiol conc. (mol/l)
Hydroxyl radical ($\cdot\text{OH}$)	10^{-9}	
Alcoxyl radical ($\text{RO}\cdot$)	10^{-6}	
Singlet oxygen ($^1\text{O}_2$)	10^{-5}	
Peroxynitrite anion (ONOO^-)	0.05 – 1.0	
Peroxyl radical ($\text{ROO}\cdot$)	7	
Nitric oxide ($\cdot\text{NO}$)	1 - 10	10^{-9}
Semiquinone radical	minutes/hours	
Hydrogen peroxide (H_2O_2)	spontan. hours/days (accelerated by enzymes)	$10^{-9} - 10^{-7}$
Superoxide anion ($\text{O}_2\cdot^-$)	spontan. hours/days (by SOD accel. to 10^{-6})	$10^{-12} - 10^{-11}$
Hypochlorous acid (HOCl)	dep. on substrate	

Free Radical Reaction Rate

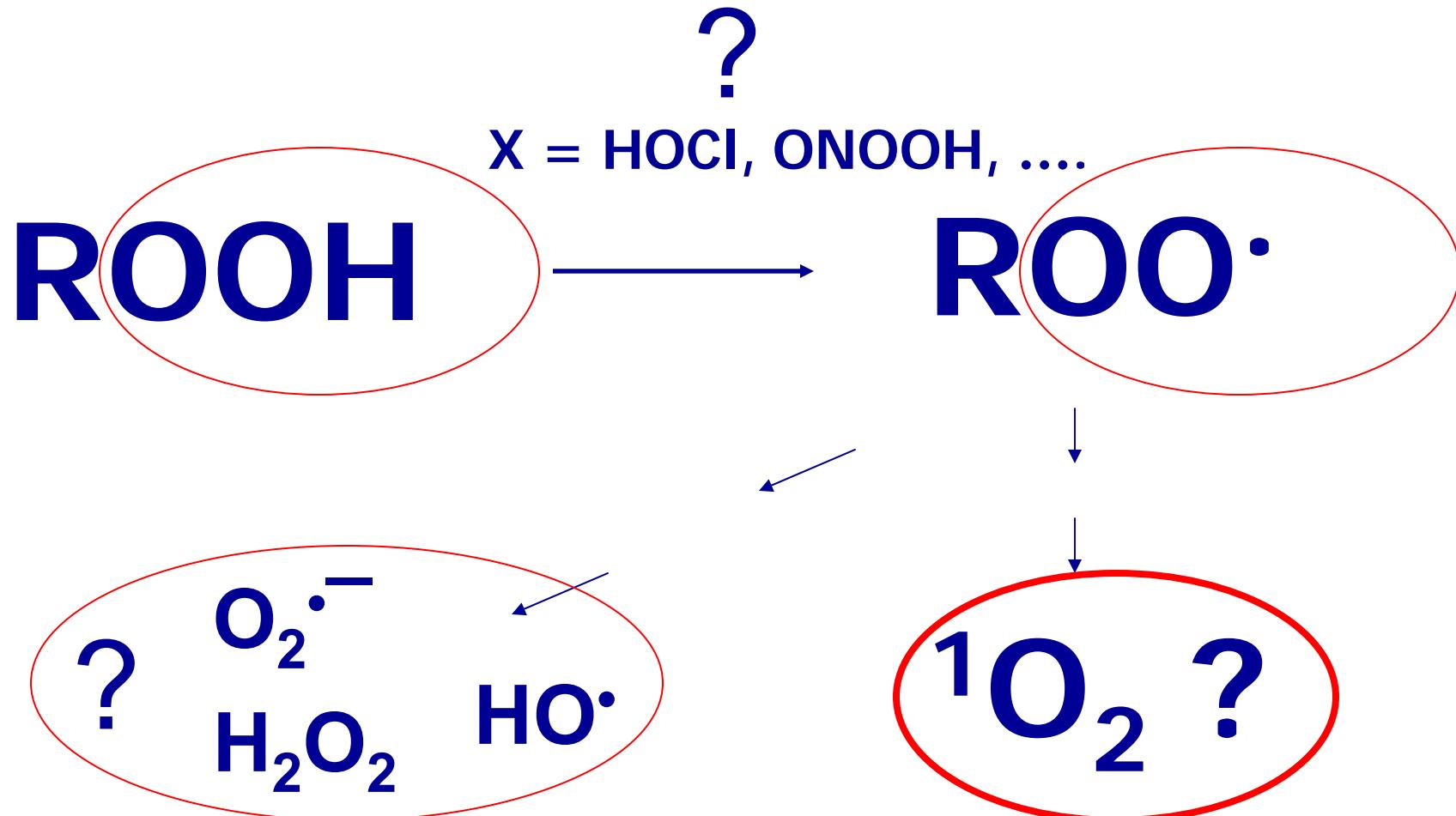


ROS generated by physical or chemical processes can damage biomolecules.
-difficulties and how to measure ROS with the singlet oxygen case



Singlet Molecular Oxygen in biological systems as an example

What's the Problem?



Singlet Molecular Oxygen in biological systems as an example

Direct

Luminescence (Light emission) +Quenchers (N_3^-), Solvent effect (Deuterated solvent, D_2O)

Indirect but specific

Isotopically labeled oxygen

Chemical trapping and HPLC-mass spectrometry

(^{18}O -labeled compounds)

EPR

Synthesis / Characterization (LAOOH, PCOOH)

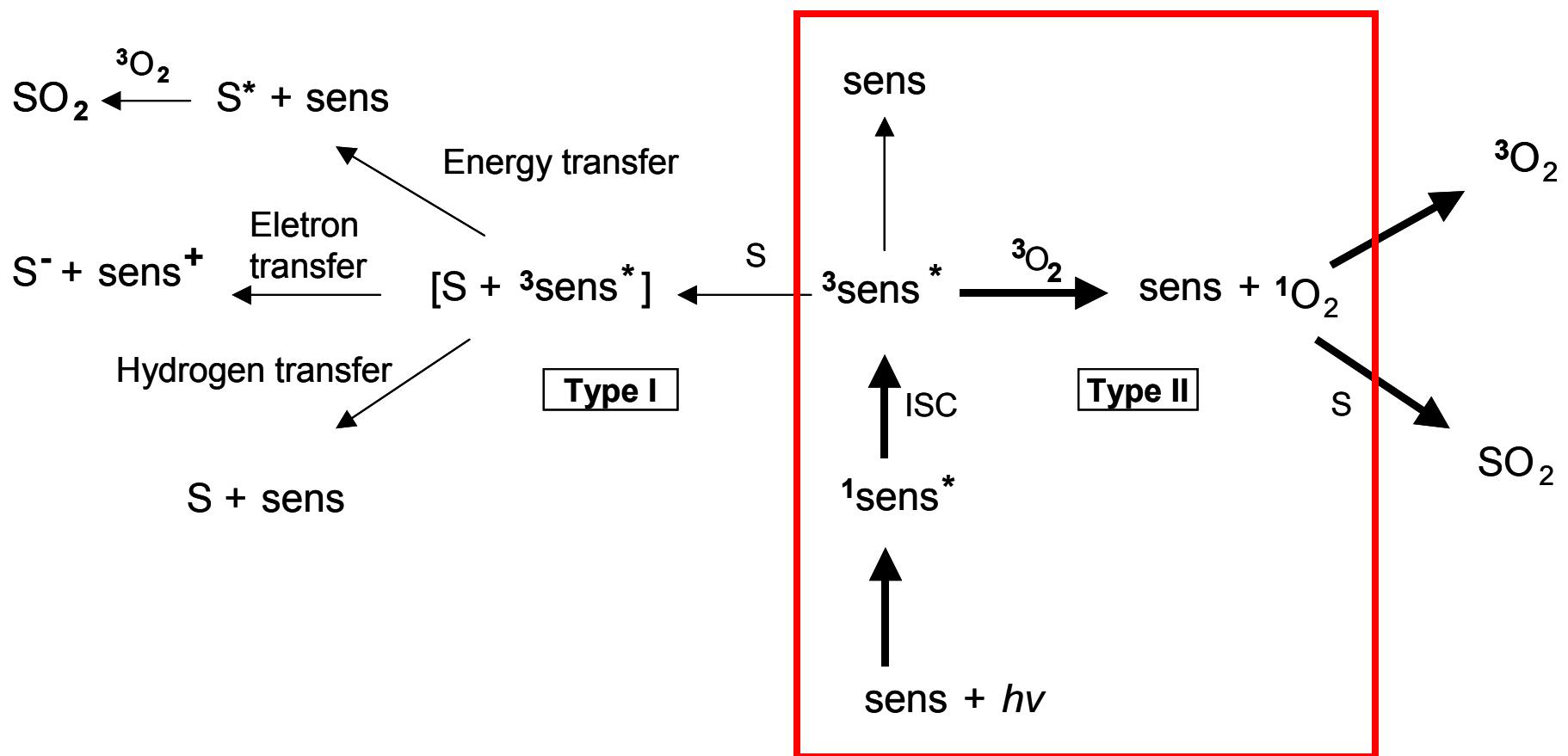
^{18}O -Labeled Compounds

Chemical source of $^{18}[^1O_2]$

**-Development of a pure source of isotopically labeled singlet oxygen,
 $^{18}[^1O_2]$**

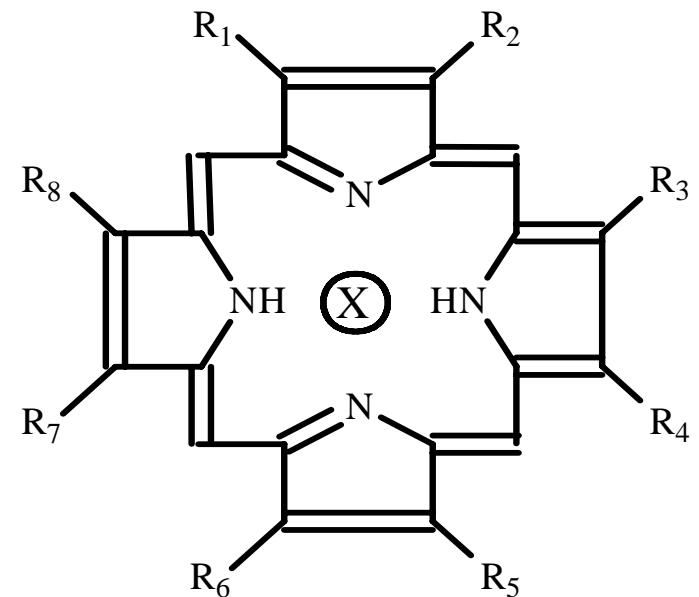
Tool for mechanistic studies !

