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Caroline Delehedde, Sophie Thétiot-Laurent, Mathieu Esgulian, Mathieu Chocry, Antal Rockenbauer, et al.. Targeting mitochondria with novel, differently vectorized linear phosphorylated nitrones:. 2020. hal-03081866v1

HAL Id: hal-03081866

<https://amu.hal.science/hal-03081866v1>

Preprint submitted on 18 Dec 2020 (v1), last revised 18 Jun 2021 (v3)

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Targeting mitochondria with novel, differently vectorized linear phosphorylated nitrones: an evaluation of spin-trapping reporter, biochemical and therapeutic properties

Vector modification strategies to improve biocompatibility of linear phosphorylated nitrones targeting mitochondria: Inhibitory effect in ROS induced apoptotic cells.

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ABSTRACT

The free radicals (FR) occurrence in biological signalling or as pathologies triggers is well-documented and the design of specific scavengers to limit oxidative stress is enthralling. Because FR become inactivated upon addition to nitrones, efforts have focused on developing alpha-phenyl-*N*-tert-butyl nitron derivatives (PBNs) with promising pre-clinical data. To improve efficacy, a new generation of nitrones derived from 2-(diethoxyphosphoryl)-*N*-(benzylidene)propan-2-amine-*N*-oxide (PPNs) incorporating an antioxidant framework, gave rise to multipotent drugs exhibiting EPR-spin-trapping properties. As mitochondrial dysfunction is involved in many disorders, efforts have been concentrated on developing tools (pH probes) or pharmacological agents targeting-mitochondria. A series of mitochondria-targeted PPNS was synthesized based on the improved biocompatibility of P-containing nitrones and the expected benefits of introducing a cationic vector. Spin-adducts EPR-parameters toward biologically-relevant FR were established. Cytotoxicity was determined on cells and mitochondria-permeation assessed in isolated rat organs. New mitochondria-targeting nitrones appeared as lower cytotoxic alternative to the commonly used mito-PBN and were safely applied for investigating the anticancer-mechanism of new drugs impacting mitochondria.

Keywords

Mitochondria-targeted nitrones, ^{31}P NMR, EPR spin trapping, mitochondrial permeability, H_2O_2 -induced apoptosis

Introduction

Mitochondria are complex, metabolically active and dynamic organelles that regulate critical cellular processes from ATP-production to intrinsic cell death. They are major oxygen-consumers with roughly 95% of the total amount of breathed O_2 undergoing, in desirable conditions, reduction to water by cytochrome *c* oxidase (complex IV) in four consecutive one-electron steps. Activation of O_2 by monovalent reduction leads to superoxide anion radical ($\text{O}_2^{\cdot-}$), a reactive oxygen species (ROS) participating in many key cellular pathways [1]. Since the 1970s, popular estimates of the steady state amount of mitochondrial $\text{O}_2^{\cdot-}$ ranged 1–2% of inhaled oxygen (i.e., 215–430 mmol of $\text{O}_2^{\cdot-}$ daily for an 80 kg human) [1a], yet recent knowledge proposed it could actually be one order of magnitude less under non pathological conditions [1b]. Nevertheless, still the mitochondrial respiratory chain is regarded, together with cytosolic NADPH oxidases (Nox), as a major cellular source for $\text{O}_2^{\cdot-}$. In mitochondria electron transport chain, $\text{O}_2^{\cdot-}$ formation involves complexes I (NADH:ubiquinone oxidoreductase) and III (cytochrome *bc₁* complex), releasing this free radical within the mitochondrial matrix and, mostly, into the intermembrane space, respectively [1c].

About thirty years ago, the production and/or regulation of mitochondrial ROS have emerged as essential mechanisms to maintain cellular functions and signaling [1d]. Impairment of these processes results in overproduction of $\text{O}_2^{\cdot-}$ and/or hydrogen peroxide (H_2O_2), the ROS specifically formed when $\text{O}_2^{\cdot-}$ is neutralized by mitochondria-specific superoxide dismutases. The following excess of H_2O_2 , if not cleared by detoxifying enzymes such as catalase (present in the mitochondria of some cell types), glutathione peroxidases (GPx) and peroxiredoxins

(Prdx), may then be converted to harmful hydroxyl radicals (HO[•]) in a general deleterious process termed as oxidative stress [2]. Mitochondrial dysfunction has been associated with a host of disease categories and metabolic conditions, including metabolic syndrome (diabetes/obesity), ischemia/reperfusion injury, inflammation, as well as chronic disorders such as cancer, neurodegeneration, Alzheimer's and Parkinson's diseases, and aging [3], even if most of them have recognized polygenic and/or environmental origins.

In this context the amount of space is expanding for chemically engineering mitochondria targeted antioxidants and free radical scavengers able to maintain the redox status of the cell and repair those dysfunctions linked to oxidative stress [4]. To this end synthetic strategies have favored hybrids made up of (i) a delocalized lipophilic cation vector for crossing the mitochondrial inner membrane by diffusion, most commonly a triphenylphosphonium cation (TPP), (ii) an aliphatic chain spacer, and (iii) a pharmacophore such as a chain-breaking antioxidant aryl moiety or an active free radical (FR) scavenging site. Because nitrones have been used for decades in electron paramagnetic resonance (EPR) spin-trapping for detection/identification purposes [5], their general common moiety C=N⁺-O⁻ was considered suitable as a FR scavenging building block in designing these new hybrid compounds [4b,6]. Moreover, the α -phenyl-*N*-tert-butyl nitron (PBN; Fig. 1A) scaffold has emerged as a privileged core for drug design in many pathologies related to FR formation. Thus a wide range of PBN- or PBN-like derivatives has been developed in the last decades (jointly named PBNs family in Fig. 1A) and many have demonstrated pharmacological action *in vivo*, assuming this may rely on the inhibition of ROS-dependent or -independent biological pathways considered as hallmarks of mitochondrial dysfunction [7,8]. Following the first reported PBN-TPP conjugate bromide, mito-PBN (**1**; Fig. 1B) [9], a number of TPP-based linear or cyclic nitrones have been synthesized, their mitochondrial versus cytosolic uptake was found increased 100–1,000 times *in vitro*, and practically all these compounds exhibited pharmacological properties to reduce mitochondrial ROS content in various situations [6]. Hence **1** has been found to inhibit cerium oxide nanoparticles toxicity in human fibroblasts by interacting with both mitochondrial

ROS formation and membrane Nox2 and Nox4 activation [10]. It has been also reported that encapsulating **1** in liposomes-lipid nanoparticles decreased $O_2^{\bullet-}$ and H_2O_2 -induced mitochondrial dysfunction in diabetic mice hepatocytes [11].

In the PBNs structure the α -*tert*-Bu group, introduced to increase the kinetic stabilization of the resulting nitroxide spin adduct and thus facilitate its EPR detection [5], brings about increased lipophilicity of the nitron but may enhance its toxicity (see [8] and references therein). In our prior approaches to discover improved spin trapping probes of oxidative stress, we repeatedly found a lower cytotoxicity when one Me of the *tert*-Bu was replaced by a diethoxyphosphoryl group $(EtO)_2P(O)$ [8,12]. On this biocompatibility basis, we developed a family of β -phosphorylated, PBN-like hybrids, i.e., based on a 2-(diethoxyphosphoryl)-*N*-(benzylidene)propan-2-amine-*N*-oxide (PPN) scaffold, bearing, for a subset, an aryl substituent inspired by those found in natural phenolic acids (PPNs family; Fig. 1A). Not only these PPNs demonstrated nitric oxide (NO)-donation properties, resulting in significant vasoprotection (at submillimolar doses) of rat aortic rings challenged by $O_2^{\bullet-}$, but those compounds bearing a methoxylated phenolic ligand showed strong antioxidant properties *in vitro*, while retaining a low toxicity in various cell lines. In contrast to many PBNs, most of these PPNs yielded EPR characterizable $O_2^{\bullet-}$ and HO^{\bullet} spin adducts in aqueous media, taking advantage of the known stabilization effect provided by α - $P(O)(OEt)_2$ substitution of linear nitrones [8].

Bearing the above in mind, we recently synthesized a series of iodides having both a TPP-grafted aryloxy substituent as for **1**, and a PPNs-type nitronyl function (mito-PPNs family; Fig. 1B). ^{31}P NMR studies in rat livers confirmed that, for the lead, para-substituted mito-PPN (**2**), the accumulation ratio in mitochondrial:cytosolic extracts was of 2:1, and its toxicity to murine fibroblasts was (i) decreased about 8 times as compared with its analogue **1**, but (ii) increased 1.6 times as compared with the ‘untargeted’ analogue where an uncharged diphenylphosphonyl group $Ph_2P(O)$ was present instead of TPP [13]. Altogether, these latter findings were considered of particular interest since they suggest the protective ‘phosphonate effect’ [12] expected in **2** may have compensated the TTP group cytotoxicity. Indeed, alkyl or aryl

phosphonium salts are known to be highly toxic to human tumor mitochondria and, as such, can exhibit chemotherapeutic potency [4b]. Furthermore, high doses of a variety of TPP conjugates have been reported to cause mitochondria membrane depolarization of normal cells, depending on structural features such as the linker chain length and the pharmacophore (e.g., quinone, vitamin E, or a stable nitroxide) [6b,14].

Given the above relative toxicity issues of TPP, alternative mitochondria specific, more biocompatible vectors to be coupled to nitrones have been proposed in the literature. Figure 1C shows typical compounds [15] such as the neuroprotective agent LPBNAH (**4**), an amide-substituted PBN vectorized by a sugar moiety, and CarnDOD-7C (**5**), a PBNs-carnitine conjugate having demonstrated good FR scavenging properties (Fig. 1C). Conceivably, other lipophilic derivatives of *N*-centered cations, as exemplified by the ammonium salt **5**, are potential mitochondriotropic candidates. Specifically considering nitrones, the preparation of PBNs pyridinium salts for spin trapping purposes has been reported decades ago [16] but only recently was a series of *N*-arylpyridinium PBNs designed as mitochondria targeted antioxidants, although their permeation property was not investigated [17].

Accordingly, the focus of this work was to tailor the general structure of the mito-PPNs class of nitrones to lower cytotoxicity while retaining good mitochondrial addressing, antioxidant power and EPR spin trapping potential. For these purposes, we synthesized eight new mito-PPNs analogues in which the aryloxy framework involved a triethylammonium (TEA; compounds **6a–d**) or a pyridinium (Py; compounds **7a–d**) lipophilic cation carrier instead of TPP. Three additional TEA or Py vectorized nitrones were prepared along an aryl framework (compounds **8–10**). To expand structural diversity, we prepared two additional mito-PPNs **11** and **12** in which the aryl or aryloxy frameworks above were coupled with the berberinium iminium cation (Ber). Berberine, a plant alkaloid used for centuries in traditional medicine, has been recently employed as a core in drug synthesis [18]. Being highly delocalized and lipophilic, Ber has been shown to be an efficient mitochondria penetrating vector [19]. The chemical structures of target compounds and vectors are outlined in Figure 2, together with

their acronyms indicating the main structural features: nature of aromatic framework, vector and counterion, *ortho/para* position with respect to the nitronyl group, and length of the alkyl spacer. Cytotoxicities were determined on human dermal fibroblasts (HDF) and compounds combining the best overall biological and EPR properties were evaluated for their mitochondrial permeation in isolated perfused rat livers and protective effect in a cellular model of H₂O₂-induced apoptosis.

