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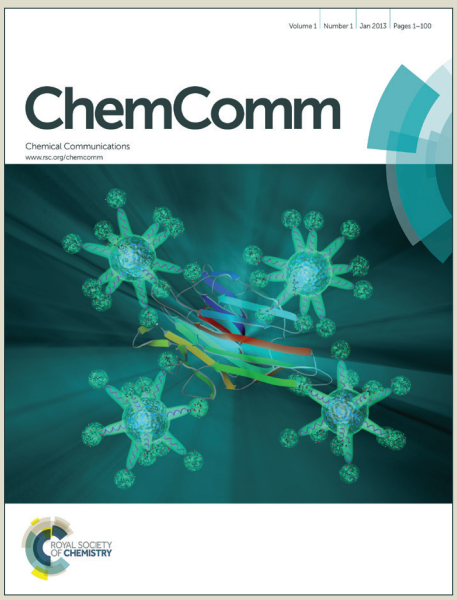

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^{19}F Magnetic Resonance Probes for Live-Cell Detection of Peroxynitrite Using an Oxidative Decarbonylation Reaction

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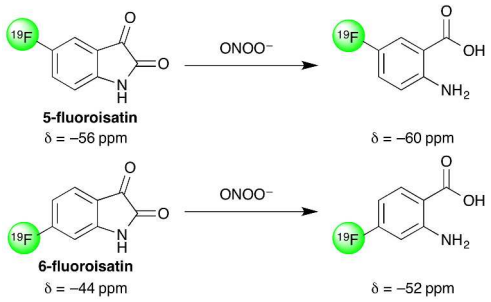
We report a newly discovered oxidative decarbonylation reaction of isatins that is selectively mediated by peroxynitrite ($ONOO^-$) to provide anthranilic acid derivatives. We have harnessed this rapid and selective transformation to develop two reaction-based probes, 5-fluoroisatin and 6-fluoroisatin, for the low-background readout of $ONOO^-$ using ^{19}F magnetic resonance spectroscopy. 5-fluoroisatin was used to non-invasively detect $ONOO^-$ formation in living lung epithelial cells stimulated with interferon-γ (IFN-γ).

Peroxynitrite ($ONOO^-$) is a highly reactive nitrogen oxide species that is formed *in vivo* from the rapid recombination of superoxide (O_2^-) and nitric oxide (NO) radicals.[1] Whereas it is recognized that NO and O_2^- are endogenously generated to mediate cellular signaling,[2] $ONOO^-$ formation is generally believed to be deleterious and is increased in a number of diseases such as cancer,[3] cardiac dysfunction,[4] asthma,[5] and aging-related pathologies. Despite its conspicuous role in many human ailments, there is currently a lack of specific methods for detecting $ONOO^-$ in biological samples due to its short lifetime, competition from endogenous scavengers, and background signal from other reactive species.[6] The current method of choice for studying $ONOO^-$ employs antibodies that stain proteins containing nitrated tyrosine residues, often considered a "footprint" of $ONOO^-$ production.[7] This method has proved valuable for increasing our comprehension of $ONOO^-$ biology, but is an indirect technique and lacks compatibility with living biological specimens. Other methods include using redox-sensitive probes dichlorodihydrofluorescein (DCHF), dihydrorhodamine 123 (DHR 123), and other recently developed fluorescent probes.[8] Although promising, these probes often suffer from a lack of selectivity and a relatively slow rate of reaction with $ONOO^-$.

^{19}F magnetic resonance has emerged as a powerful technique for the detection and imaging of biological analytes due to low background in human cells and tissues.[9] The ^{19}F isotope is present at nearly 100% abundance in terrestrial fluorine and has a gyromagnetic ratio that endows it with sensitivity comparable with the ^{1}H nucleus. Indeed, ^{19}F NMR and MRI have been used for *in vitro* and *in vivo* detection of pH,[10] metal ions,[11] hypochlorite,[12] NO,[13] tissue oxygenation,[14] hypoxia,[15] enzymatic activity,[16] and other proteins of interest.[17] These advantages prompted us to develop two reaction-based ^{19}F magnetic resonance probes, 5-fluoroisatin and 6-fluoroisatin, for the detection of $ONOO^-$ using newly discovered oxidative decarbonylation chemistry (Scheme 1).



Scheme 1. $ONOO^-$-mediated oxidative decarbonylation of 5-fluoroisatin and 6-fluoroisatin.

The oxidative *decarboxylation* of α-ketoacids has been employed as a chemoselective and biologically compatible reaction for detecting H_2O_2 by hyperpolarized ^{13}C MRI.[18] This reaction is entropically favoured and is thought to proceed by a two-electron mechanism.[19] $ONOO^-$, on the other hand, can access one-electron oxidation pathways after forming adducts with carbonyl compounds,[20] offering an opportunity to program selectivity versus other reactive oxygen species. We reasoned that the α-ketocarbonyl of isatins would be stable towards two-electron cleavage of the carbonyl–carbonyl bond, but might be vulnerable to one-electron decarbonylation mechanisms. In