Photosensitized generation of singlet O₂



Porphyria Patient

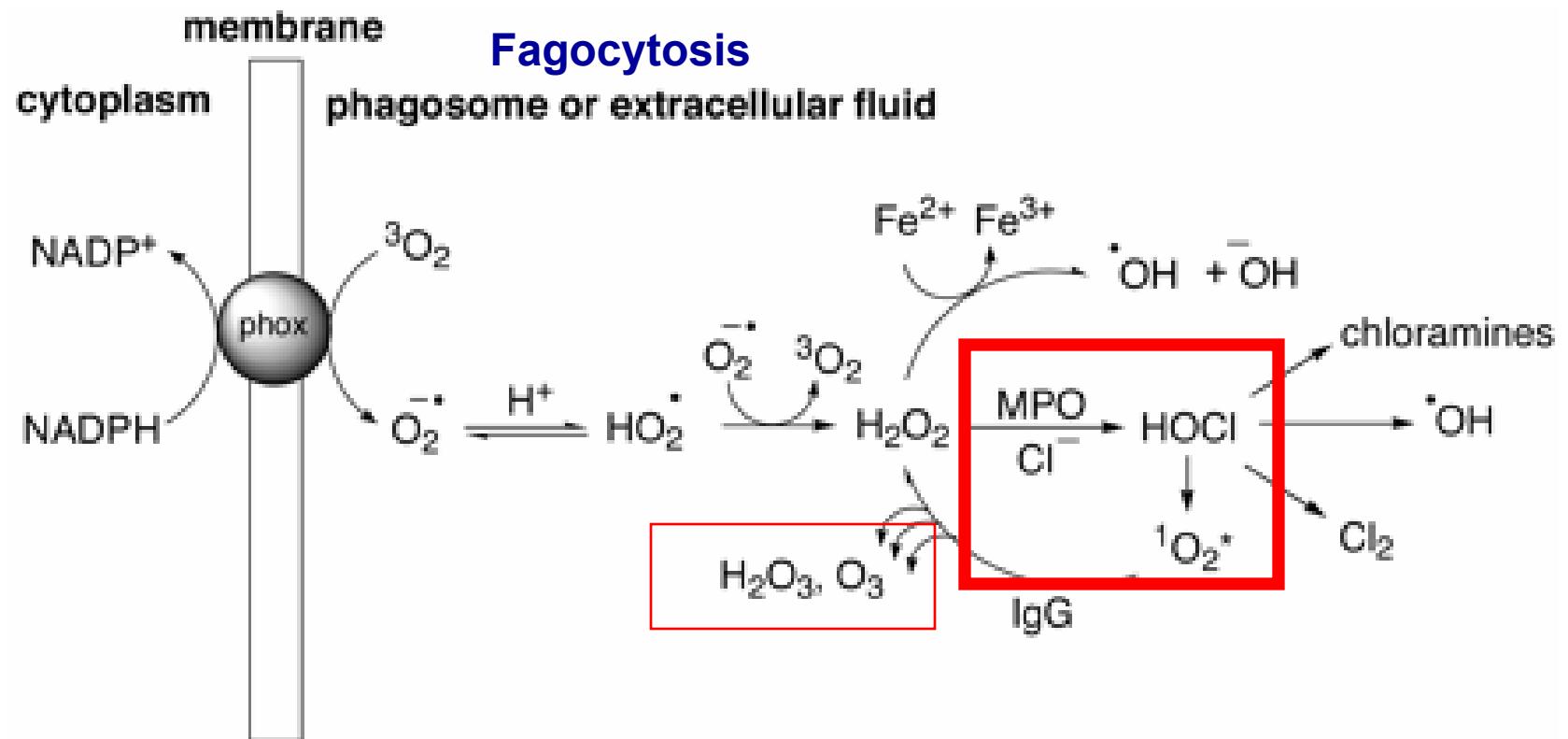
Porphyria is a disease in which pigments called porphyrins accumulate in the skin, bones and teeth.



Porphyrin Nucleus

Activated leukocytes

Bacterial killing: Respiratory burst



Steinbeck *et al.* (1992) *J. Biol. Chem.*, 267, 13425

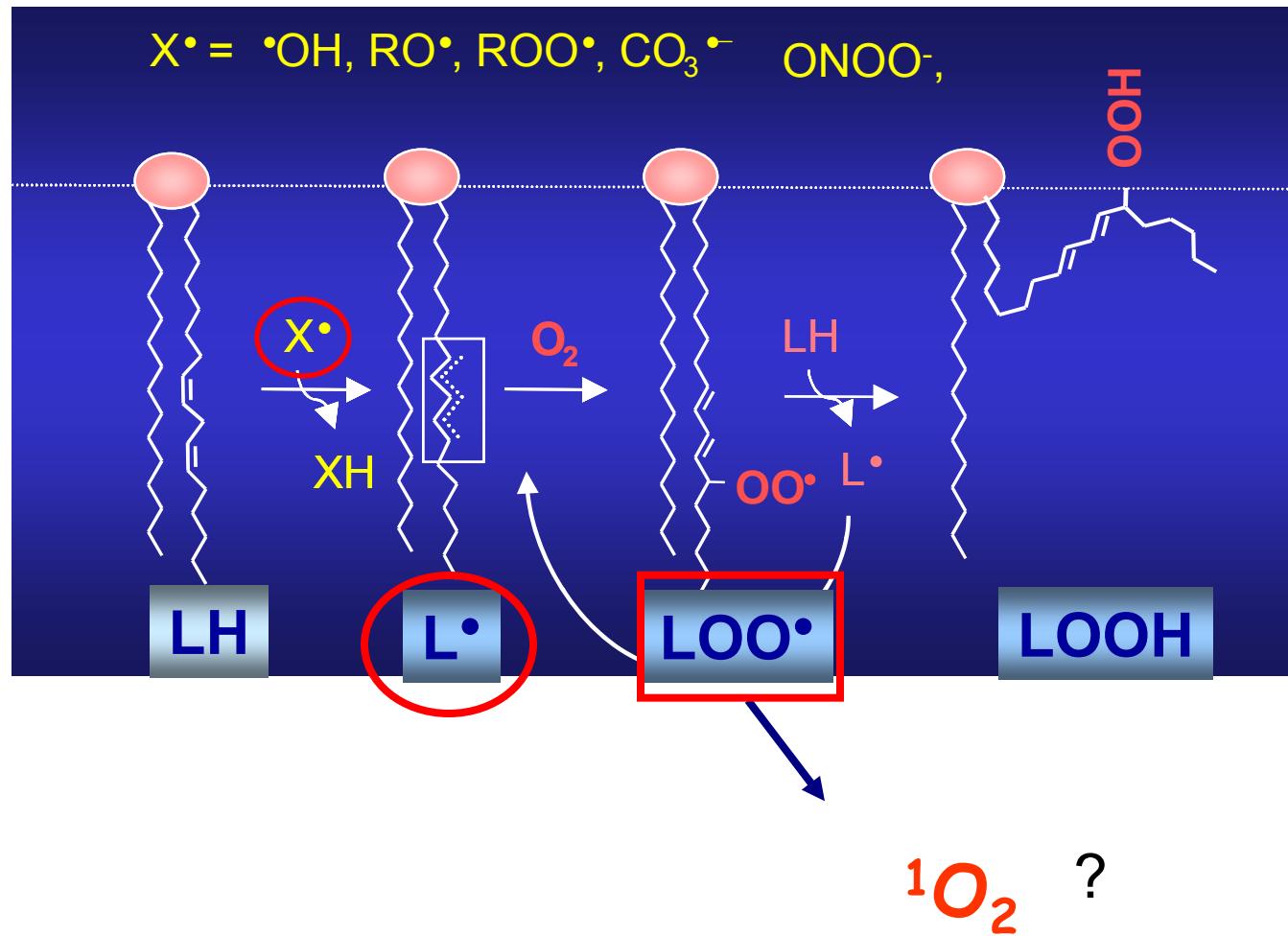
Wentworth *et al.* (2002) *Science*, 298, 2195.

Babior *et al.* (2003) *Proc. Natl. Acad. Sci. USA*, 100, 3031.

Lipid peroxidation

Russell Mechanism?

Is singlet oxygen generated from lipid hydroperoxides?



Generation of Singlet Oxygen by the “Russell Mechanism”

1957

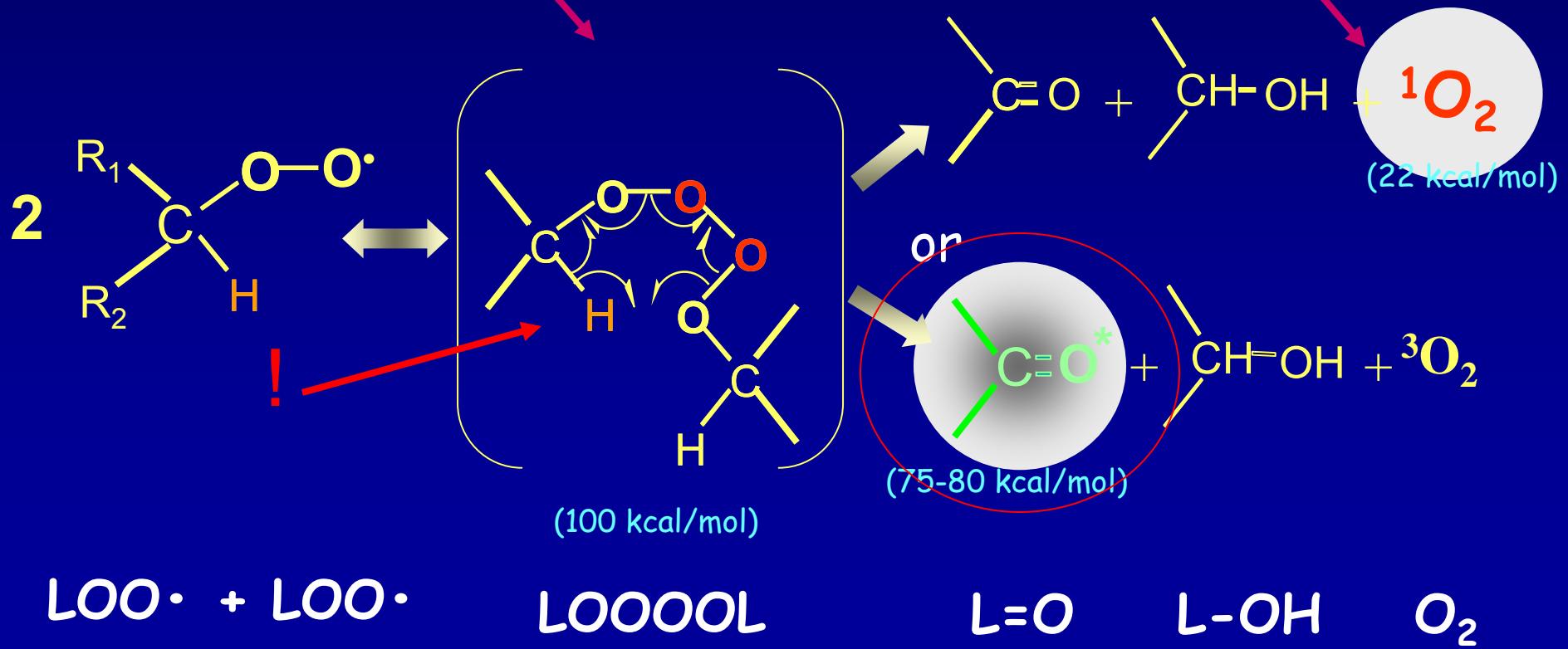
Russell proposed that termination reaction of 2 peroxy radicals involves a tetraoxide intermediary state.

Russel J. Am. Chem. Soc., 79, 3871, 1957

1968

Howard & Ingold showed the formation of singlet oxygen in the reaction of sec-butylhydroperoxide with Ce^{4+} .

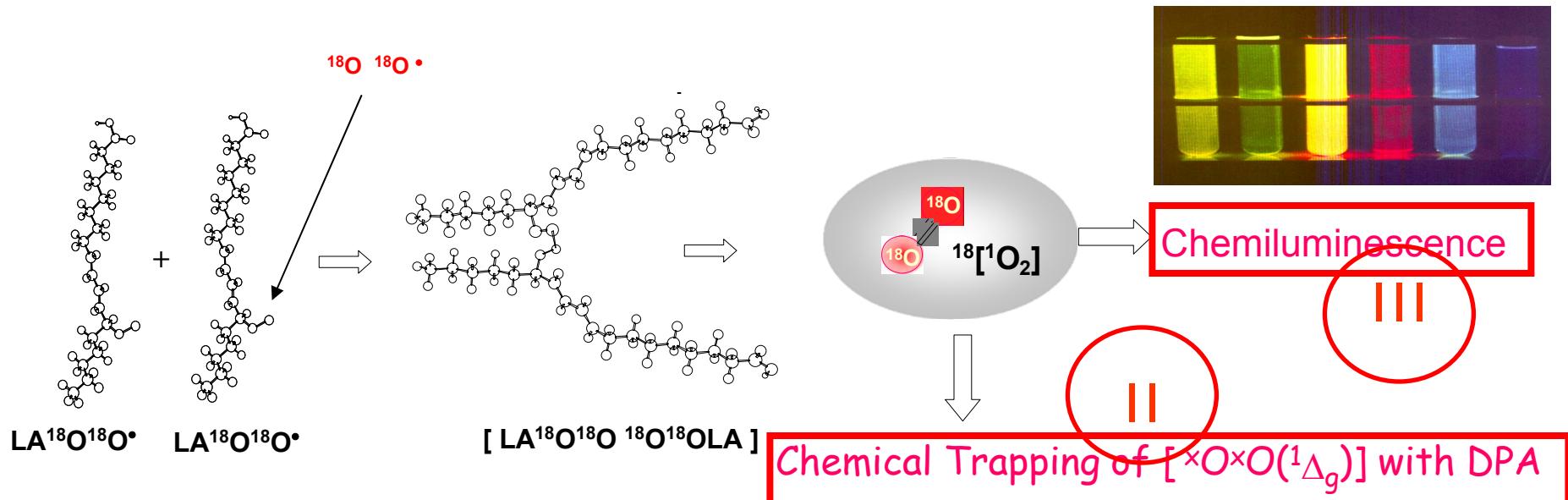
Howard & Ingold J. Am. Chem. Soc., 90, 1057, 1968