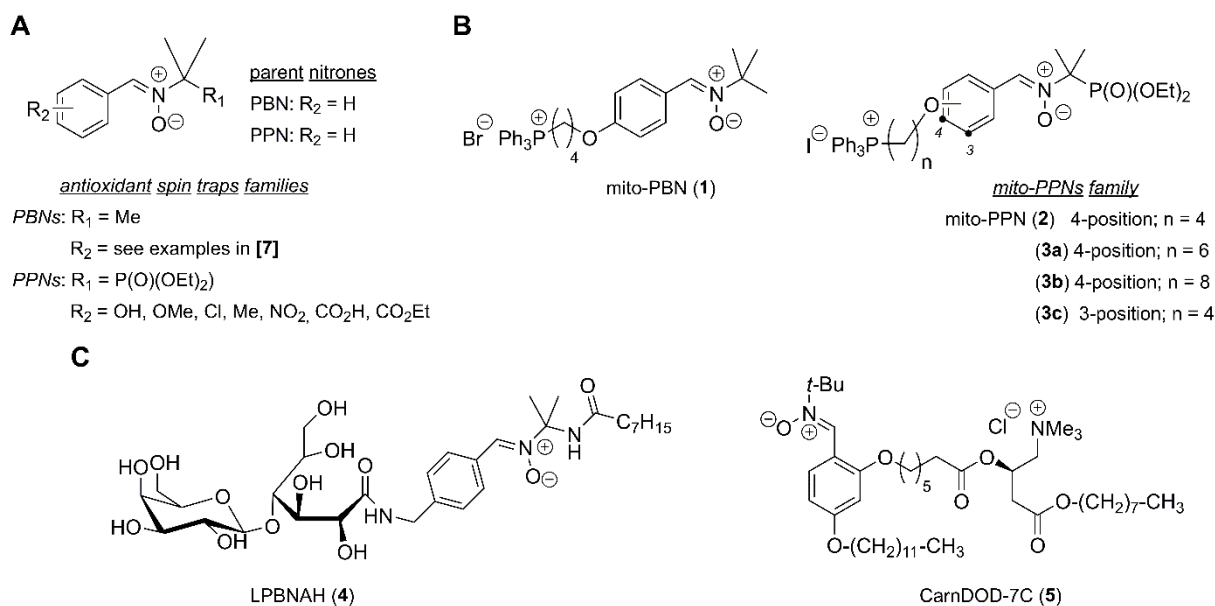


Figure 1. General structures of noncyclic nitrones and related families exhibiting antioxidant and/or pharmacological activity (A) deriving from PBN and PPN [7b,8], or targeted to the mitochondrion (B) by a TPP cation [6a,8] or (C) using alternative vectors [15].

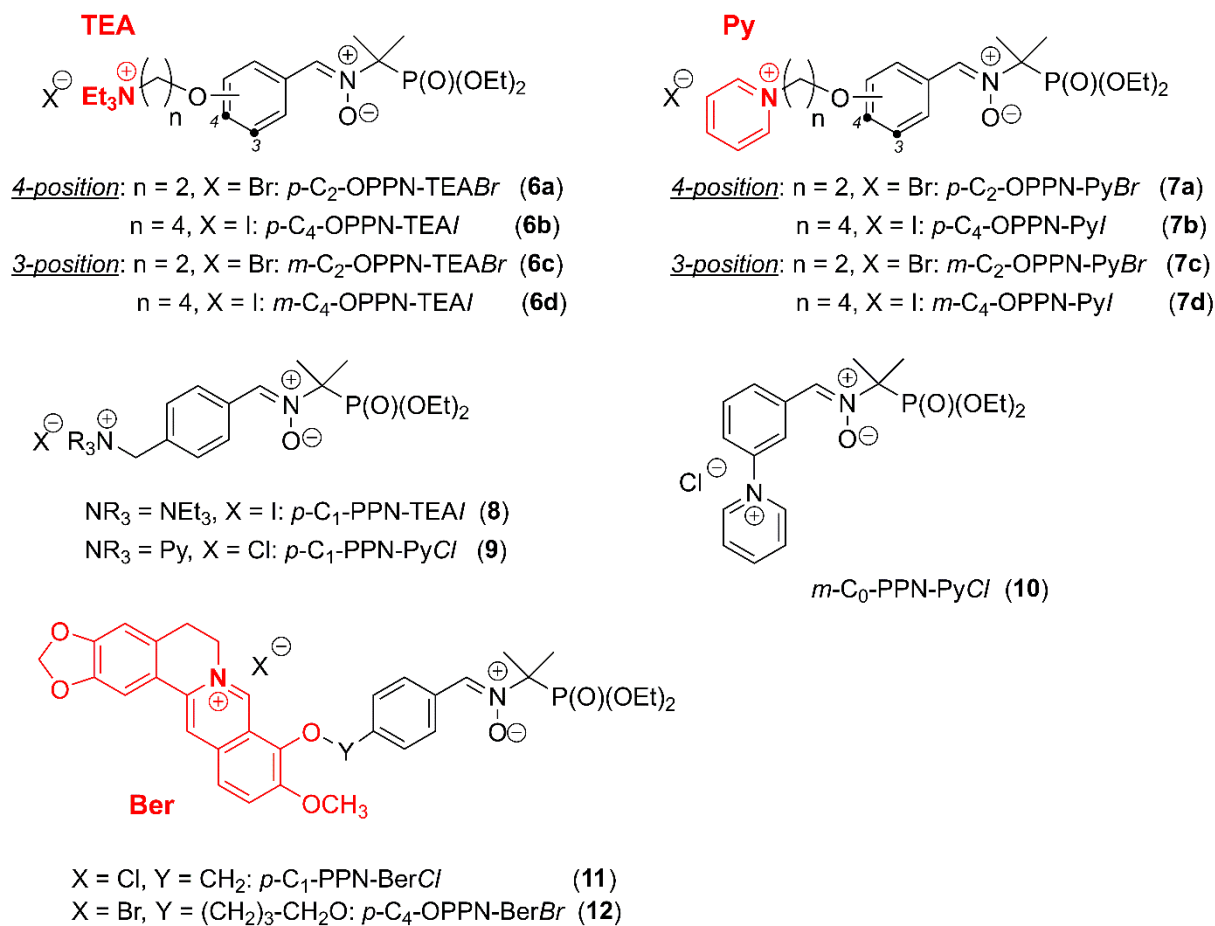


Fig. 2. Structures of new mitochondria targeted PPNs 6–12 showing TEA, Py and Ber vectors.

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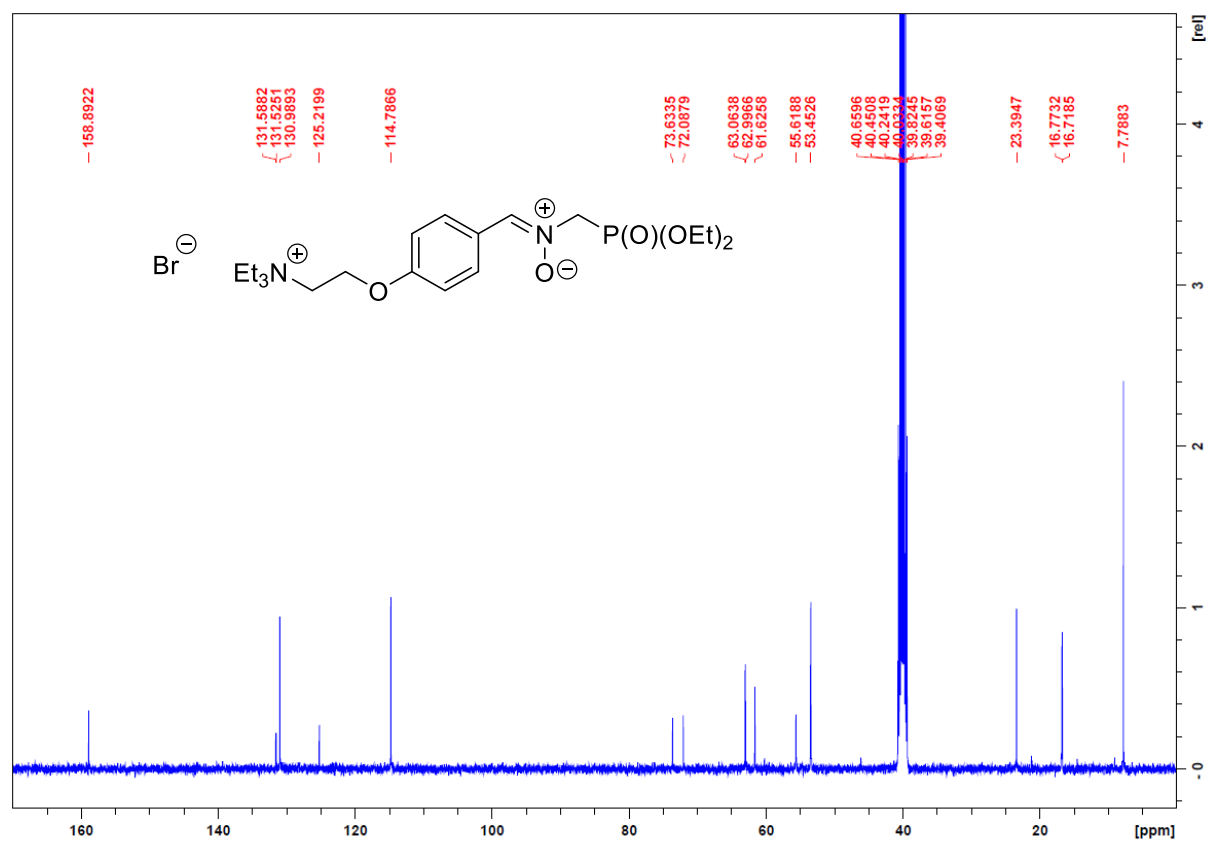
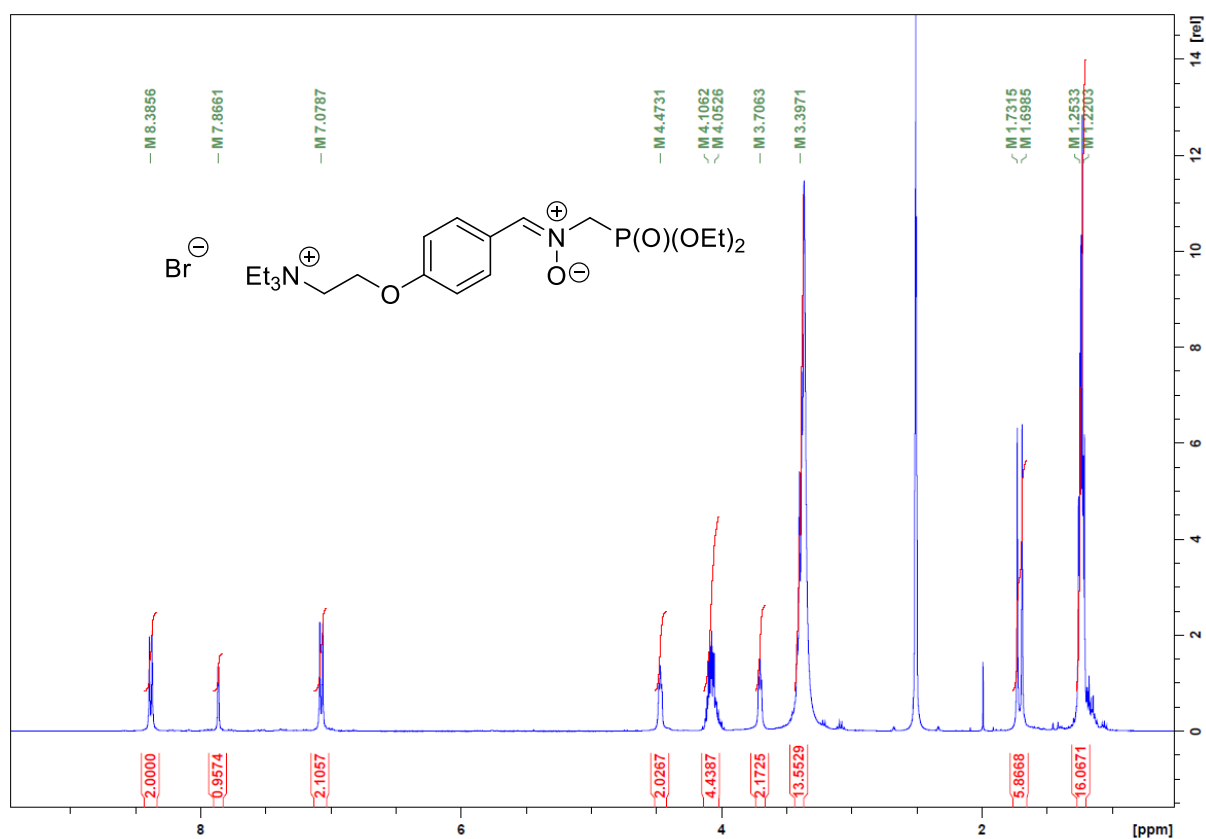
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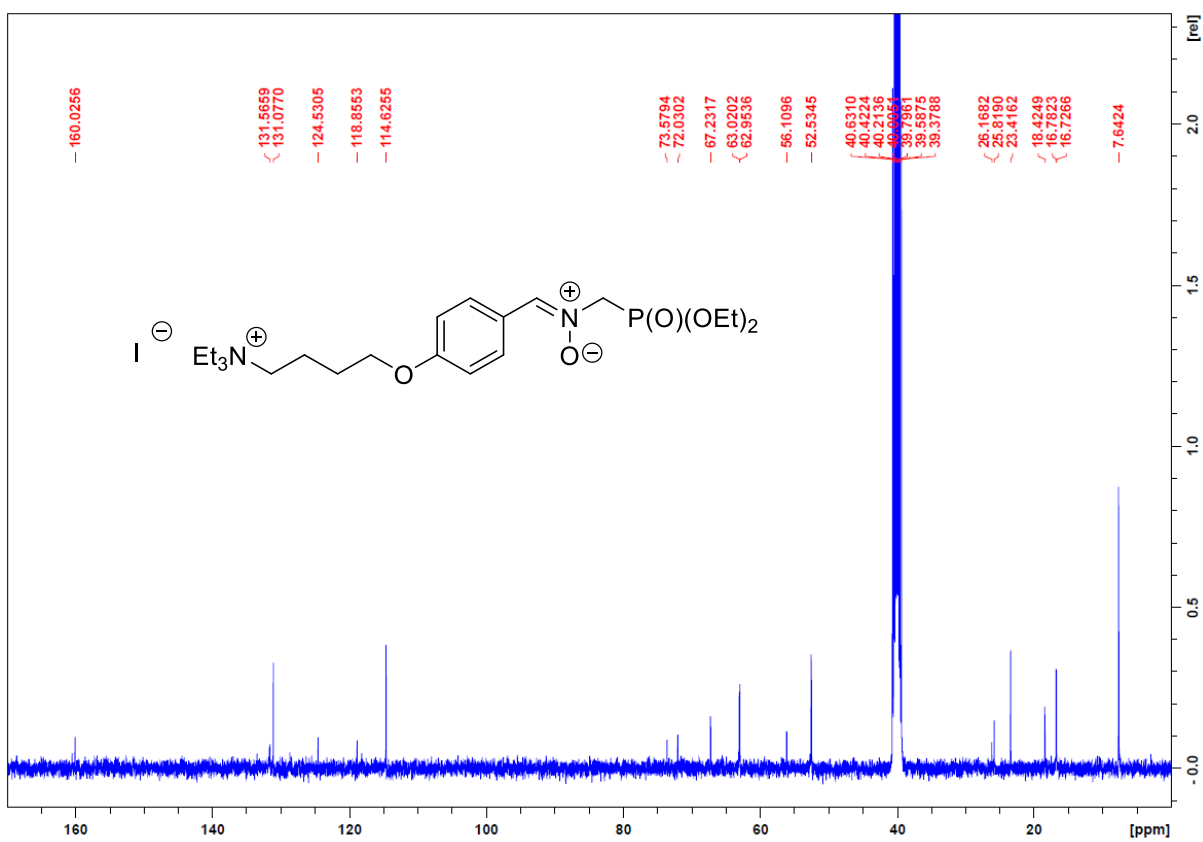
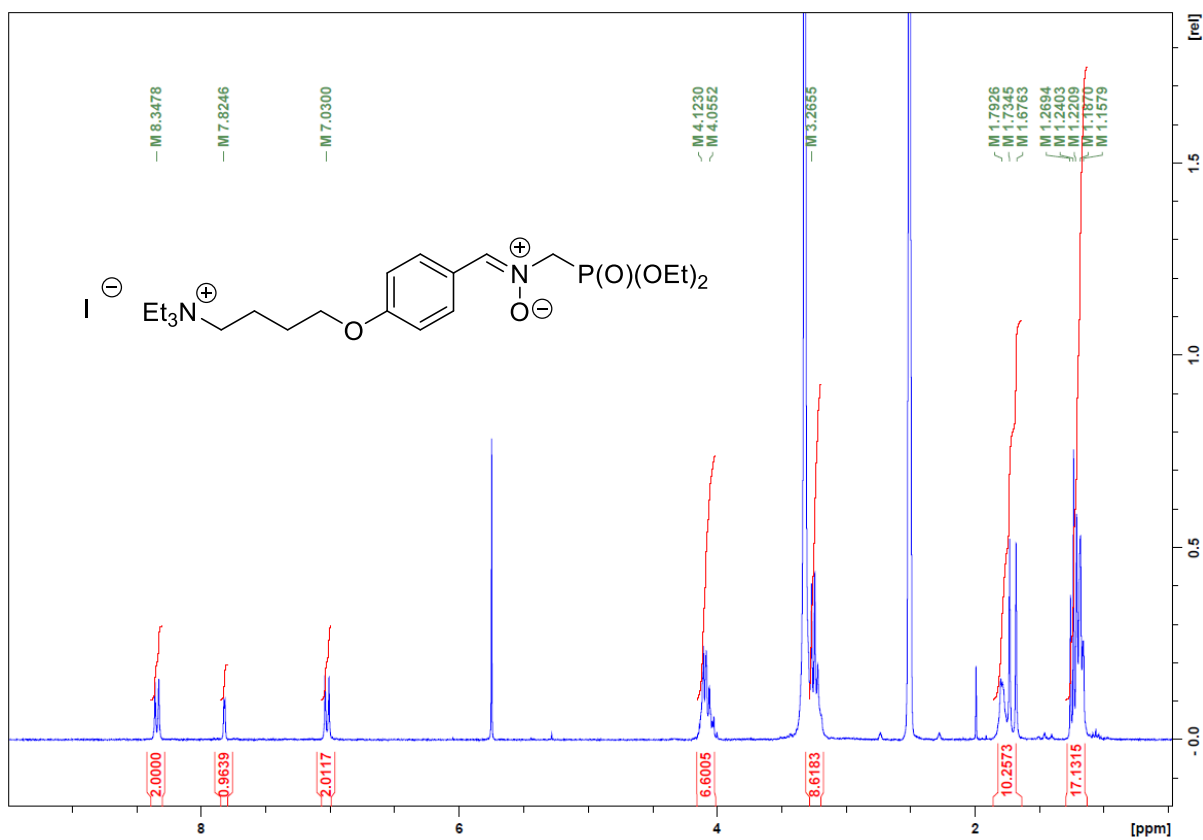
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Supplementary Material