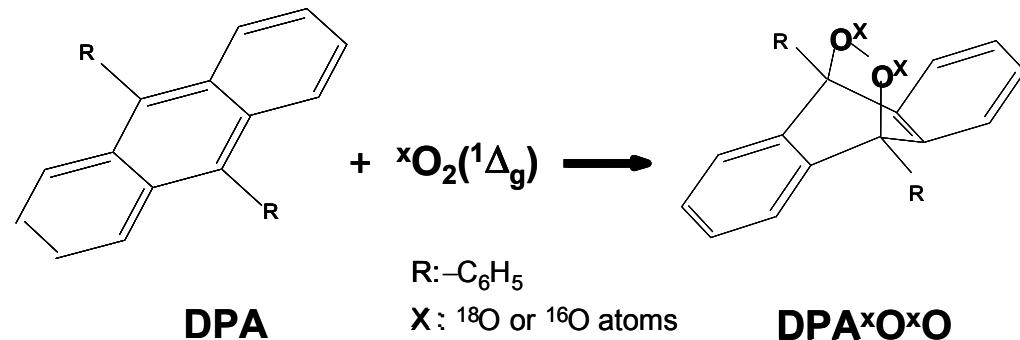
Experimental Strategy

I- Synthesis of LA¹⁸O¹⁸OH

Incubation: LA¹⁸O¹⁸OH + Metal ion (Cerium, Iron, peroxy nitrite) → LA¹⁸O¹⁸O[•]



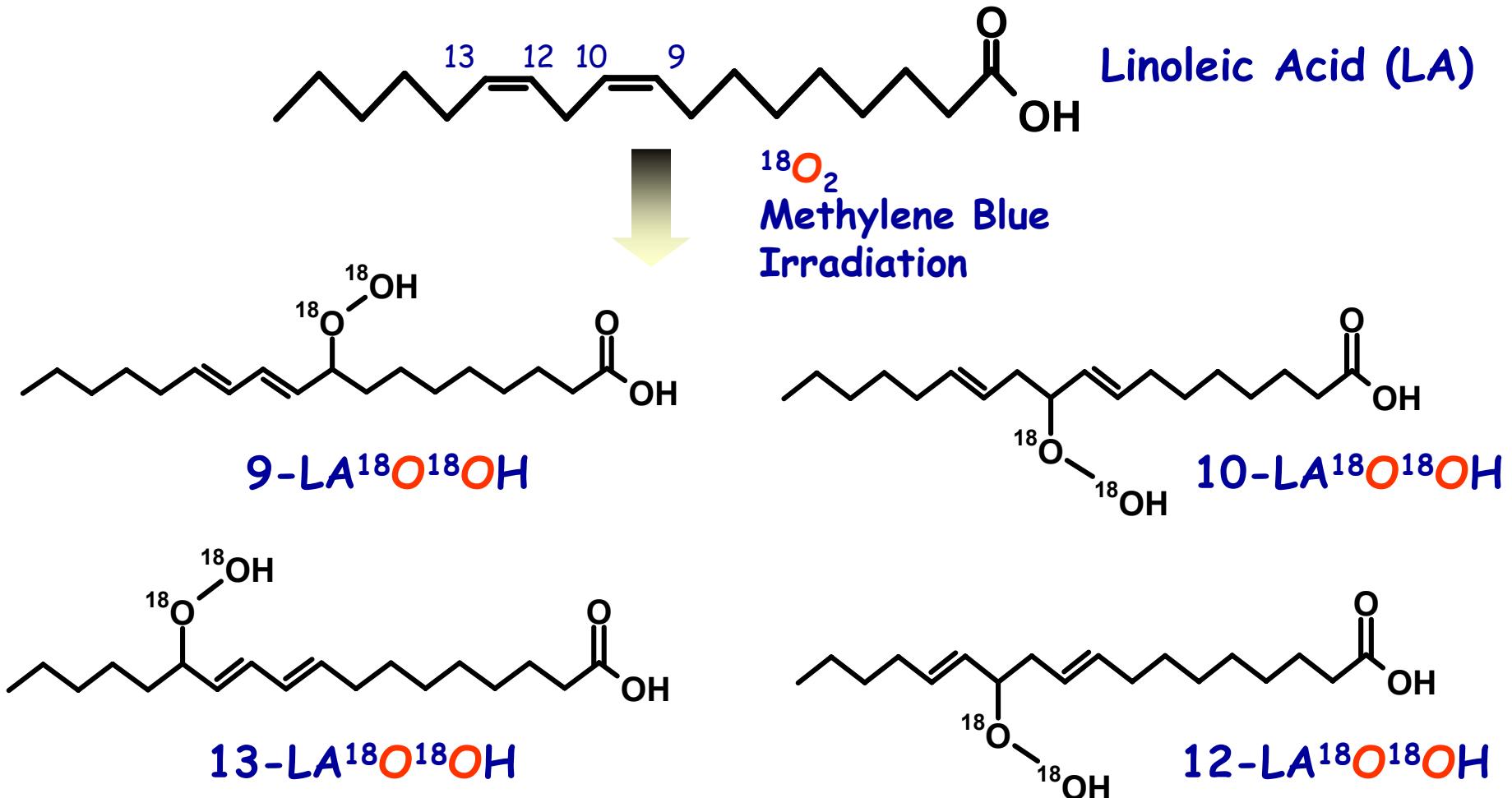
IV- EPR



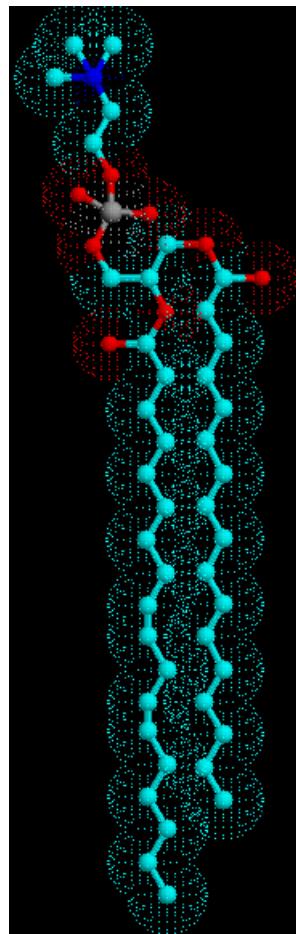
Synthesis of ^{18}O -Labelled Linoleic Acid Hydroperoxide ($\text{LA}^{18}\text{O}^{18}\text{OH}$)

Part I

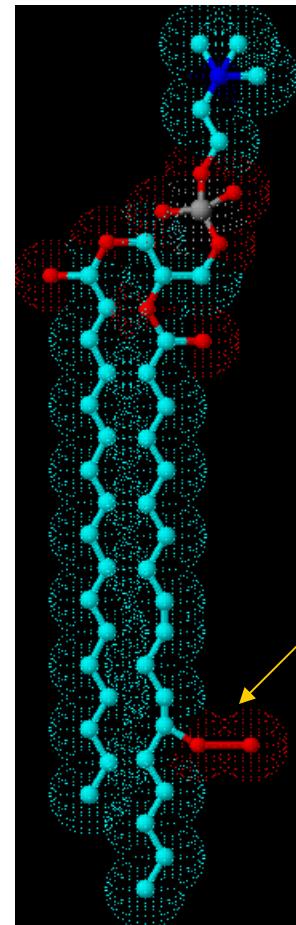
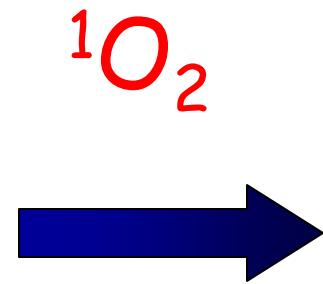
Structures of the 4 LAOOH isomers generated from linoleic acid photooxidation under $^{18}\text{O}_2$ atmosphere



Synthesis of Phosphatidylcholine Hydroperoxides by Photooxidation using Methylene Blue as a Photosensitizer

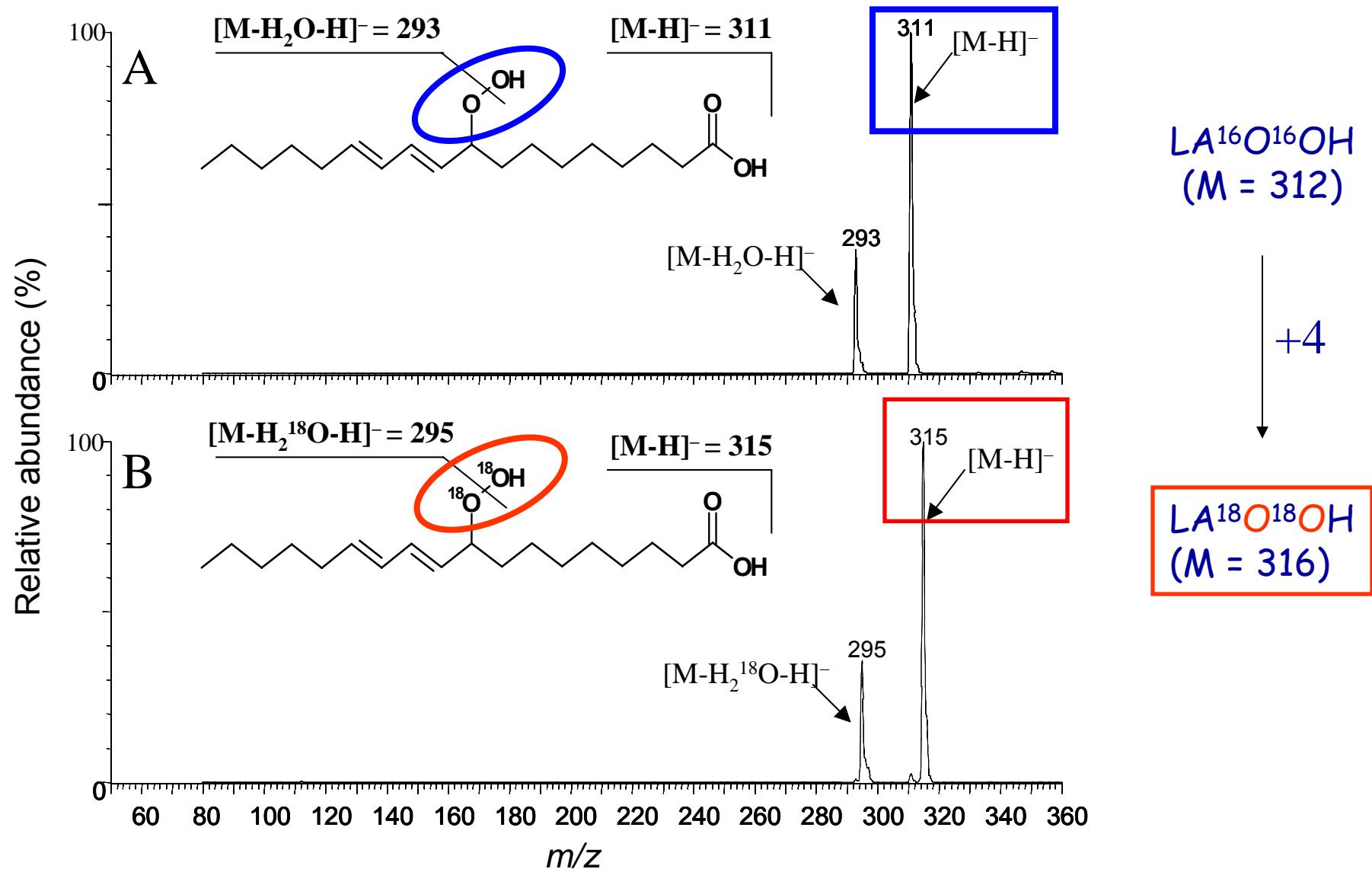


Phosphatidylcholine
(PC)