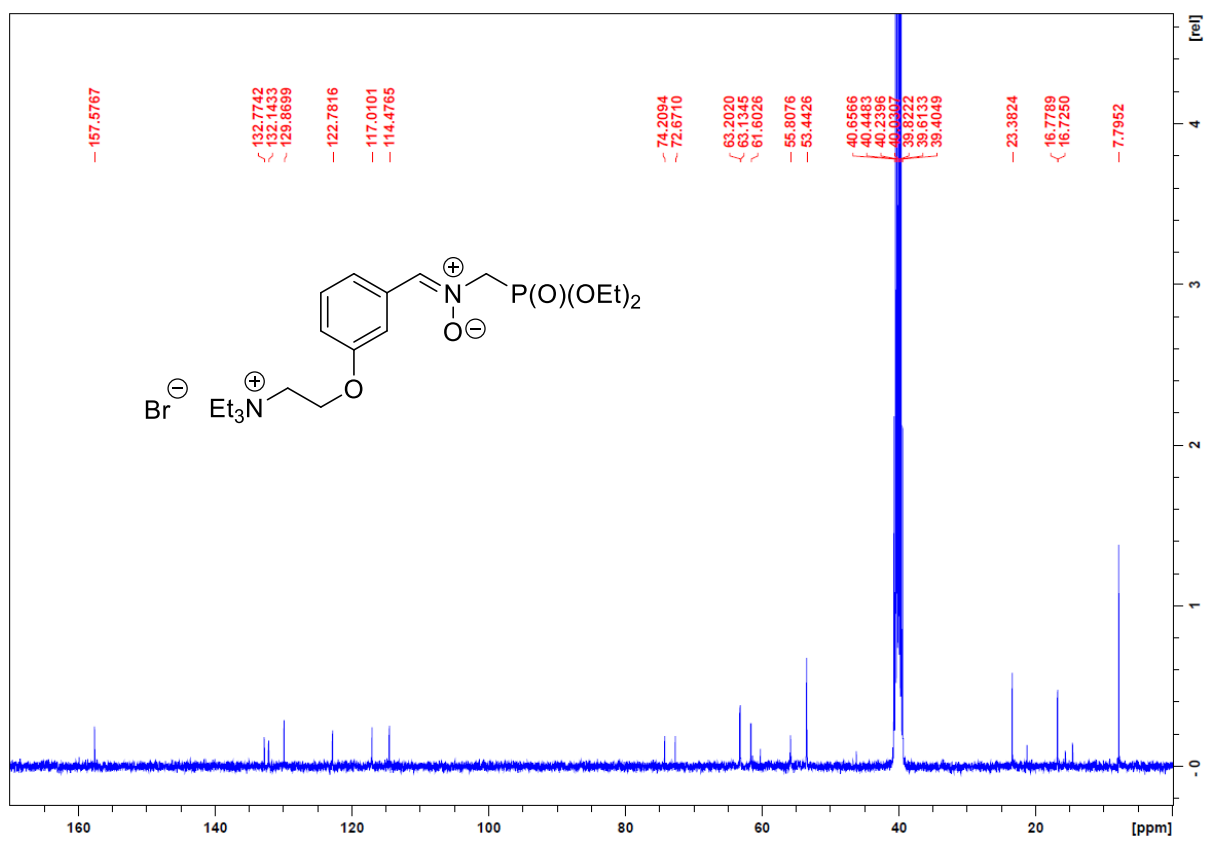
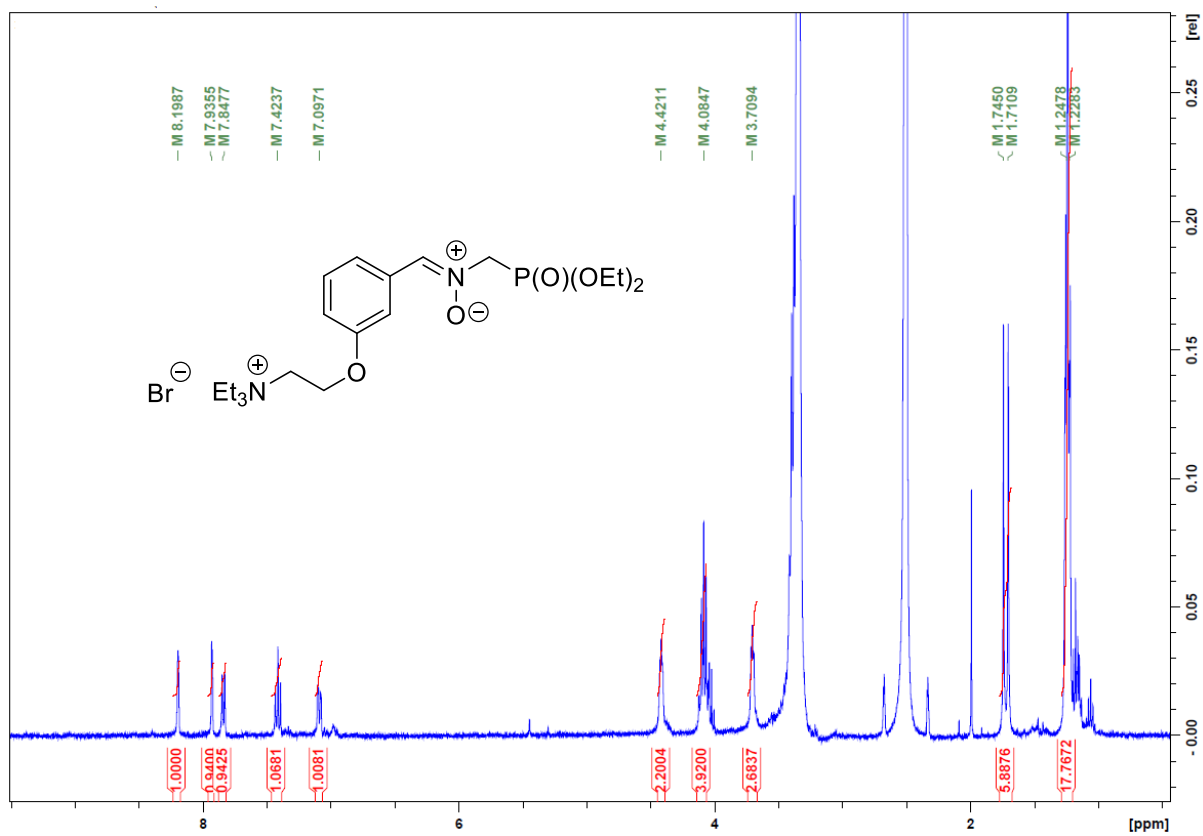
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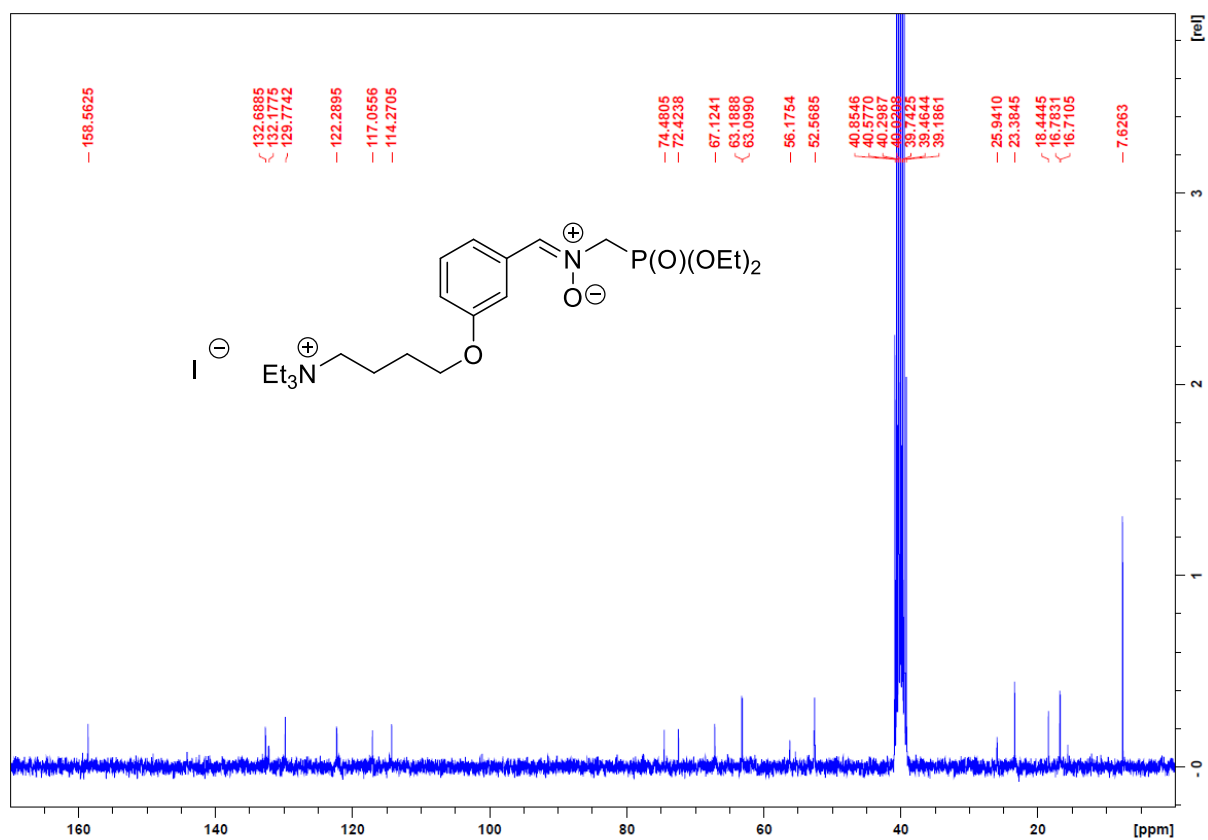
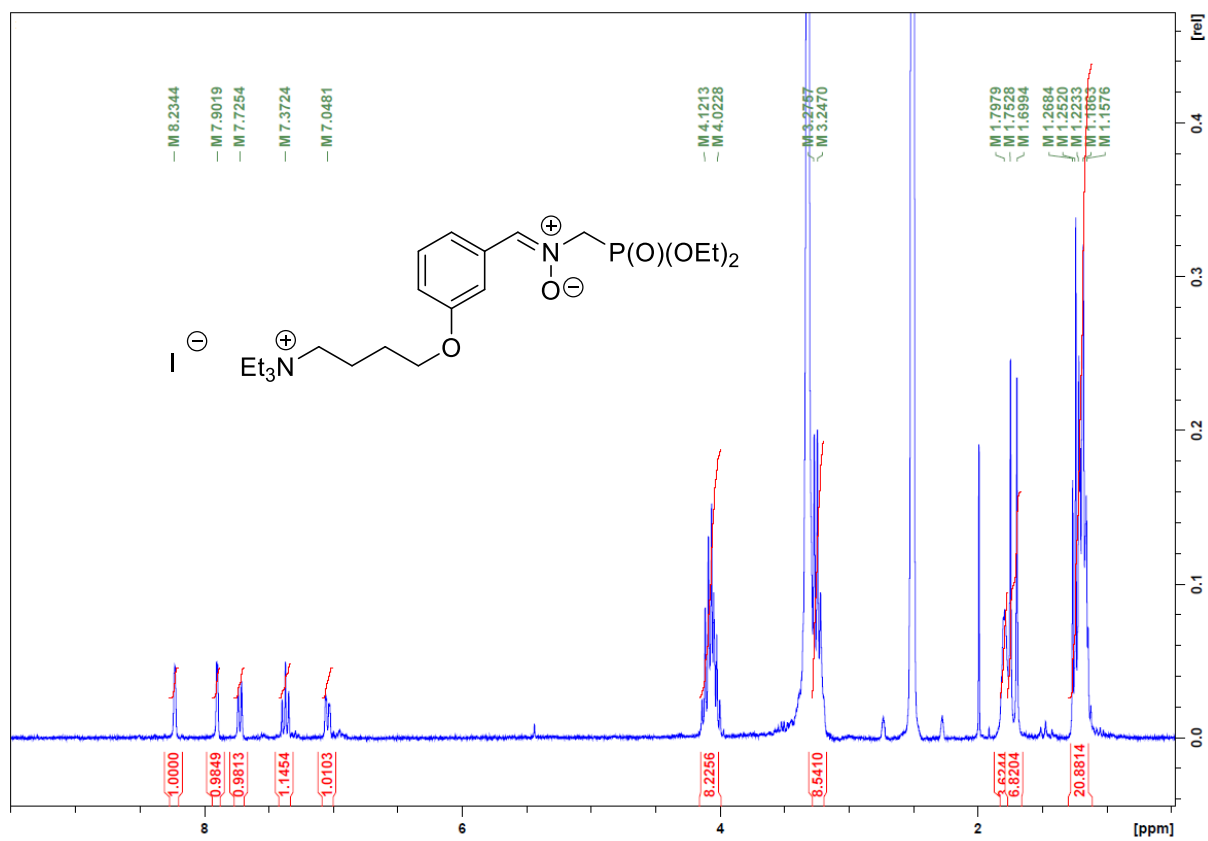
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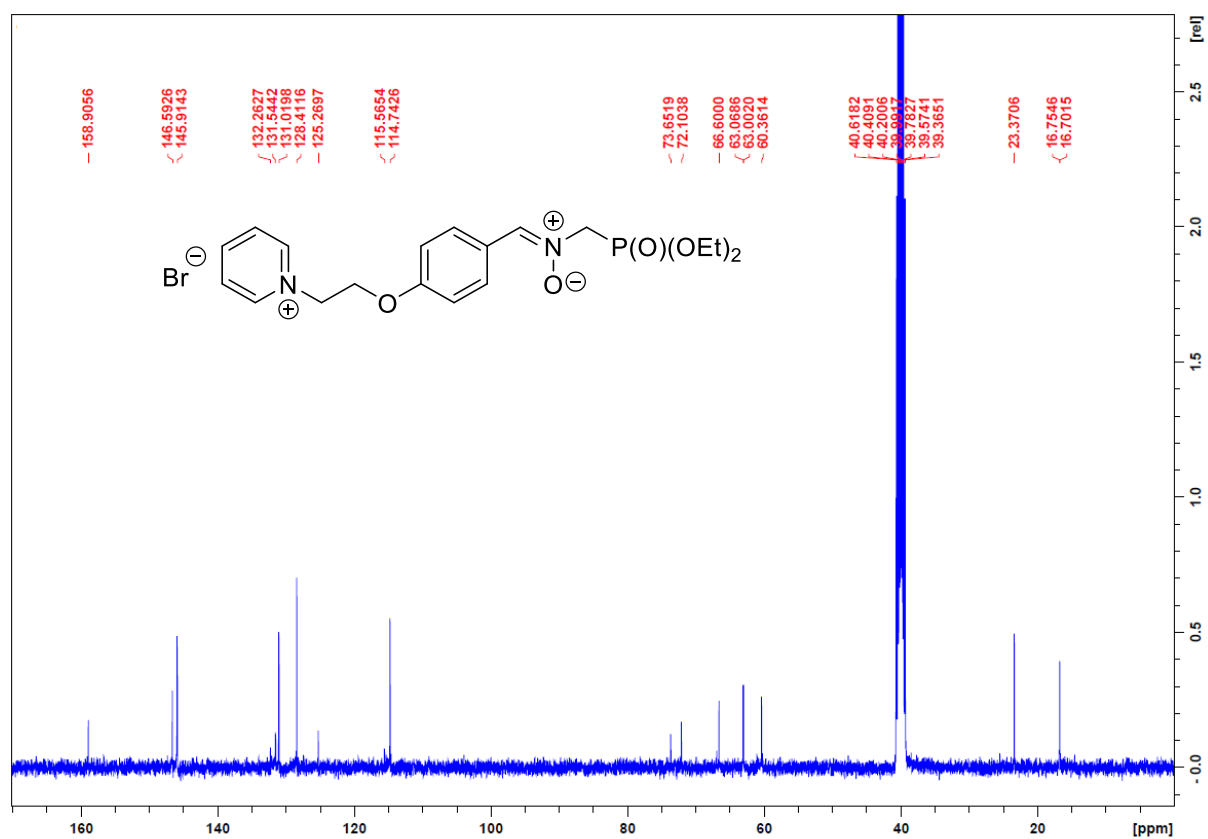
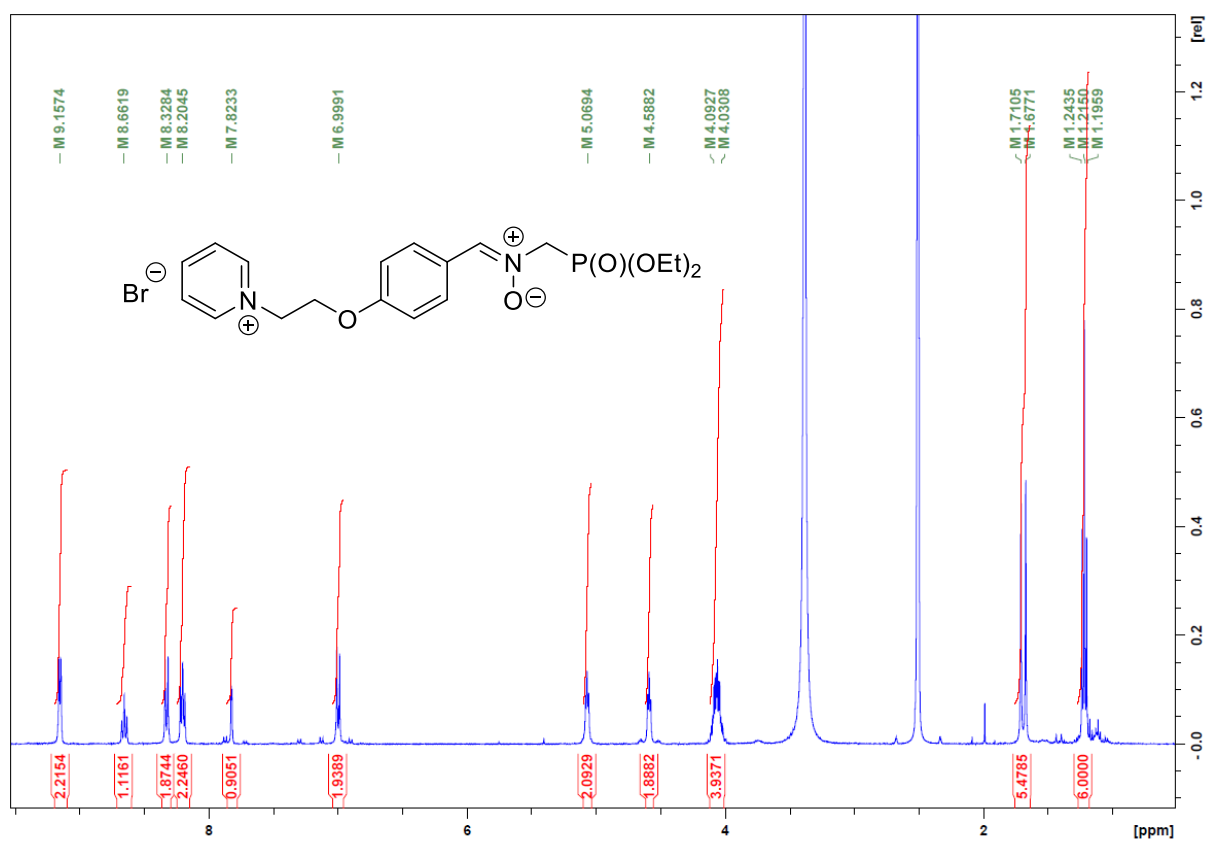
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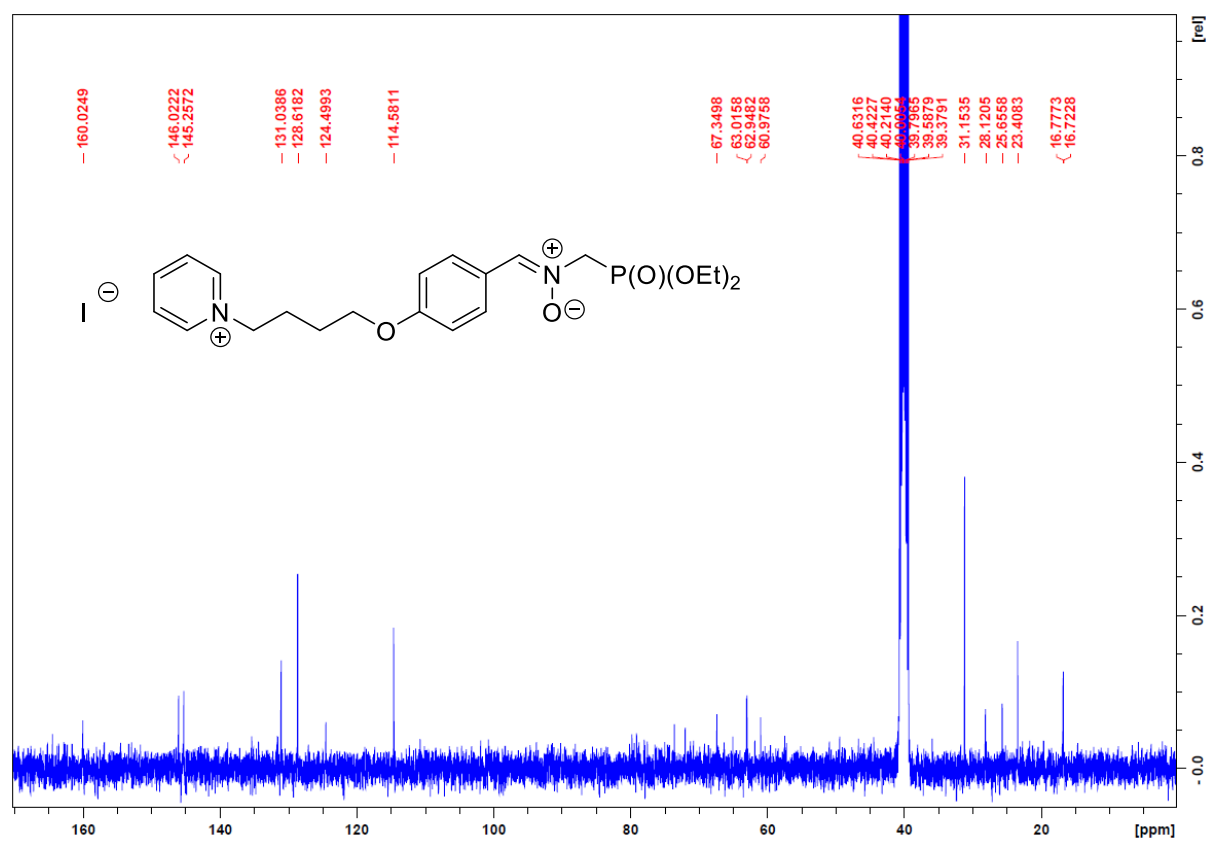
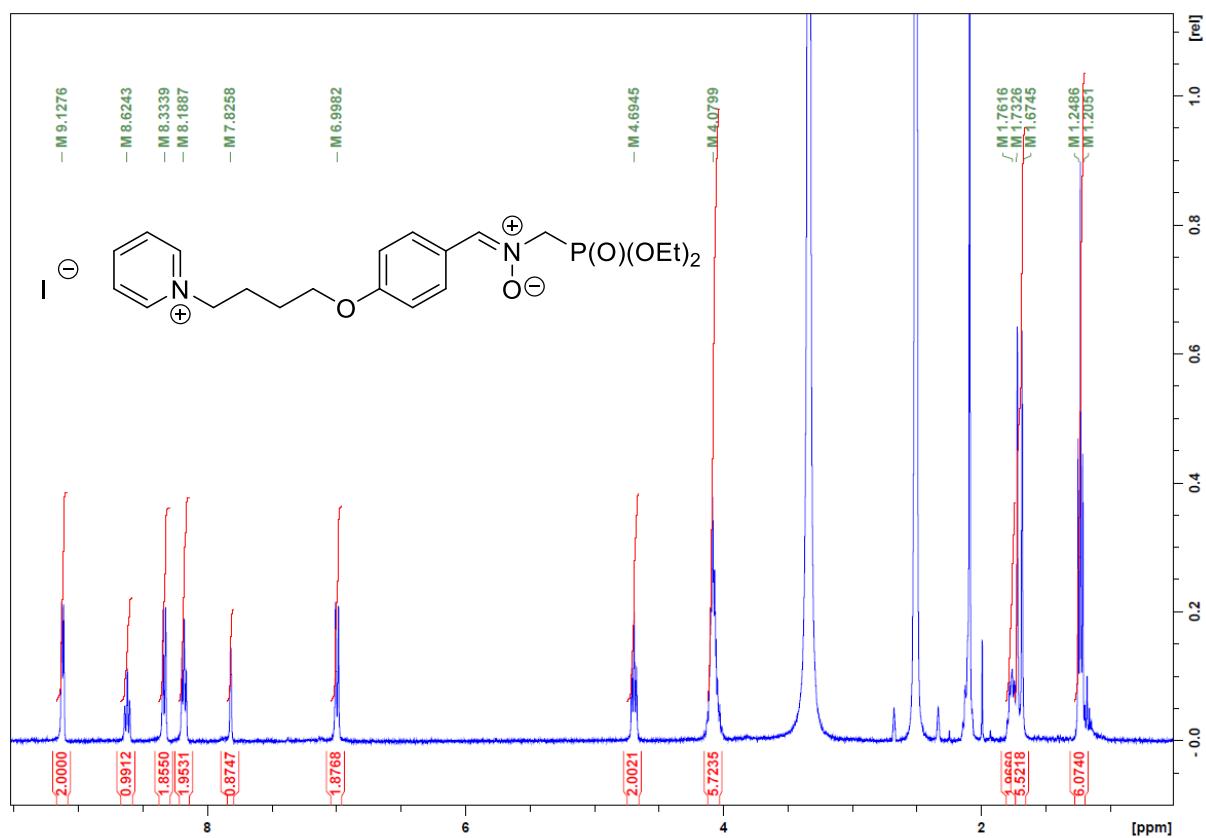
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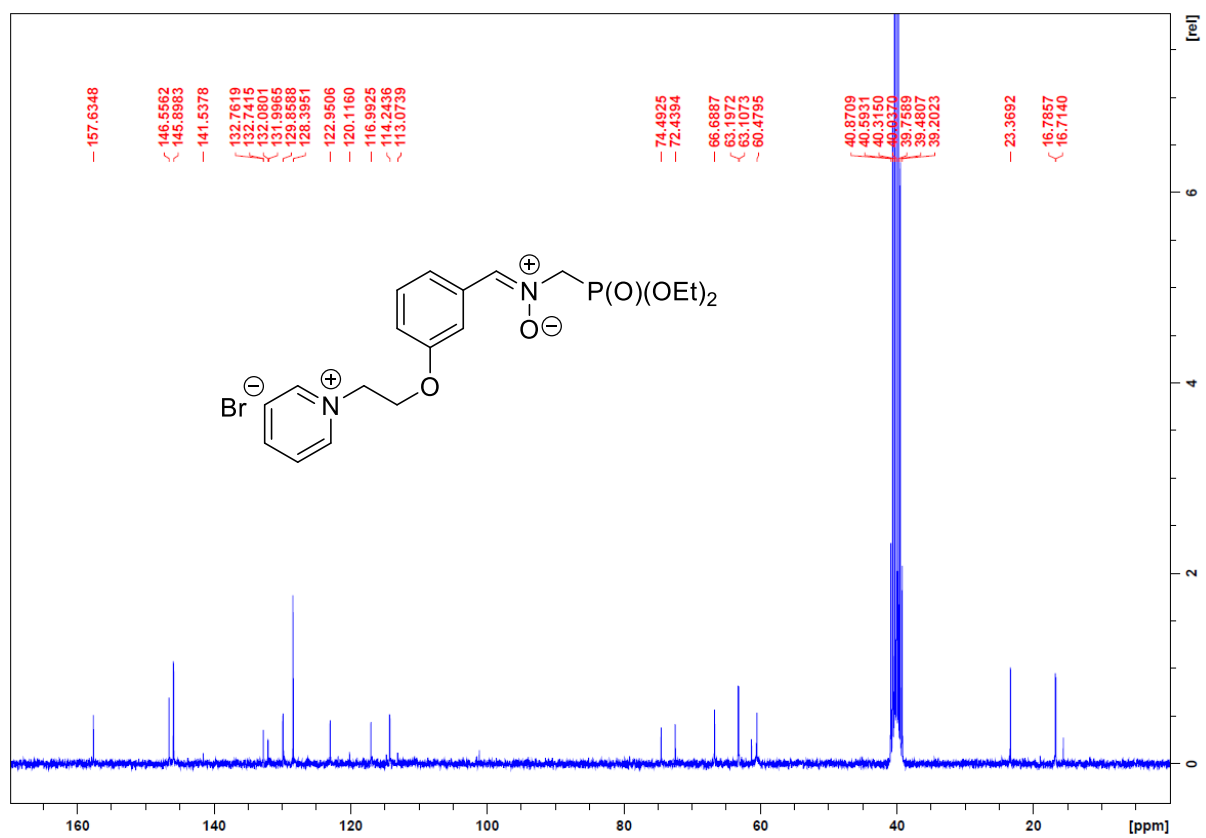
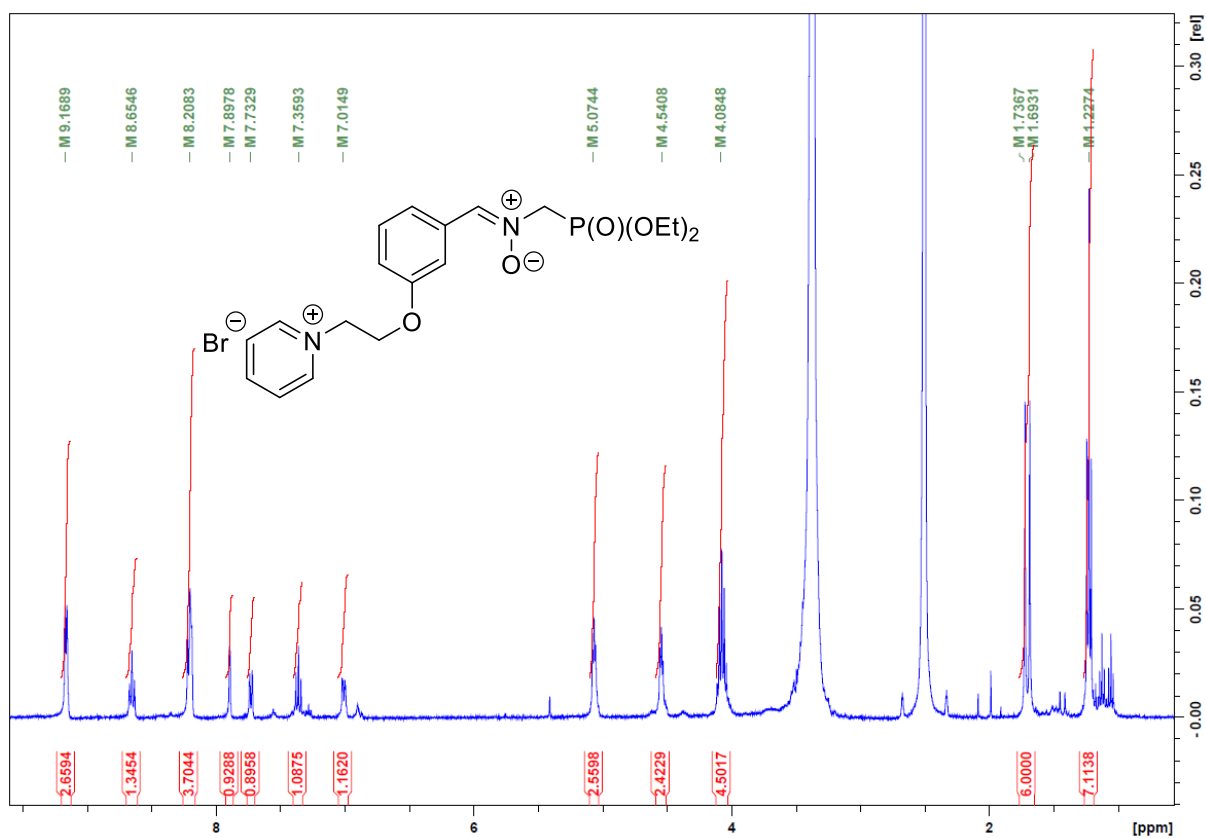
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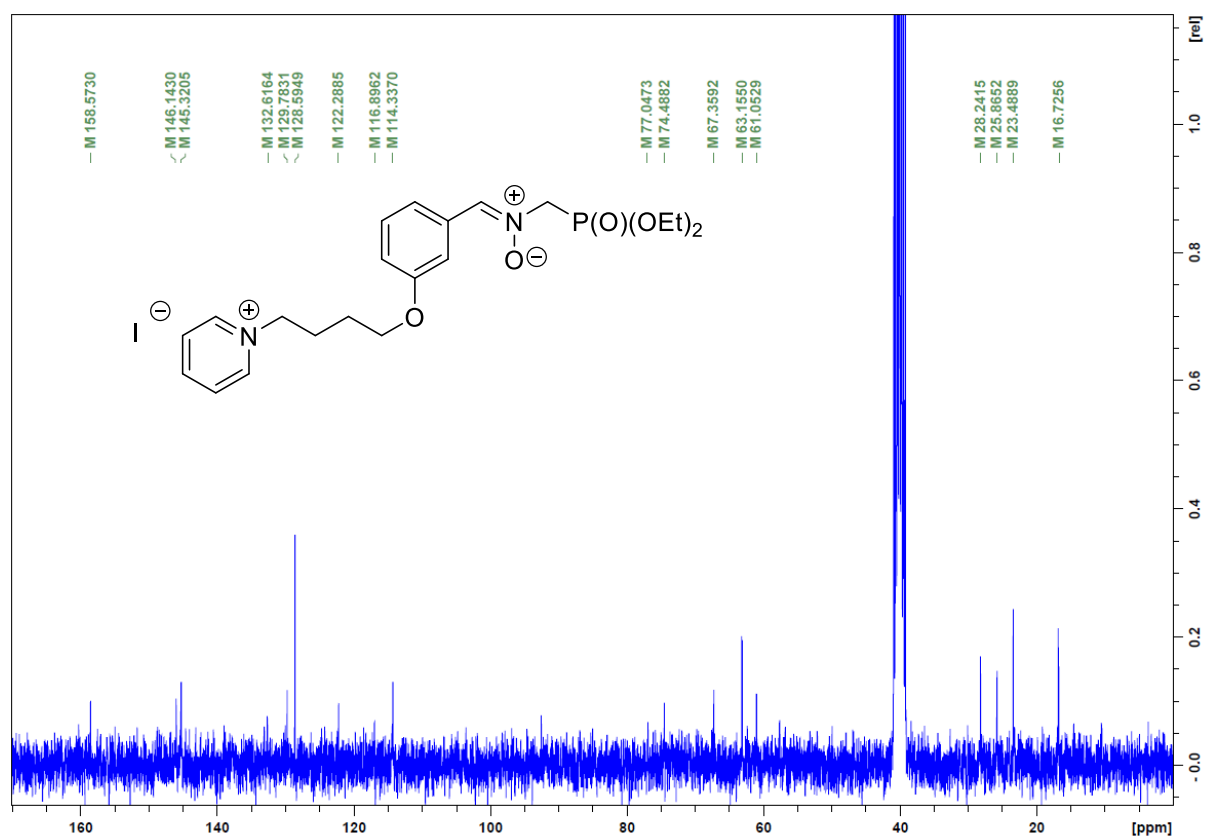
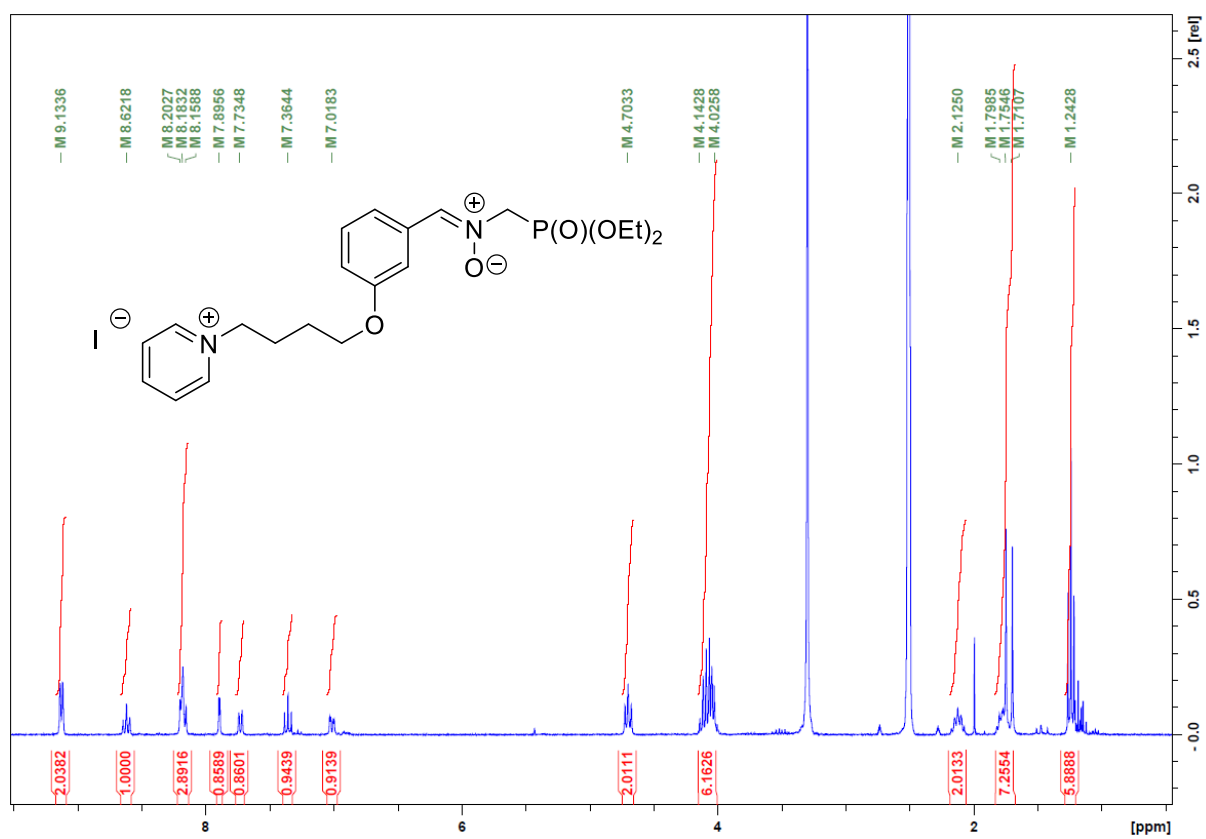
6. Spectra of nitron *12b*