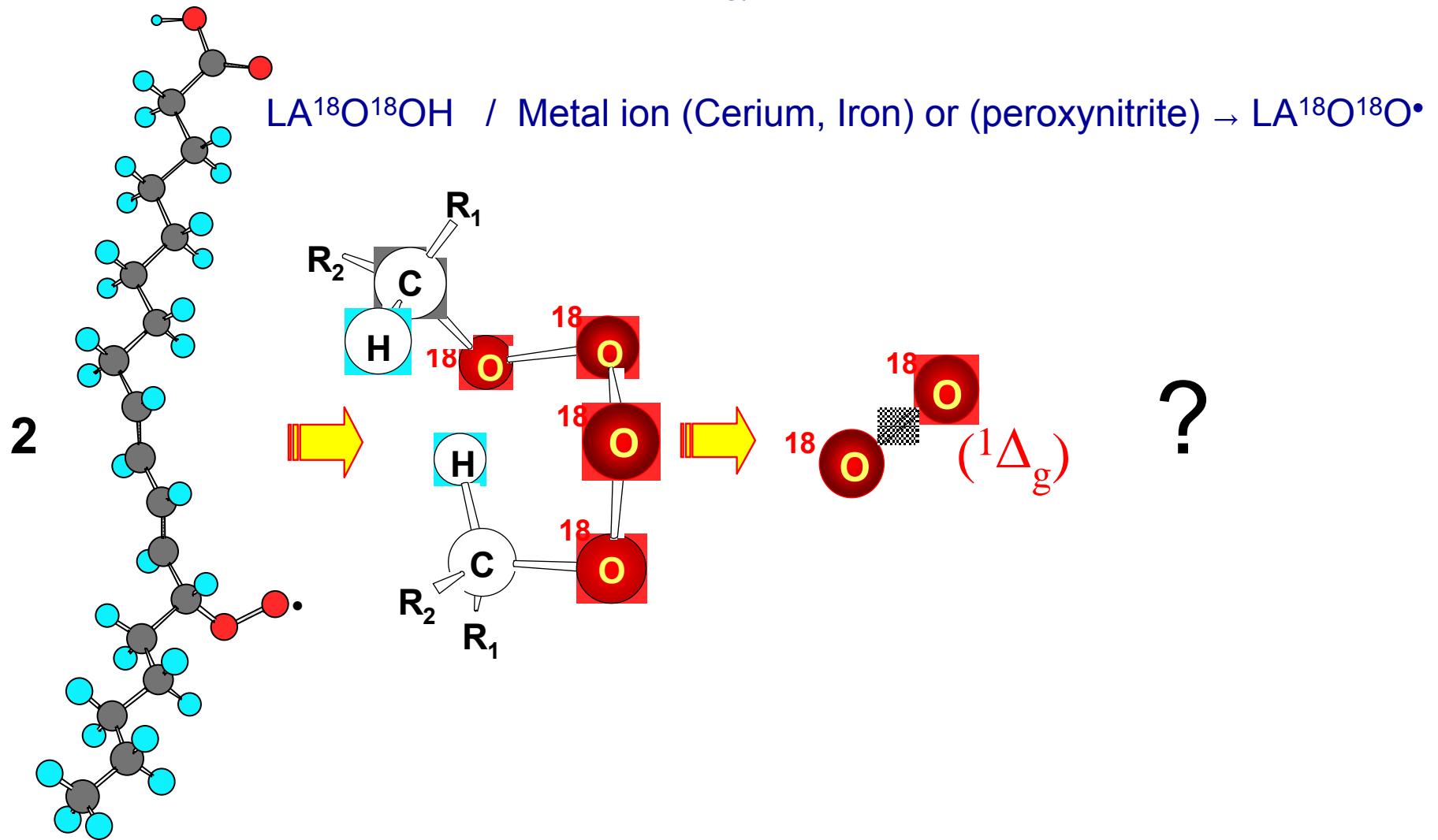


Phosphatidylcholine Hydroperoxides
(PCOOH)

Electrospray ionization mass spectra of LAOOH and $\text{LA}^{18}\text{O}^{18}\text{OH}$ obtained in the negative ion mode.



Generation of [$^{18}(^1O_2)$] from LA $^{18}O^{18}O^\bullet$



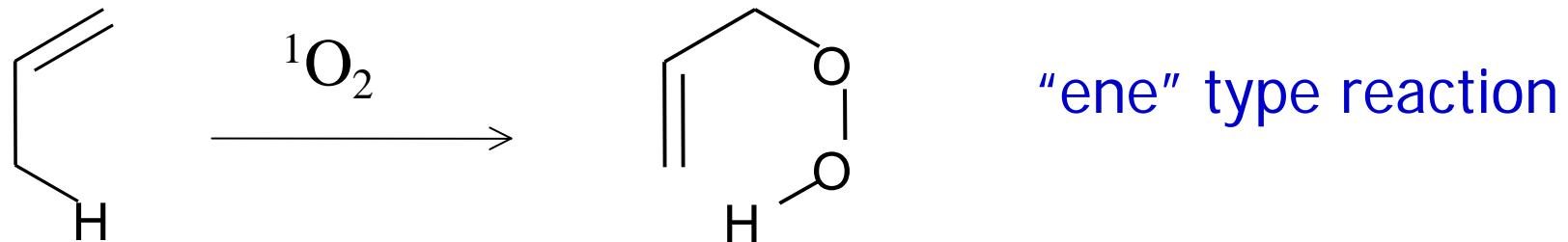
Part II

Detection and quantification of ${}^1\text{O}_2$ by chemical trapping with **9,10-diphenylanthracene (DPA)**

Mass spectrometry

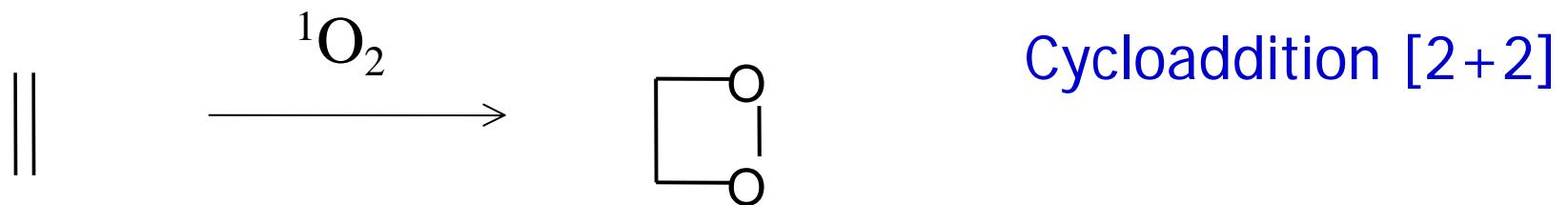
HPLC/MS-MS

(${}^{18}\text{O}$ -labeled compounds)

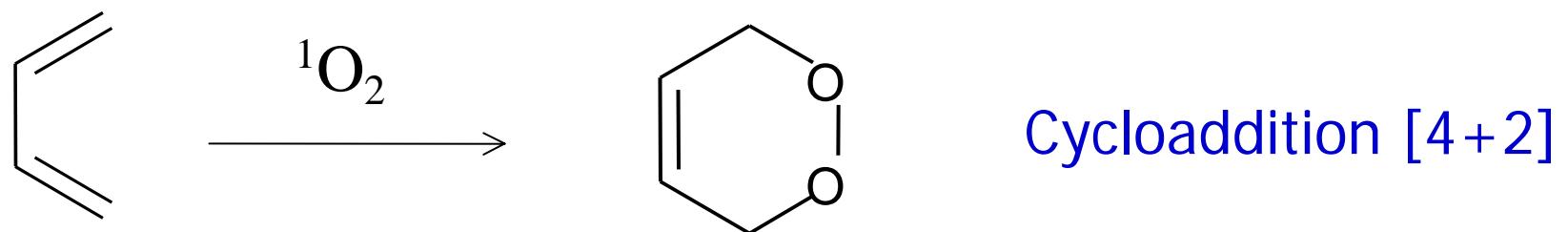


"ene" type reaction

Cycloaddition of Singlet Oxygen



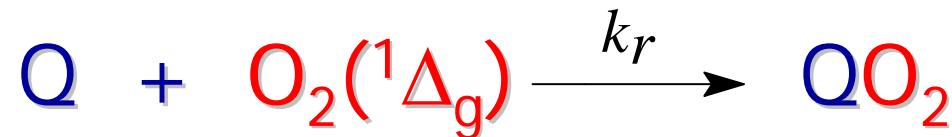
Cycloaddition $[2+2]$



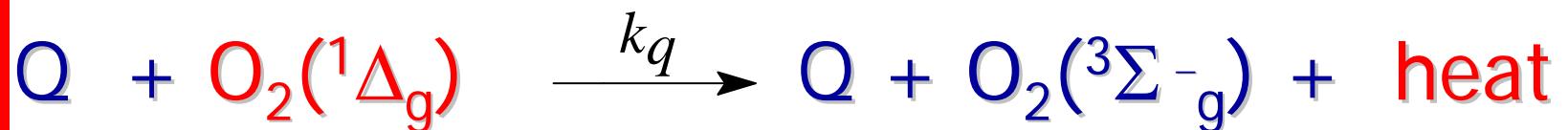
Cycloaddition $[4+2]$

Singlet Molecular Oxygen Quenching

Chemical Quenching

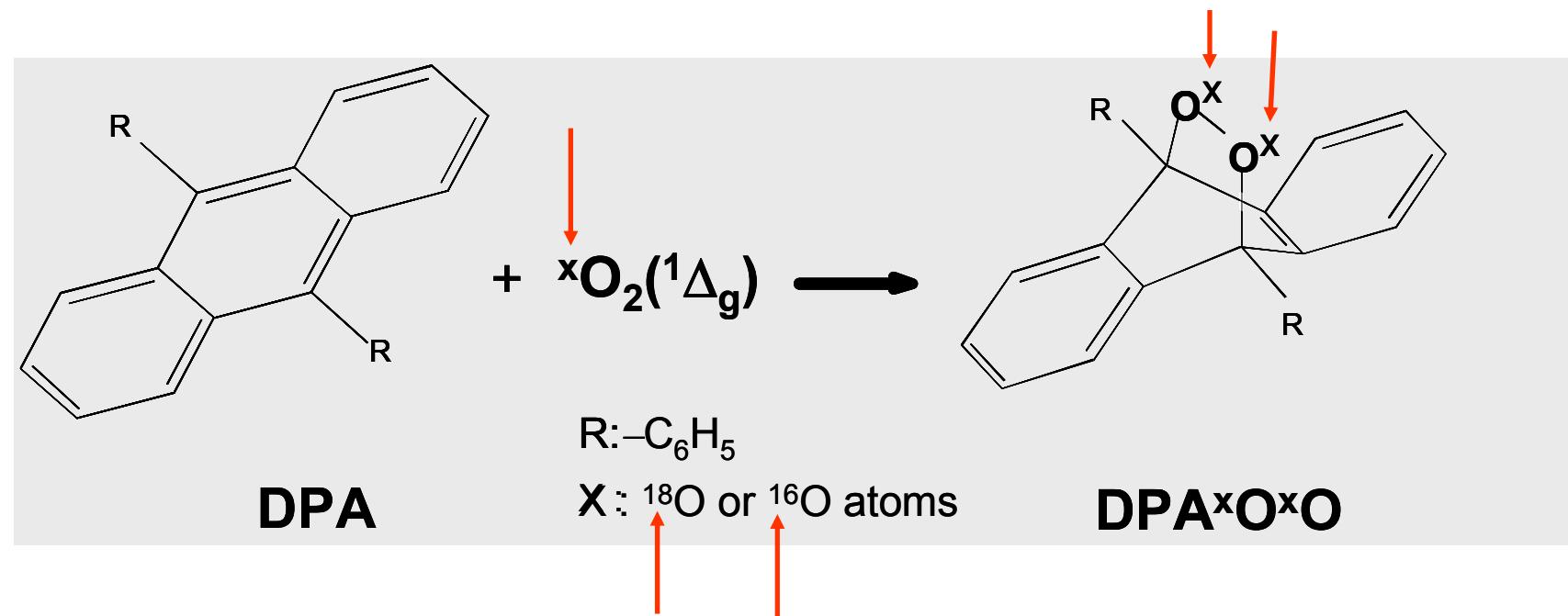
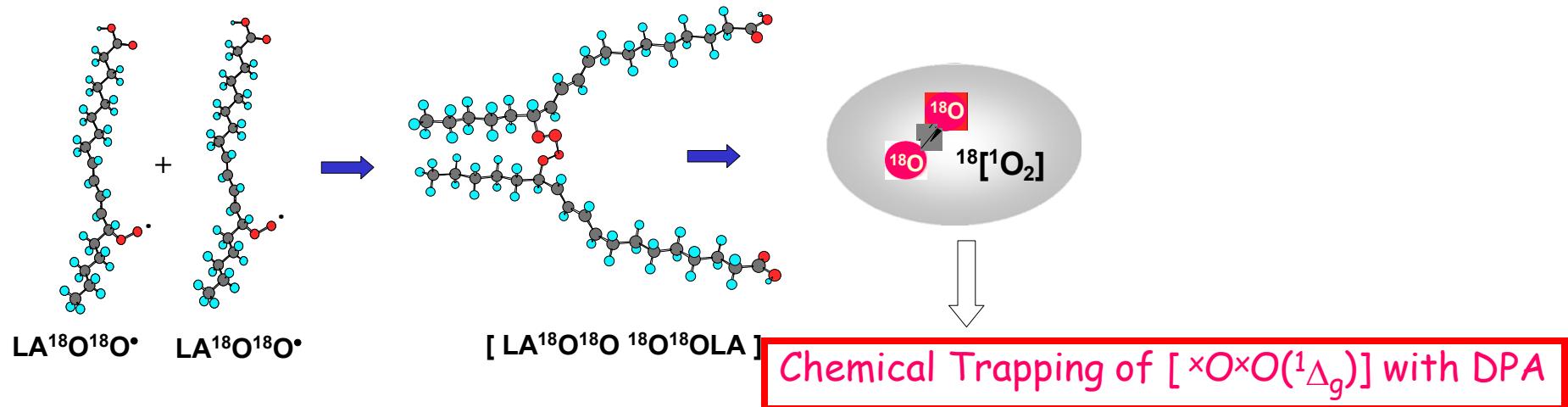