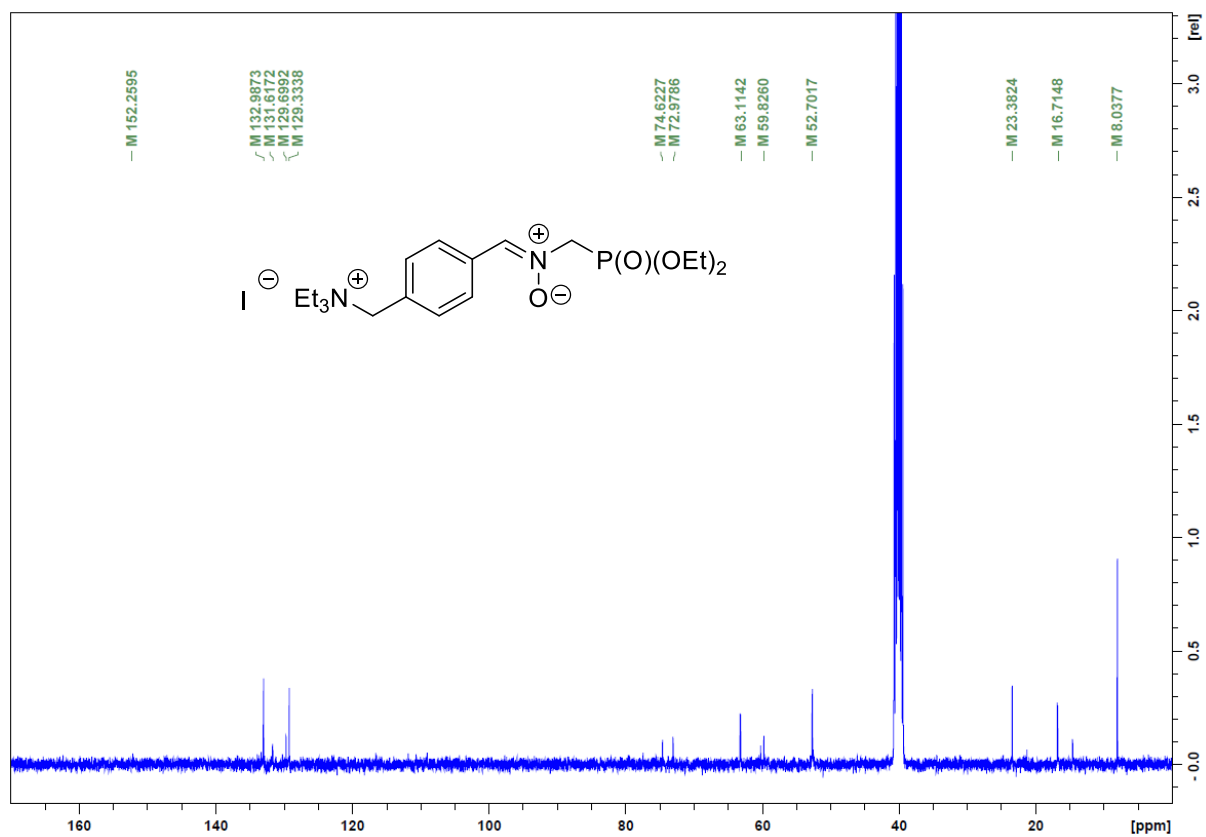
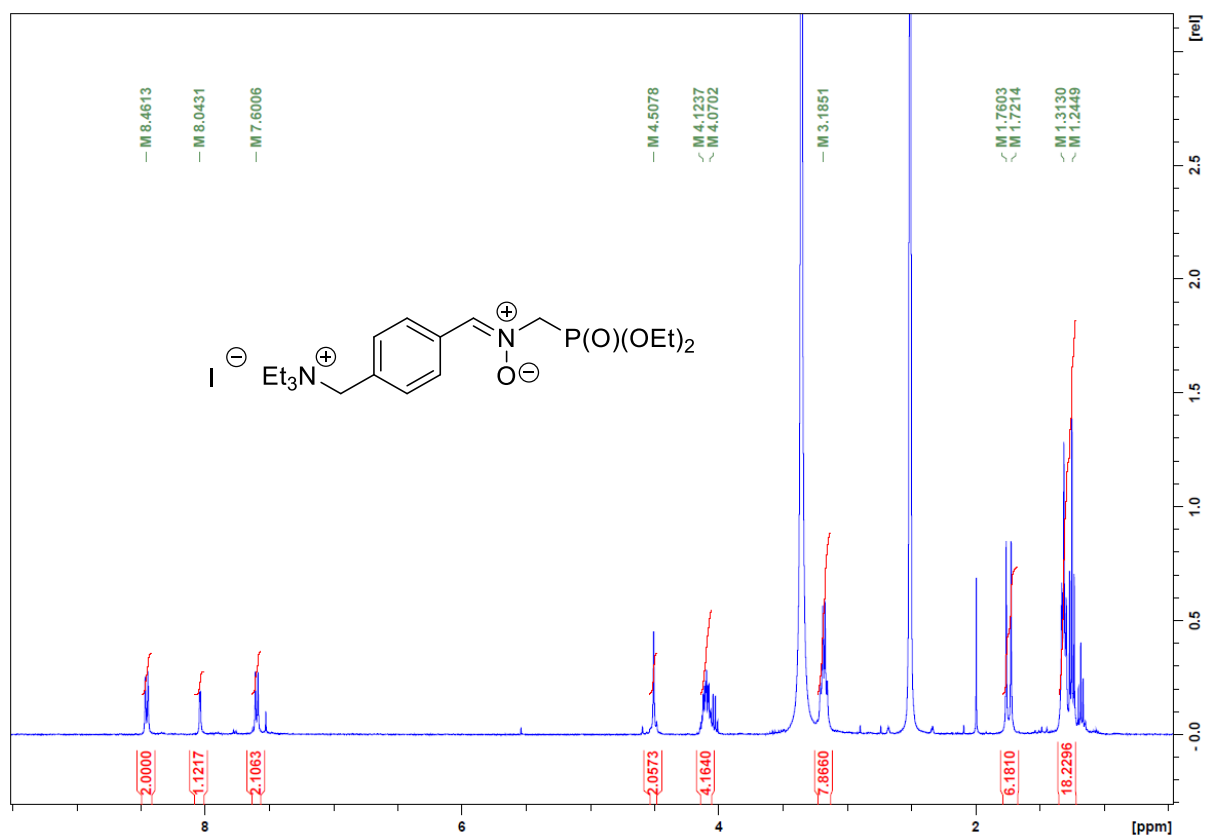
7. Spectra of nitron 12c