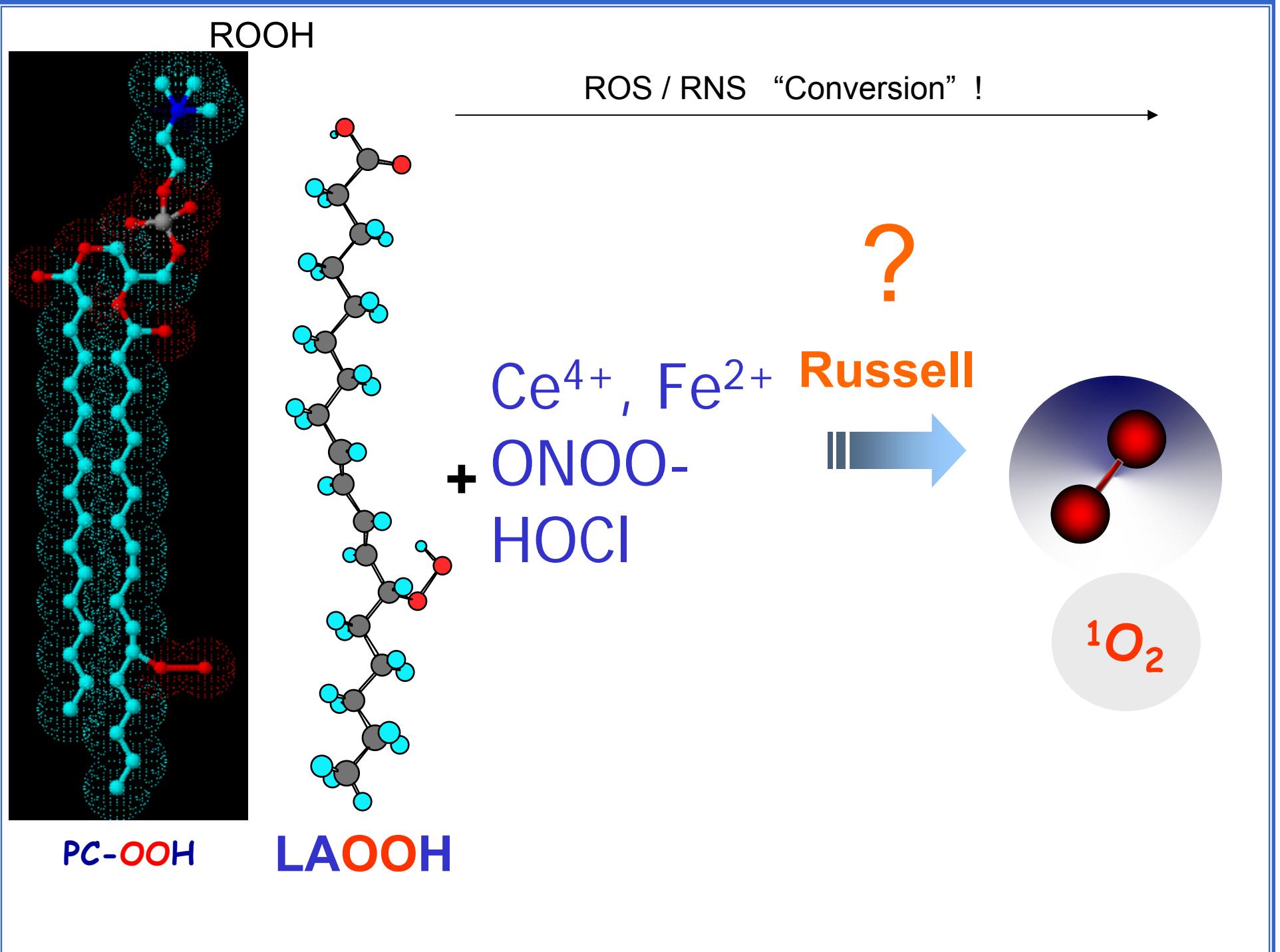
Physical Quenching



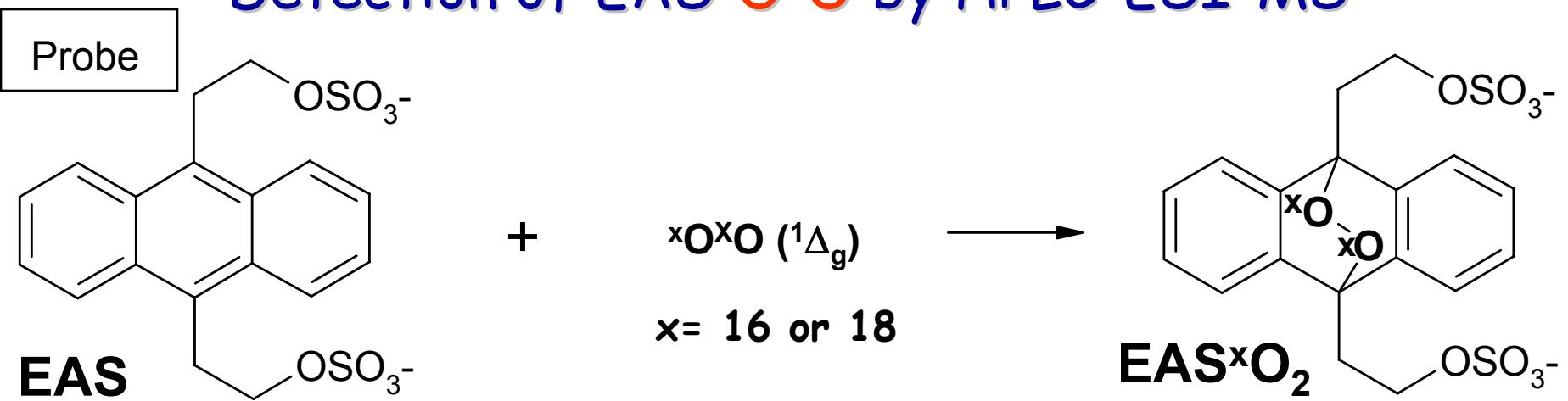
Lycopene

Chemical Trapping of [$\text{xO}^{\text{x}}\text{O}(^1\Delta_g)$] with DPA



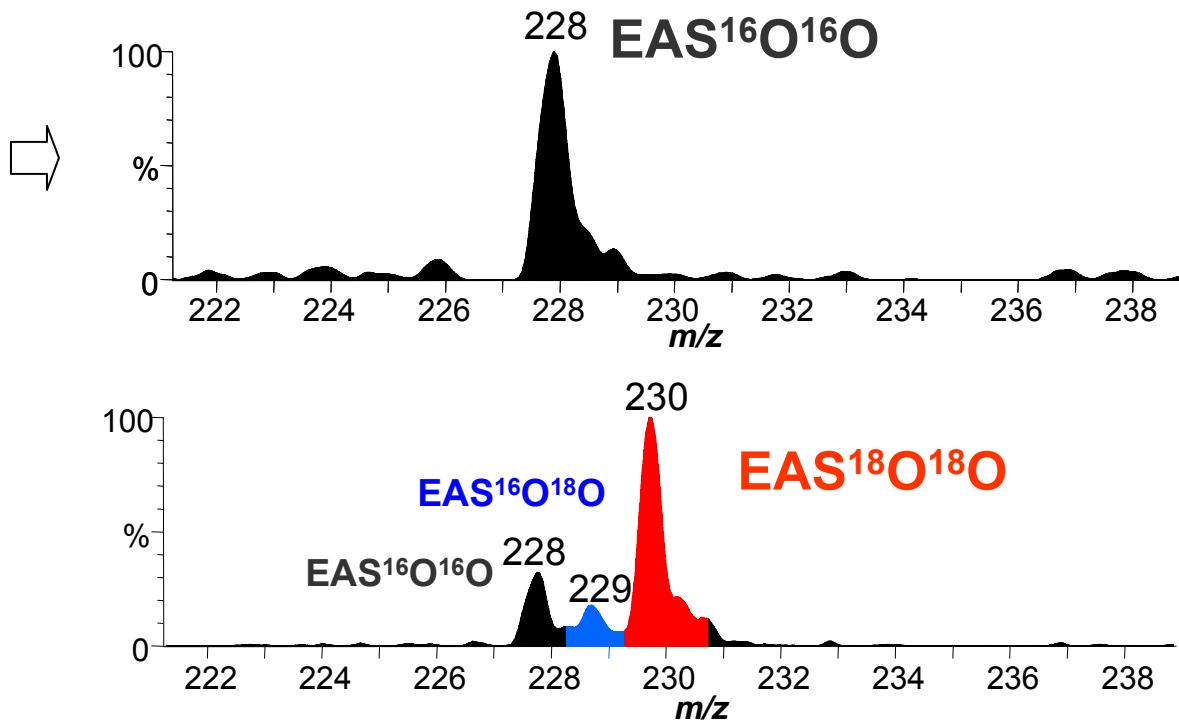


Chemical Trapping of [${}^x\text{O}{}^x\text{O}({}^1\Delta_g)$] by EAS and Detection of EAS ${}^x\text{O}{}^x\text{O}$ by HPLC-ESI-MS



$\text{LA}^{16}\text{O}^{16}\text{OH}$
+ HOCl

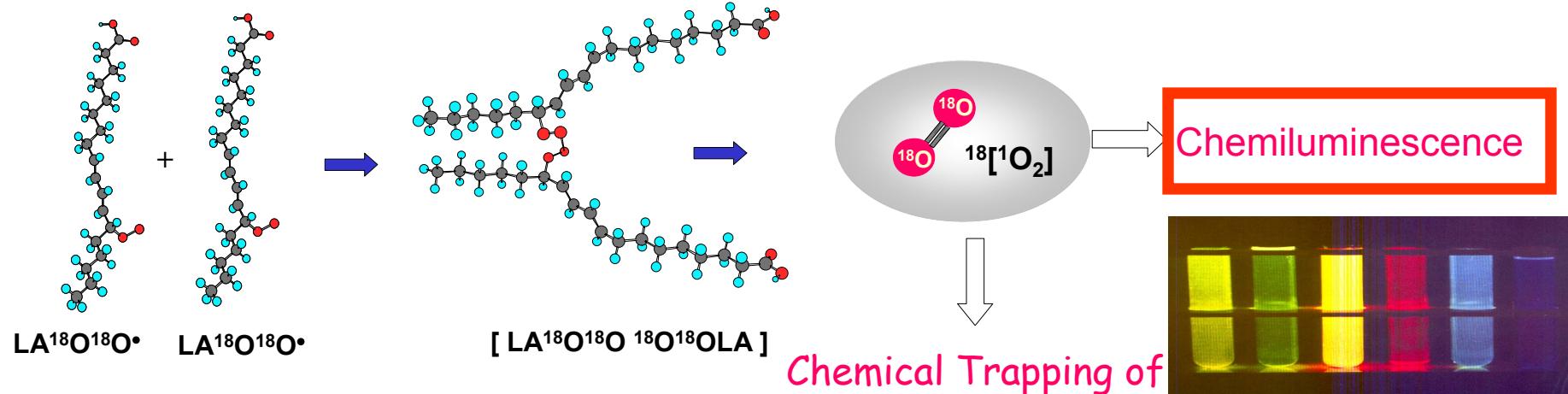
$\text{LA}^{18}\text{O}^{18}\text{OH}$
+ HOCl



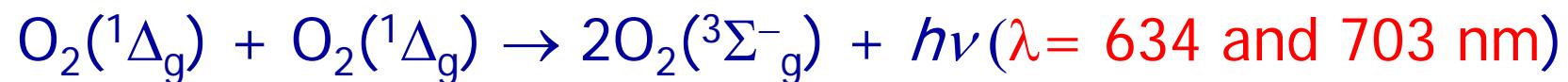
Part III

Luminescence

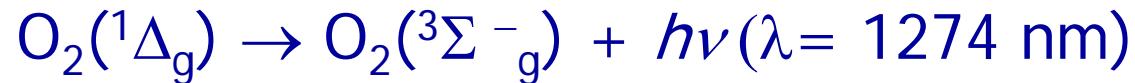
Light Emission (*spectroscopic investigation*)



Dimol emission



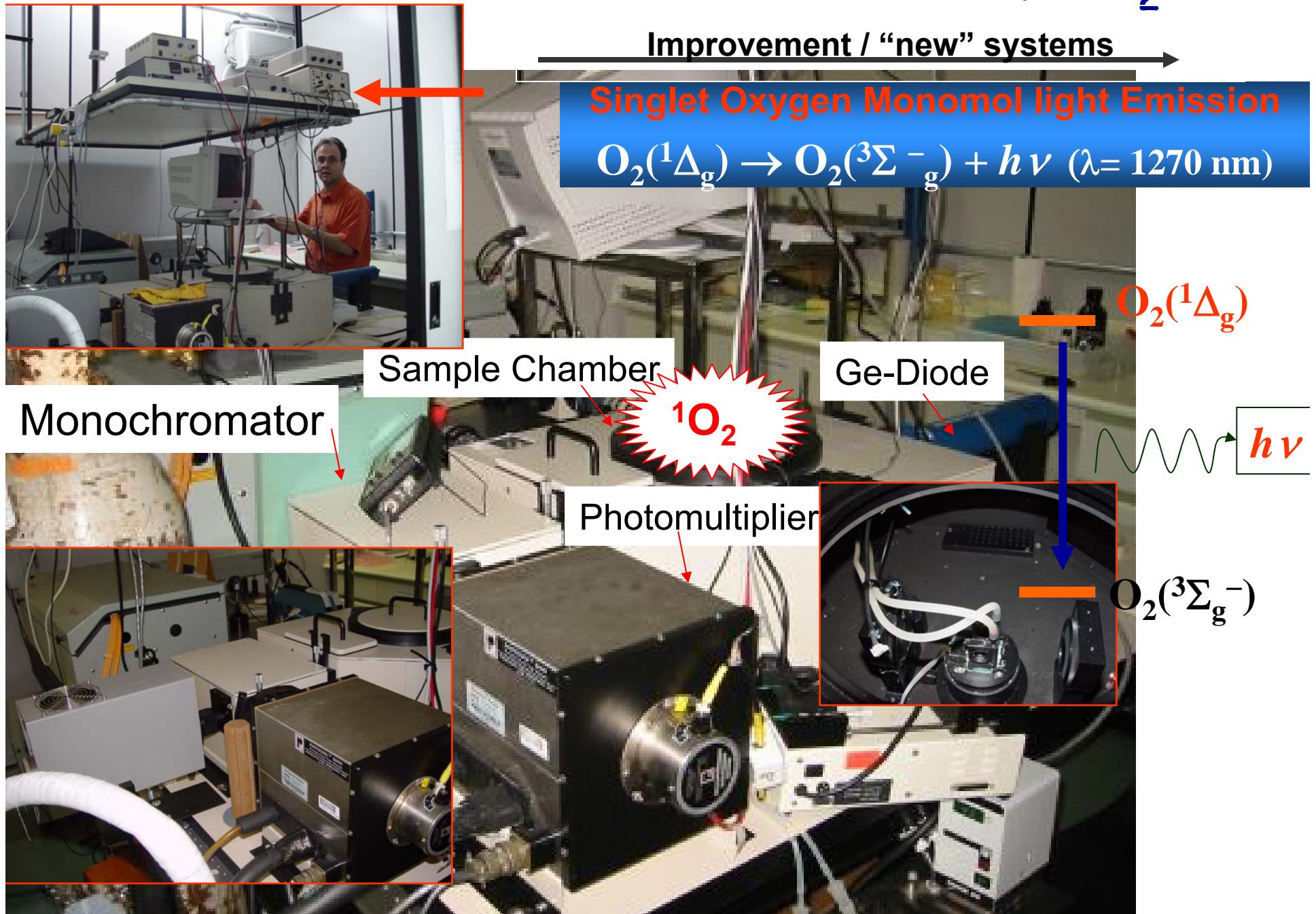
Monomol emission

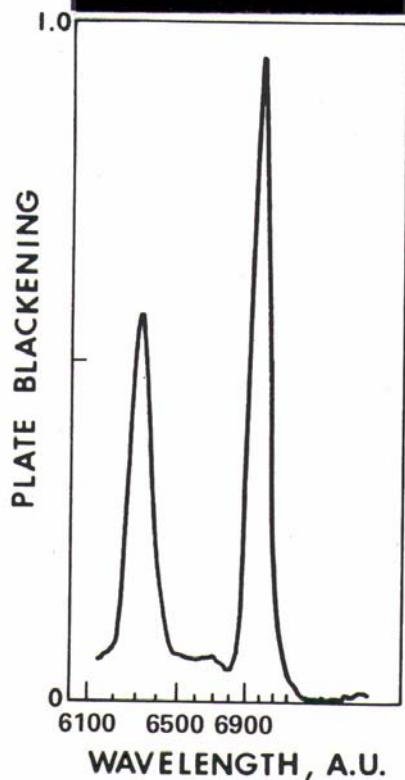
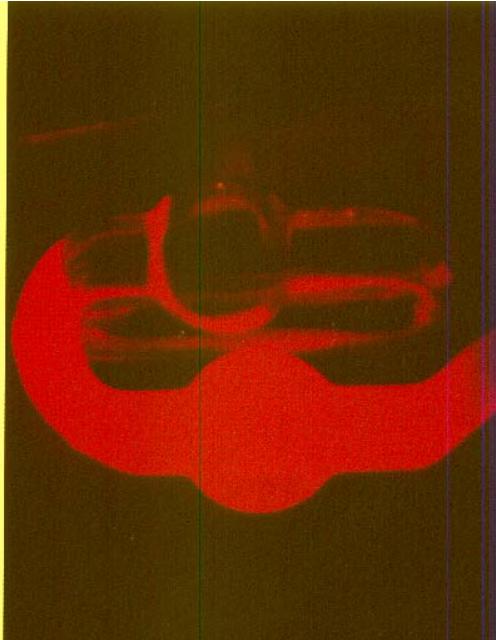