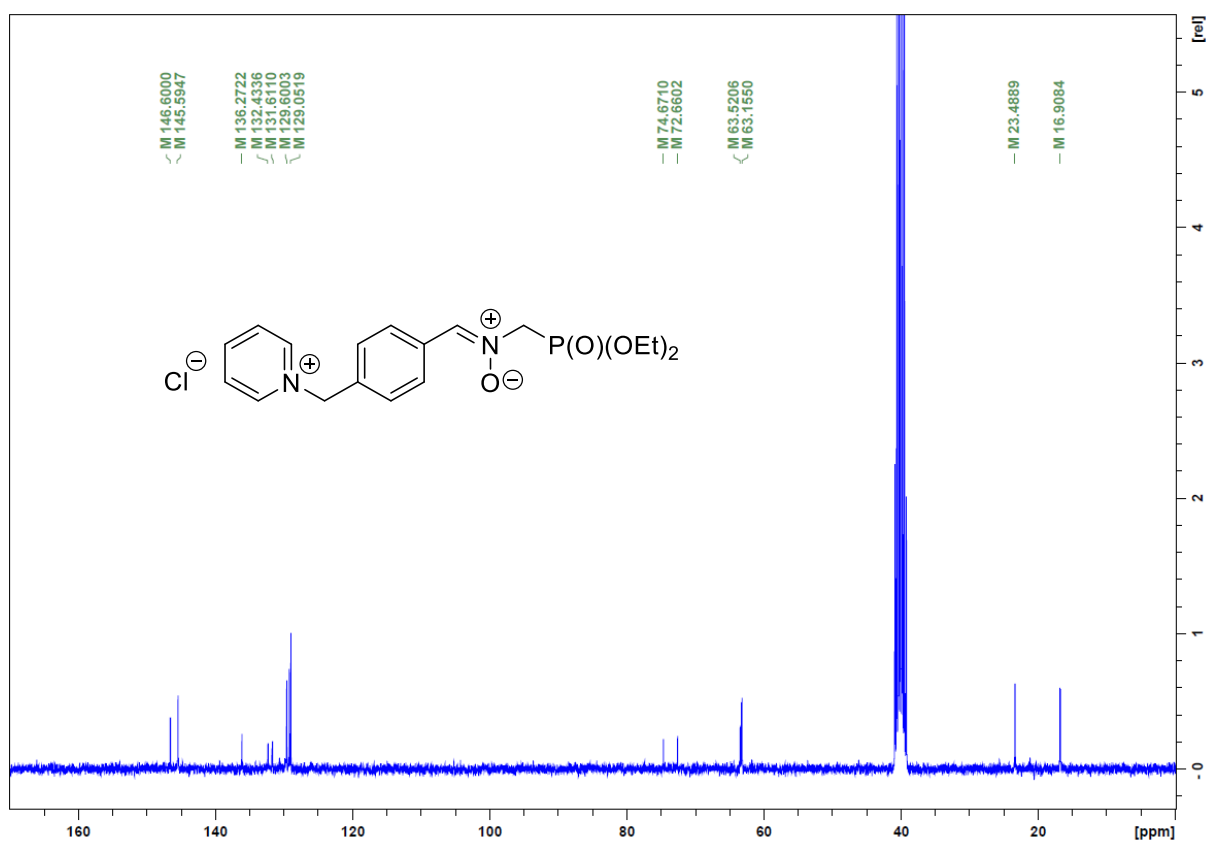
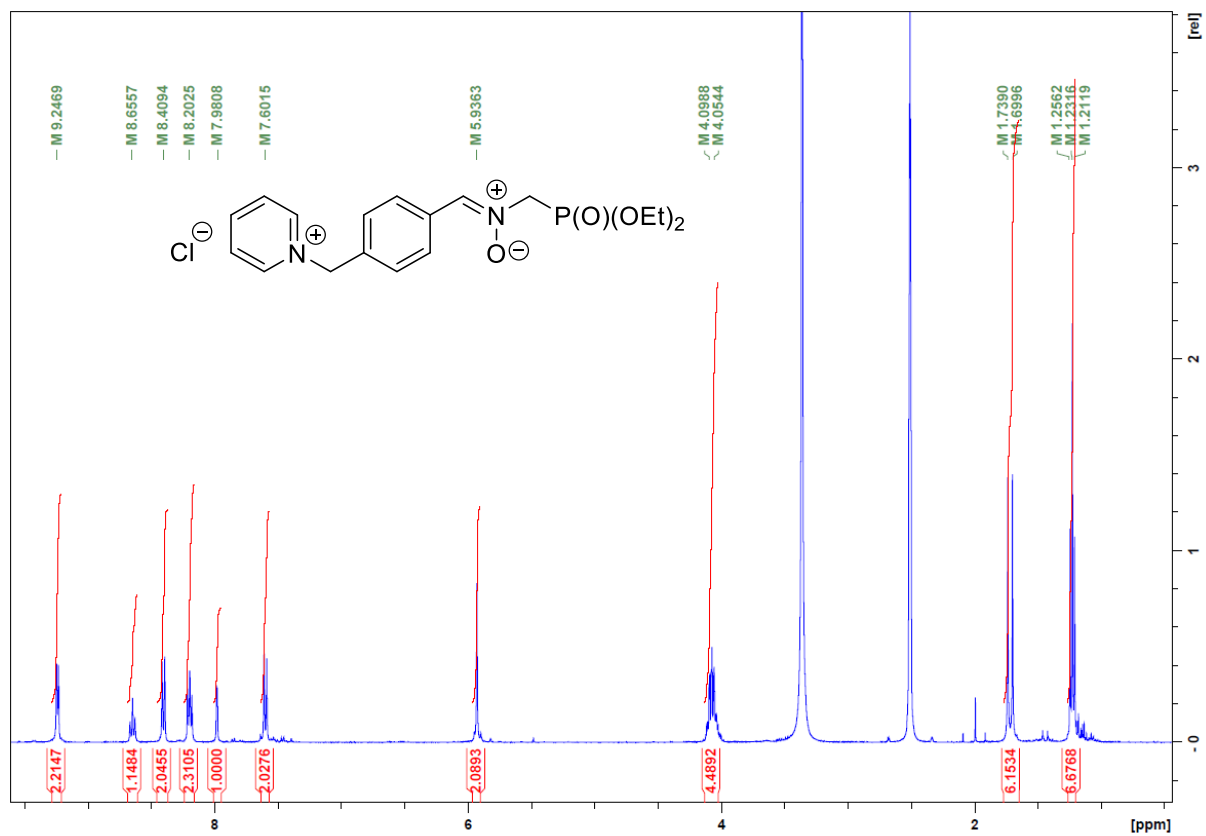
8. Spectra of nitron *12d*