Detection and Characterization of 1O_2

Improvement / “new” systems

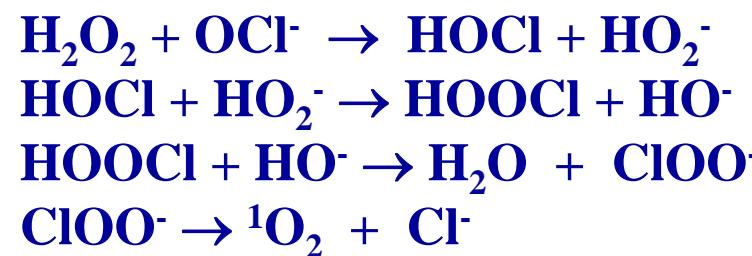
Singlet Oxygen Monomol light Emission



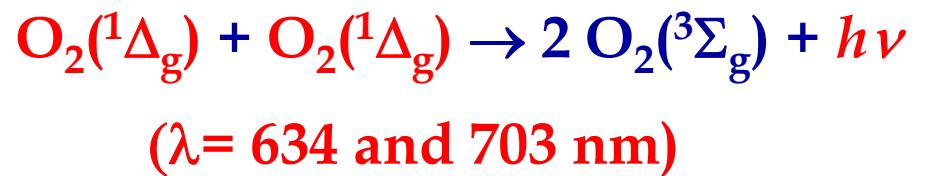


Khan and Kasha, 1963

Emission spectrum of the hydrogen peroxide-hypochlorite reaction

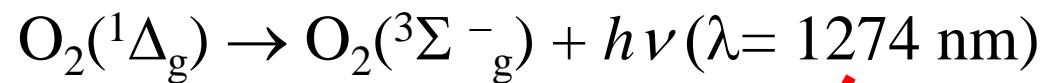


Dimol emission

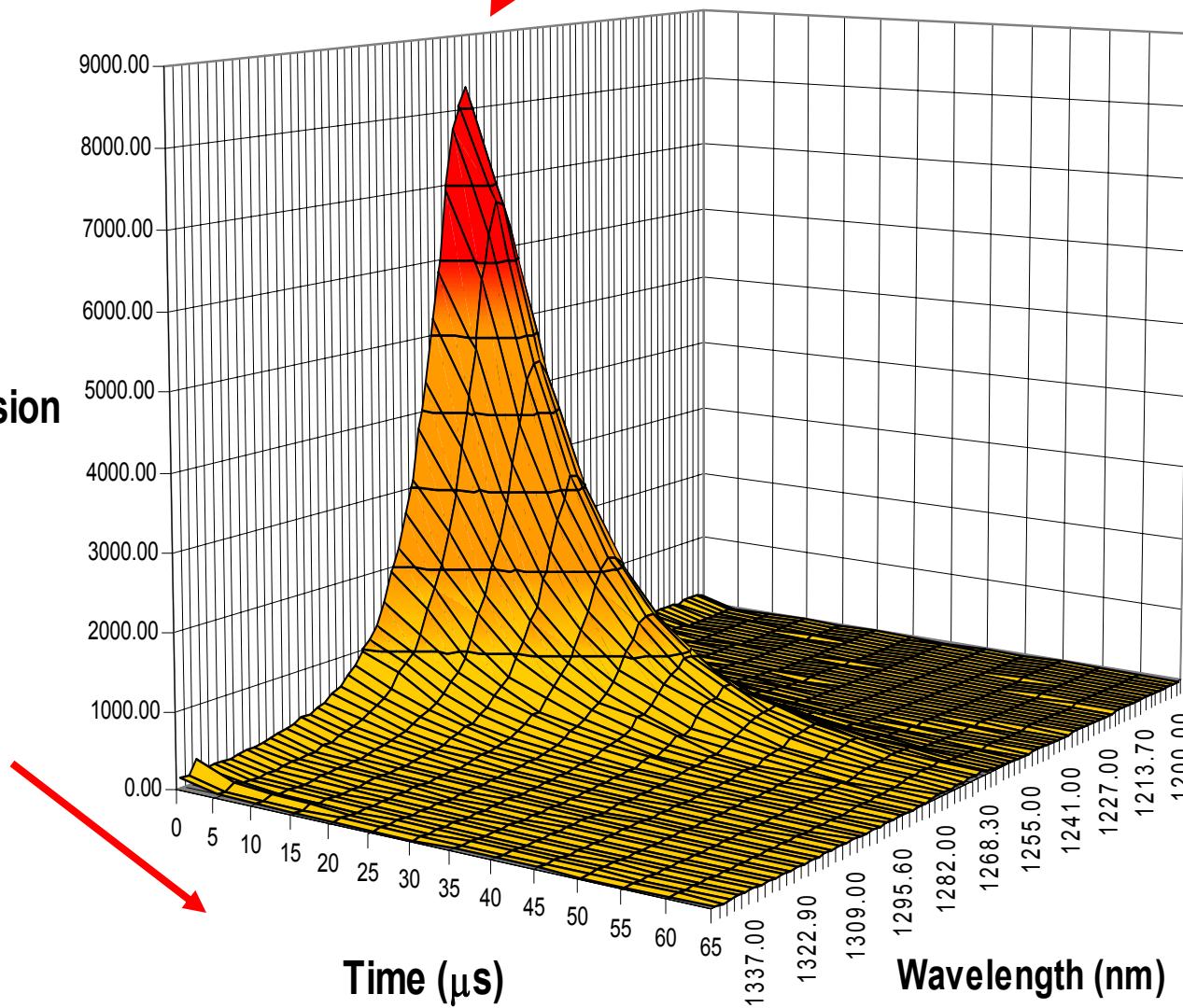


Monomol emission

Near-IR

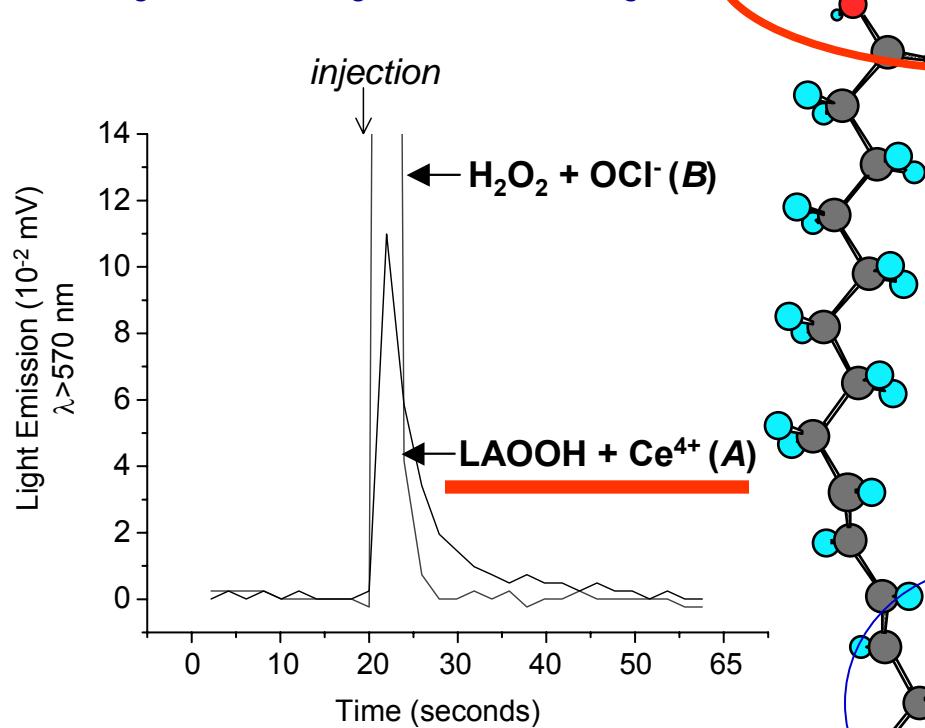
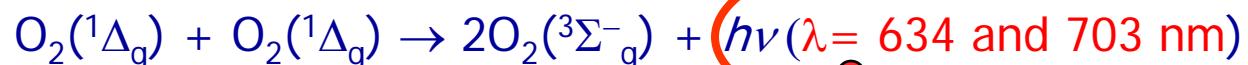


Light Emission
(cps)

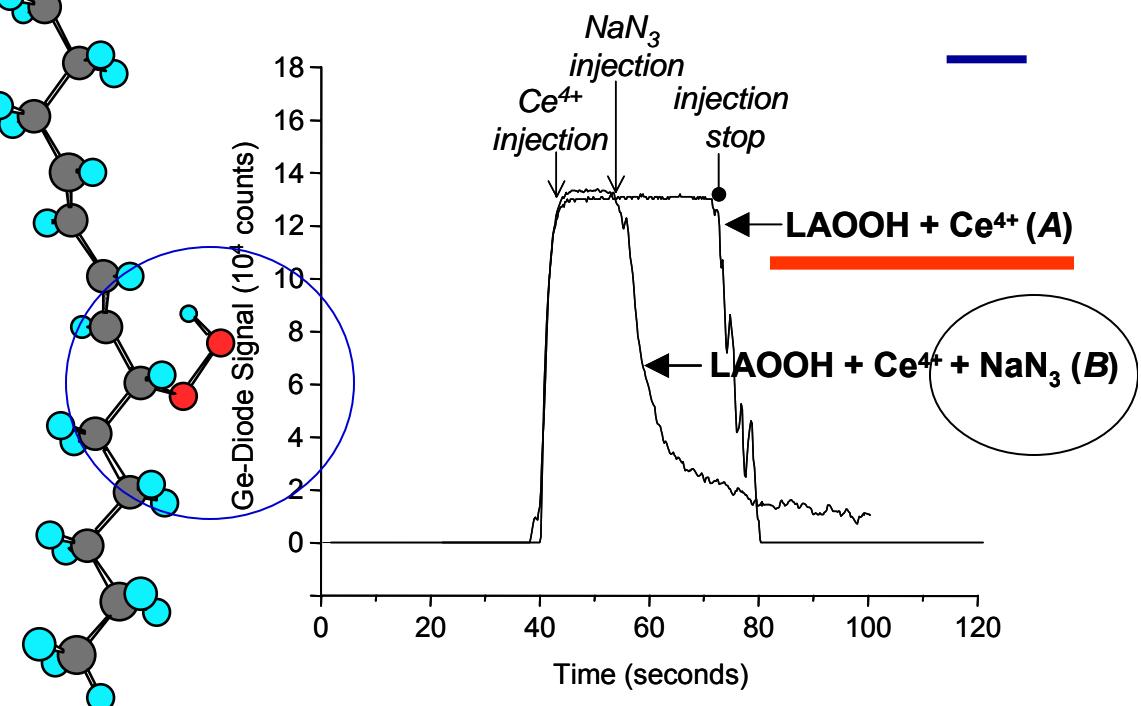
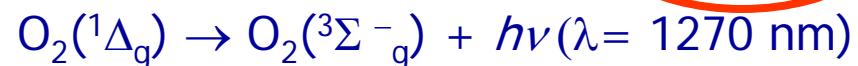


Characterization of singlet oxygen generated in the reaction of LAOOH and Ceric ion by chemiluminescence

Dimol light emission at $\lambda > 570$ nm

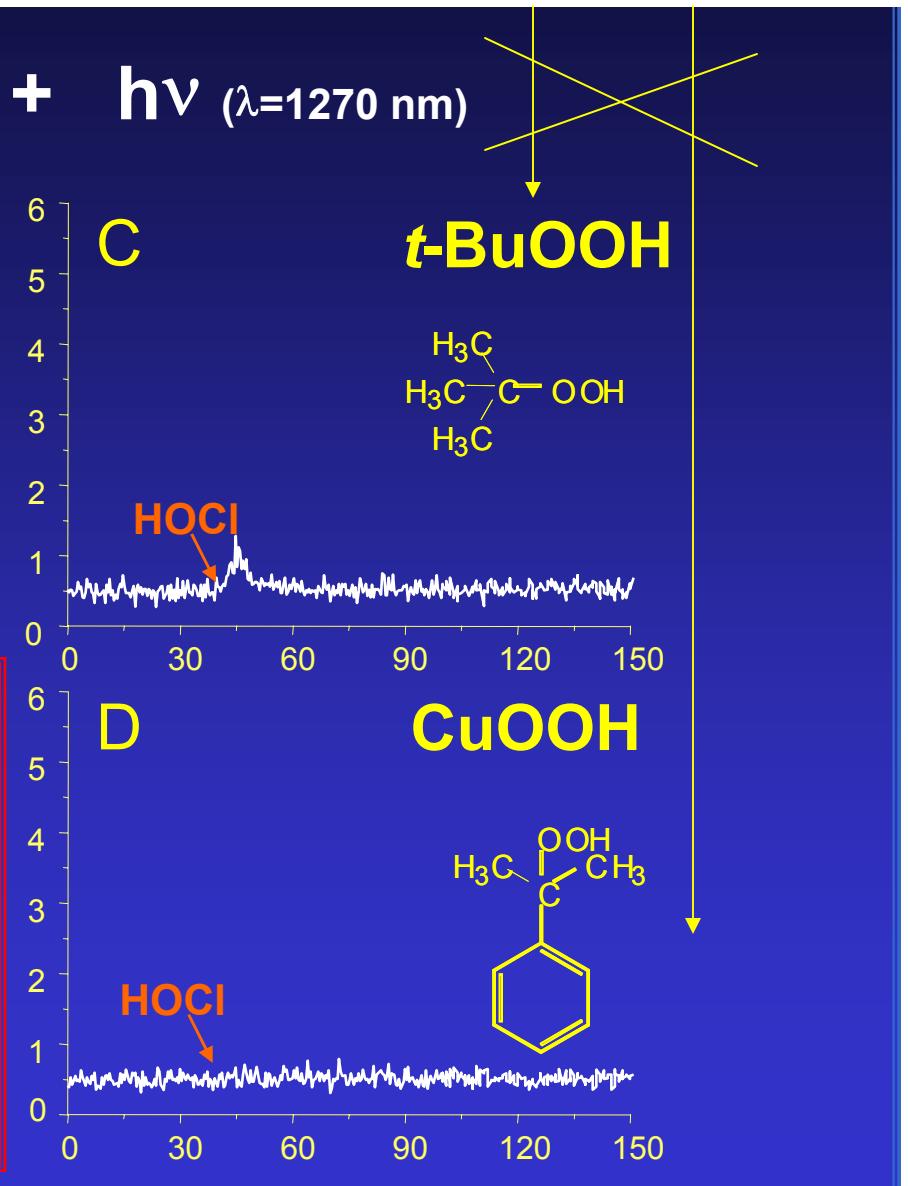
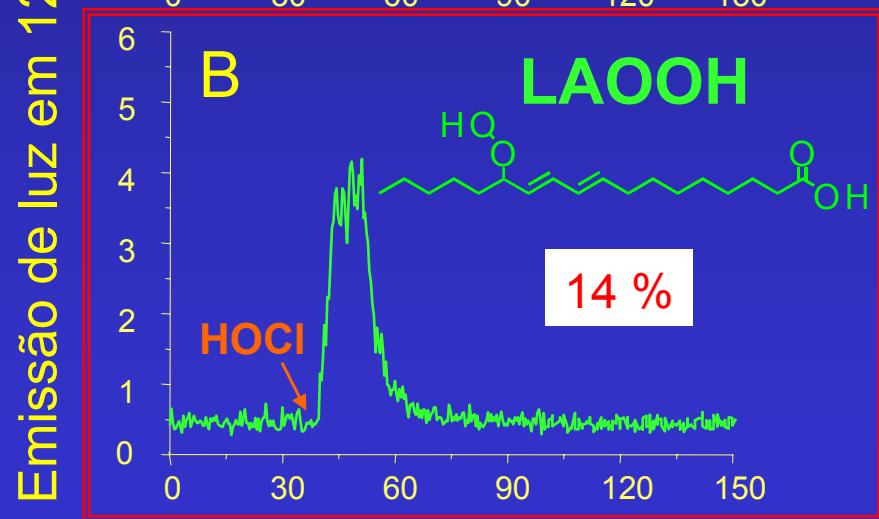
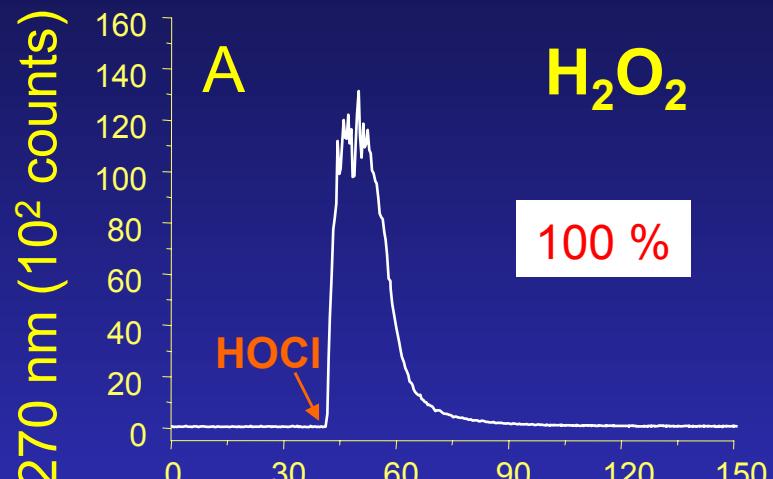