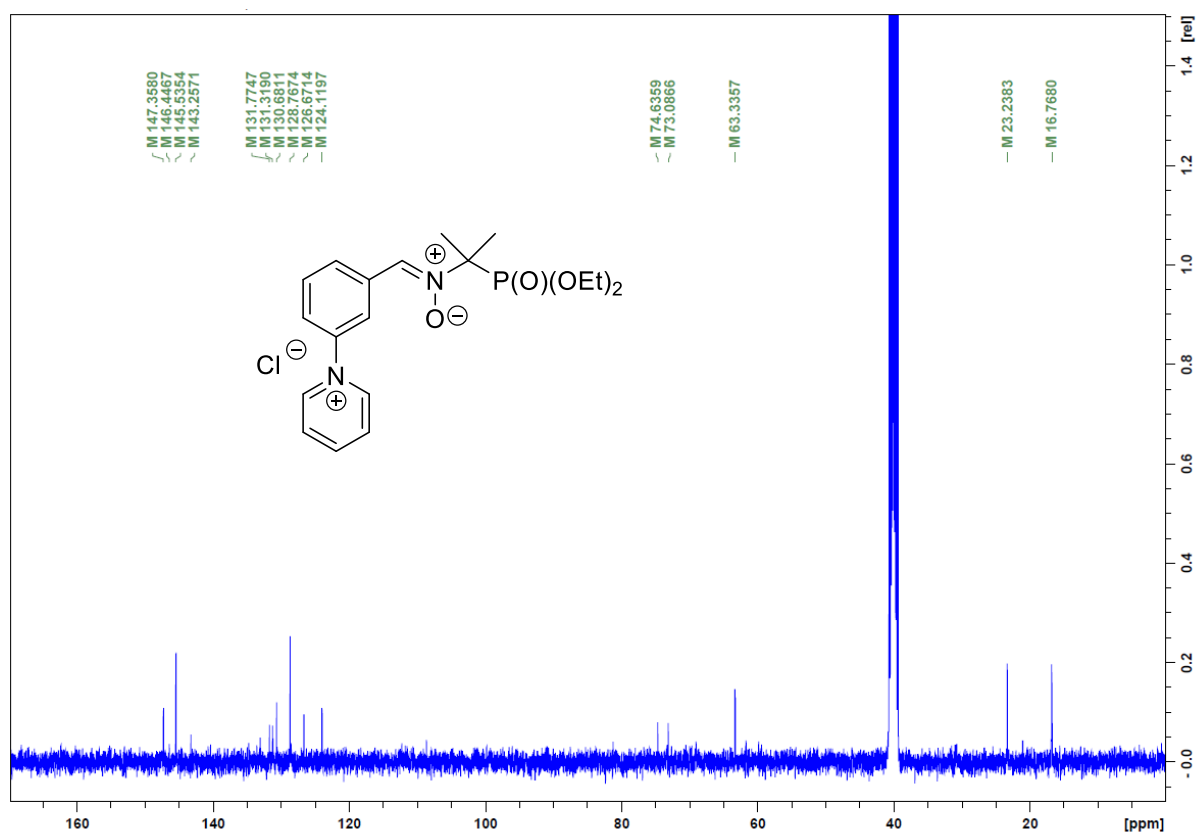
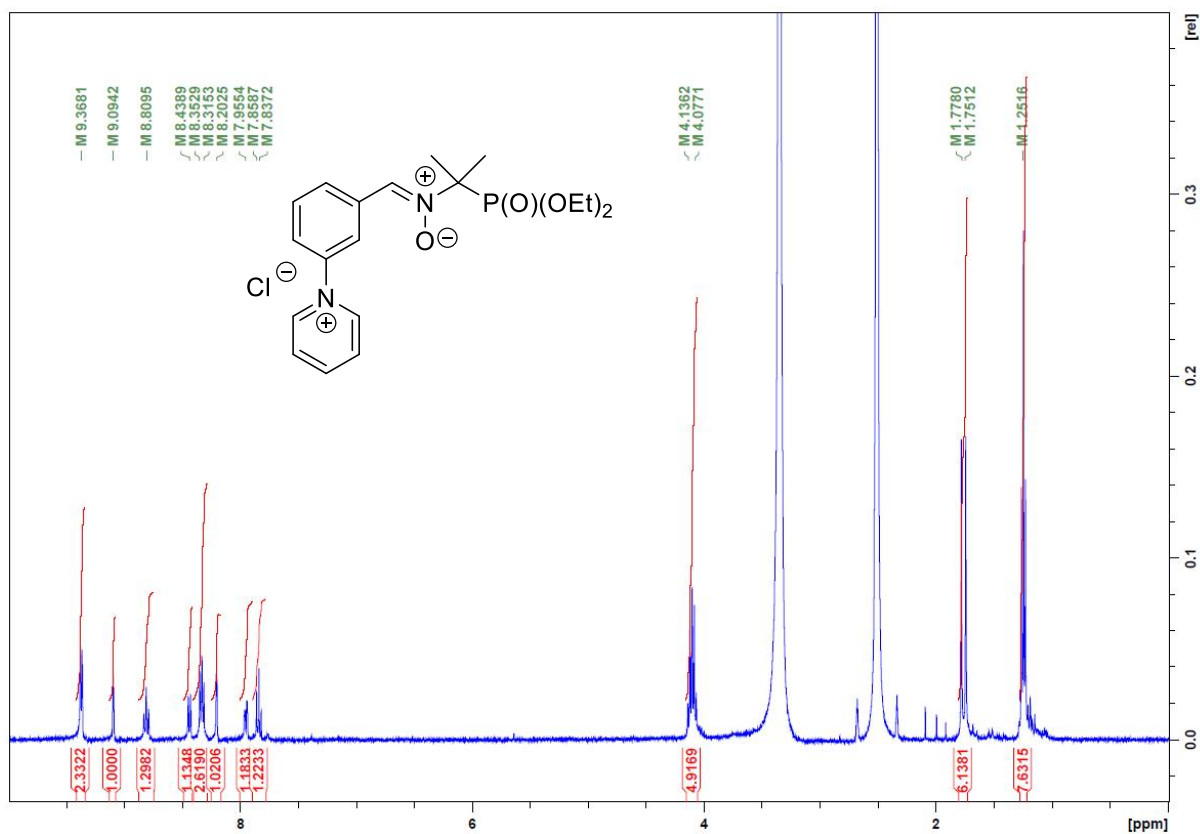
9. Spectra of nitron 13