Monomol light emission at $\lambda = 1270$ nm

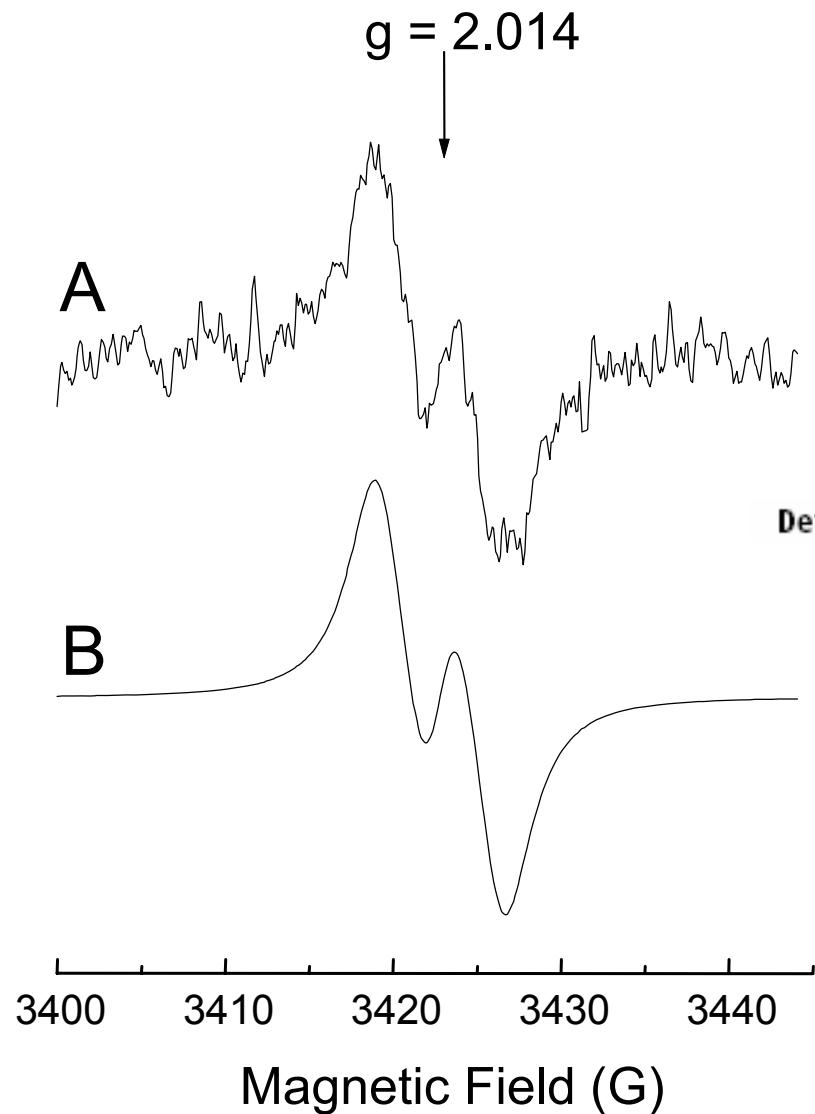


ROOH / HOCl

The presence of a hydrogen- α at the carbon to which the hydroperoxide is attached is essential for the generation of $O_2 ({}^1\Delta_g)$.



EPR spectrum of linoleate peroxy radicals, LAOO[•]. Reaction of LAOOH with HOCl



Part IV

Direct detection

Detection of Peroxyl Radical by Continuous-Flow EPR.

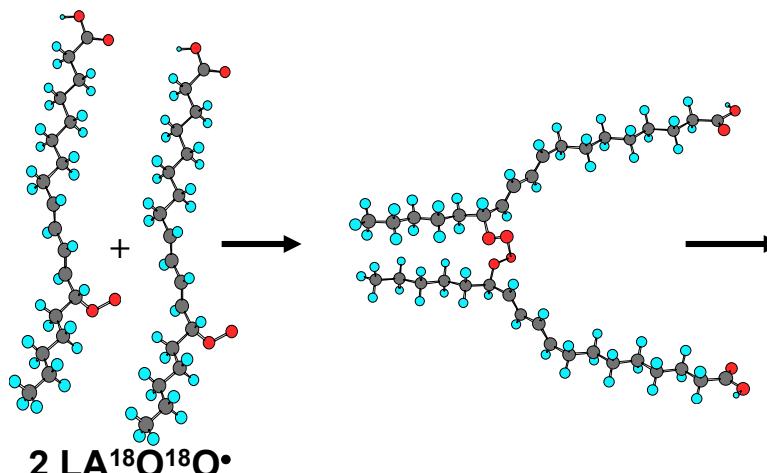
Take-Home Message

Conclusion: Combination of Different Techniques !

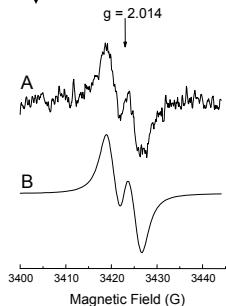
Source

For example, lipids?

I- Synthesis of ^{18}O -labeled source

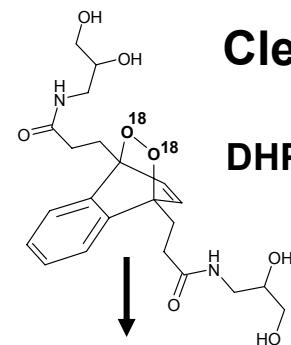


EPR



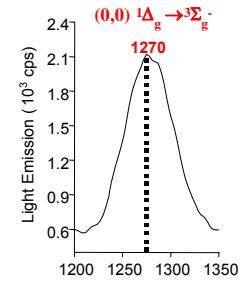
IV- EPR

V- Clean chemical source of ^{18}O -labeled reactive species, $[^{18}\text{O}_2]$

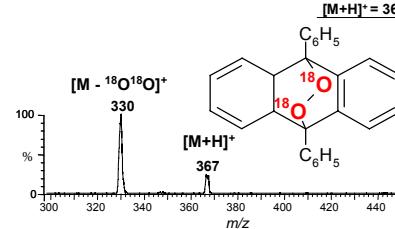


Clean source
DHPN $^{18}\text{O}_2$

II- Chemiluminescence Near-IR and Visible



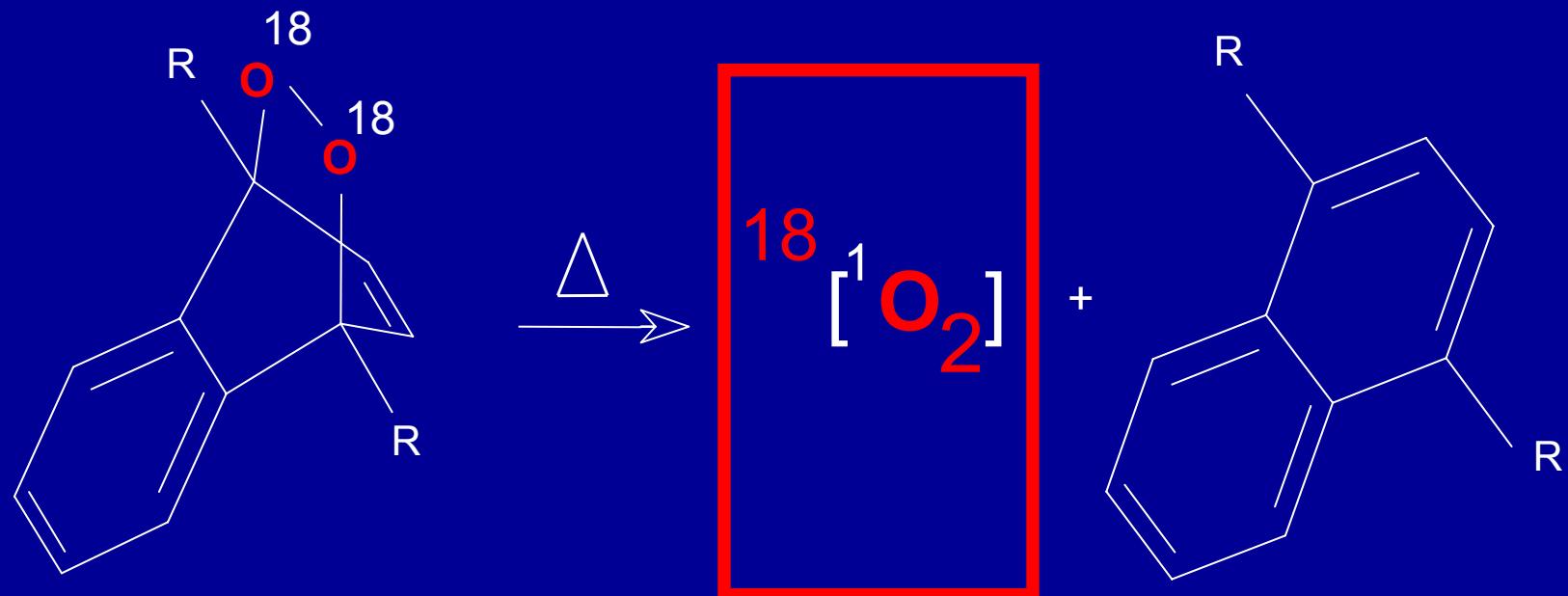
HPLC-MS/MS



III- Mass Spectrometry

and Visible
trapping

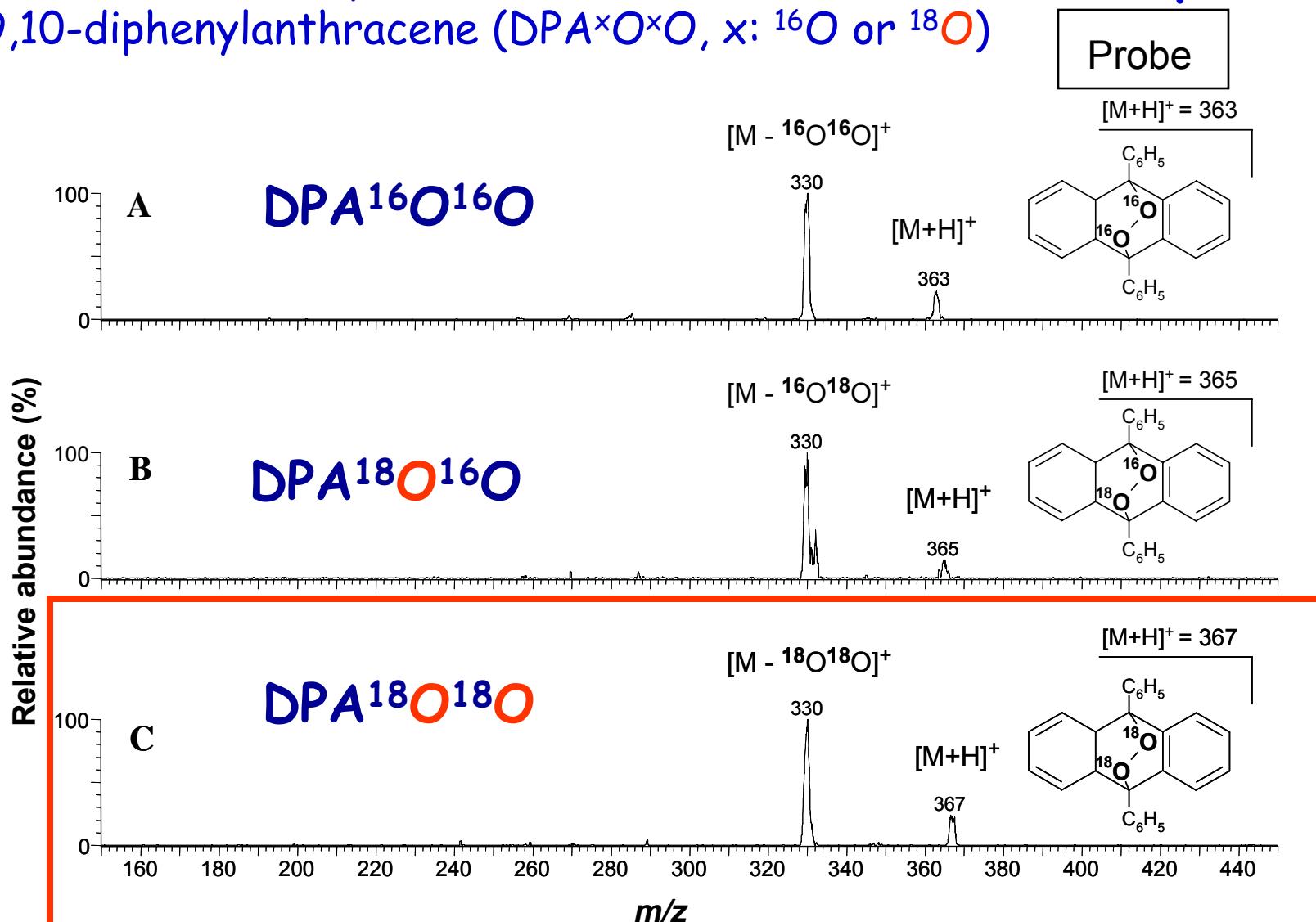
Chemical source of [^{18}O] isotopically labeled singlet oxygen $^{18}[{}^1\text{O}_2]$



? ..to elucidate mechanism of ${}^1\text{O}_2$ reaction
towards biological target

Excellent tool (+MS) for mechanistic studies of the reaction of singlet molecular oxygen in biological media.

Positive APCI-mass spectra in the MS/MS mode of the **endoperoxides** of 9,10-diphenylanthracene (DPA \times O \times O, x: ^{16}O or ^{18}O)



25 mM LA $^{18}\text{O}^{18}\text{OH}$ + 25 mM Ce $^{4+}$ + 60 mM DPA/37°C, 1 hour

Energy transfer between singlet (${}^1\Delta_g$) and triplet (${}^3\Sigma_g^-$) molecular oxygen in aqueous solution.