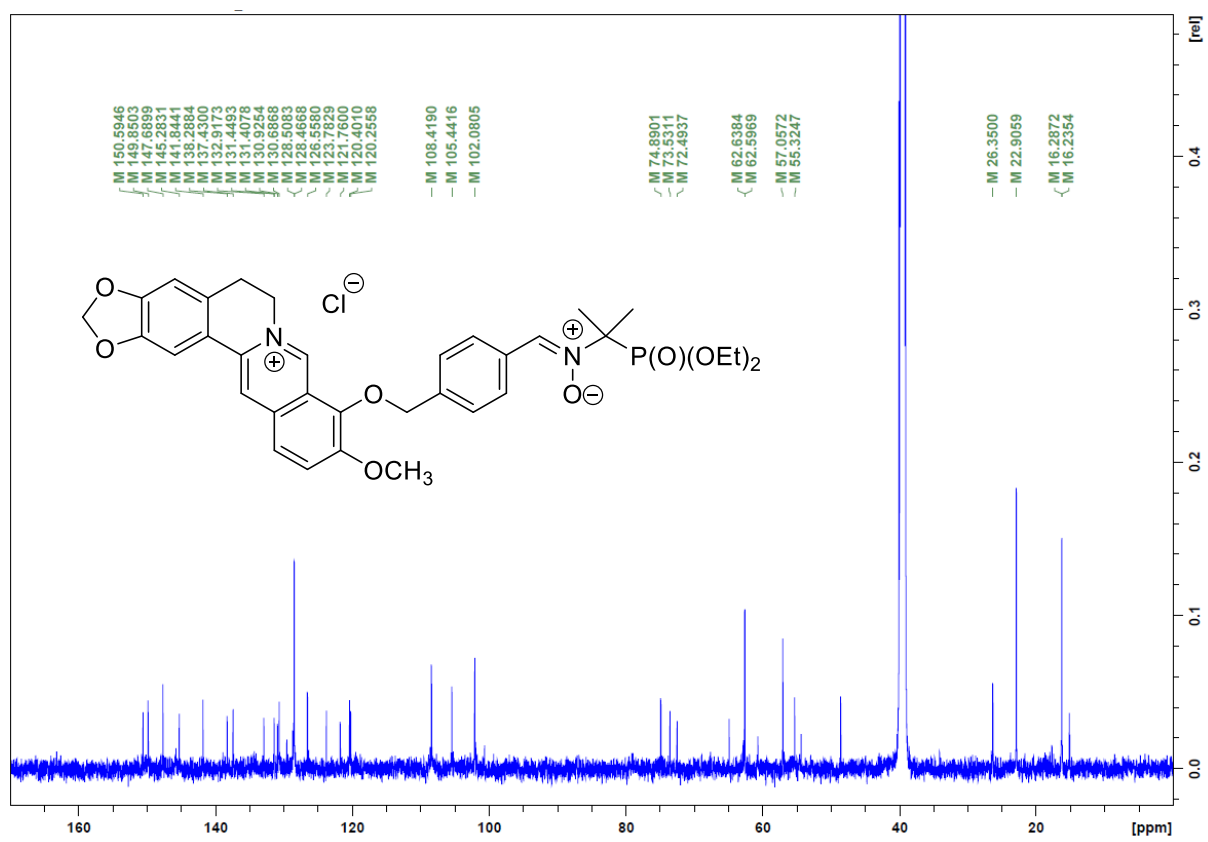
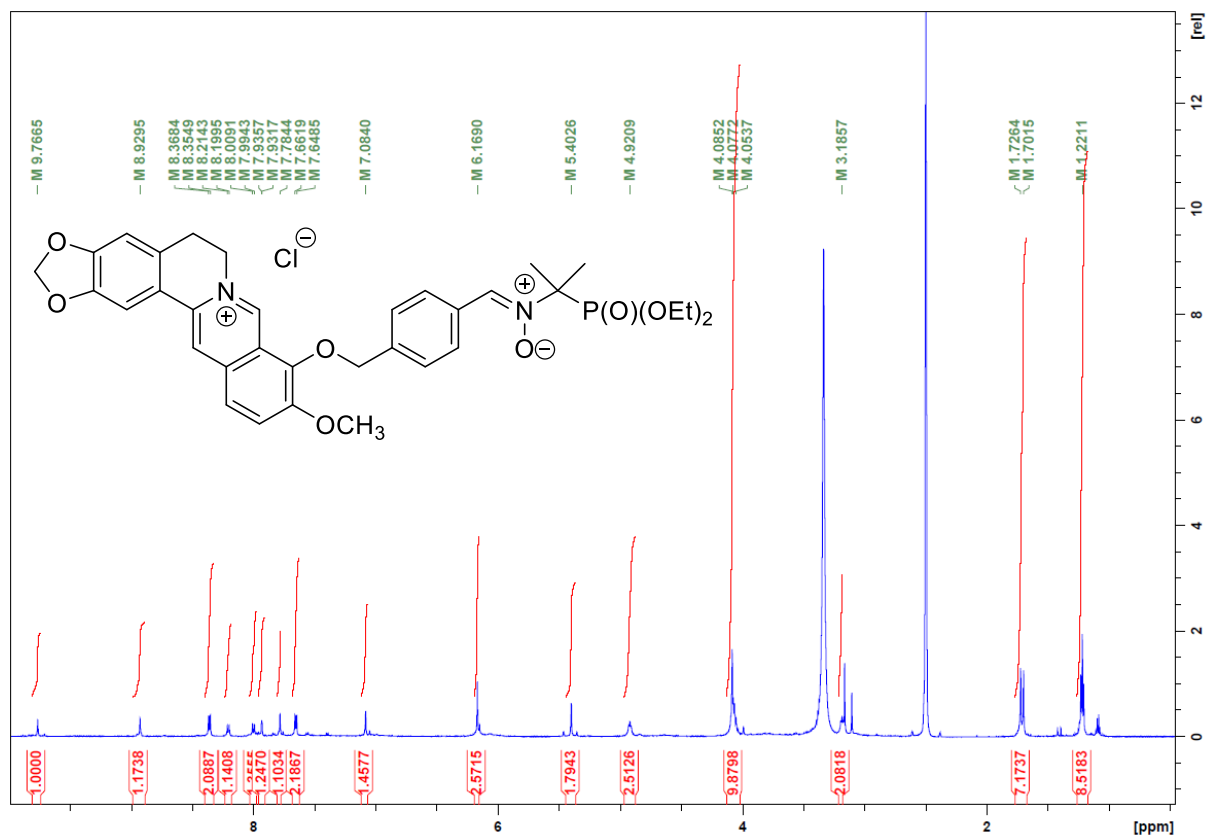
10. Spectra of nitron 14



11. Spectra of nitron 15



12. Spectra of nitron 16



13. Spectra of nitron 17