support of this reasoning, isatins are inert to biological levels of H_2O_2, but react instantly with $ONOO^-$ to form a fluorescent anthranilic acid product with an emission maximum centred at 405 nm (Figure 1). We measured an observed second order reaction rate constant of isatin with $ONOO^-$ of $3.5 \pm 0.1 \times 10^4$ $M^{-1}s^{-1}$ at 21 ºC using glutathione (GSH) as a reaction competitor (Figure 1).[21] The combination of the rate constant (k) and the substrate concentration [T], k[T], can be used to compare the reactivity versus other molecules. For instance, for 1 mM isatin at 21 ºC, k[T] = 35 s^{-1}, comparing favourably to CO_2, a key scavenger of $ONOO^-$ in biological systems, which has k[T] = 60–100 s^{-1} under physiological conditions.[22] Encouraged by these properties, we synthesized fluorinated isatin compounds using a previously disclosed sulfur ylide methodology.[23] We confirmed the production of 5-fluoroanthranilic acid in 86% yield from the reaction of 5-fluoroisatin with 1.5 equivalents of $ONOO^-$ by ^{19}F NMR, ^1H NMR, GC/MS, and HPLC analysis (Figure S1).

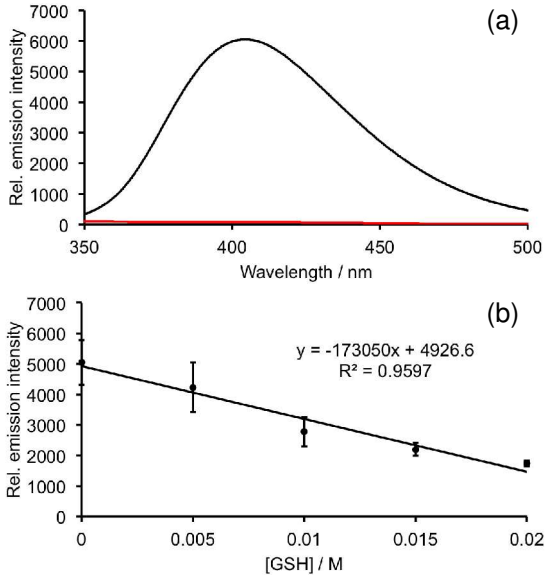


Figure 1. Oxidative decarbonylation of isatin with $ONOO^-$. (a) Fluorescence spectra of 200 µM isatin with 0 µM $ONOO^-$ (red trace) and 20 µM $ONOO^-$ (black trace). (b) 200 µM isatin and 0, 5, 10, 15, or 20 mM GSH was reacted with 20 µM $ONOO^-$ in 100 mM HEPES at pH 7.4 and 21 ºC. Emission spectra were acquired with λ_{ex} = 312 nm and the intensity was measured at 405 nm.

We tested our first generation probes, 5-fluoroisatin and 6-fluoroisatin, for their ^{19}F NMR responses to synthetic $ONOO^-$ (Figure 2).[24] An insert tube containing a 0.1% solution of trifluoroacetic acid (TFA) in D_2O provided a reference signal at –35.6 ppm. With increasing 250 µM increments of $ONOO^-$, the chemical shift changes from –56 to –60 ppm for 5-fluoroisatin ($\Delta\delta$ = 4 ppm) and from –44 to –52 ppm for 6-fluoroisatin ($\Delta\delta$ = 8 ppm). Complete conversion of isatin to anthranilic acid was observed at 1.5 equivalents of $ONOO^-$. Integration of the ^{19}F NMR peaks of the fluoroanthranilic acids and fluoroisatins provide a quantifiable evaluation of the reaction with increasing $ONOO^-$ concentration (Figure S2 and S3).

The ^{19}F NMR responses of these probes were then evaluated for their selectivity for $ONOO^-$ against other reactive

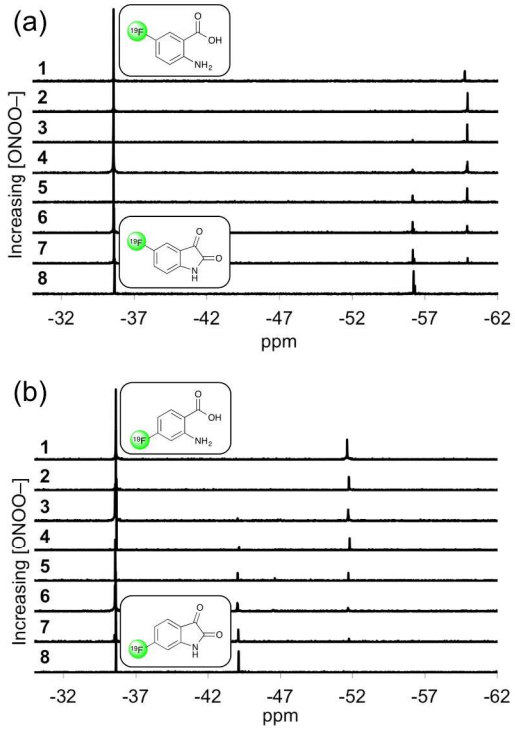


Figure 2. Responses of **(a)** 5-fluoroisatin and **(b)** 6-fluoroisatin to ONOO–. **1.** 1 mM fluoroanthranilic acid. (**2–7**) Reaction of 1 mM fluoroisatin with **2.** 1.5 mM $ONOO^-$ **3.** 1.25 mM $ONOO^-$ **4.** 1.0 mM $ONOO^-$ **5.** 0.75 mM $ONOO^-$ **6.** 0.5 mM $ONOO^-$ **7.** 0.25 mM $ONOO^-$ **8.** 1 mM fluoroisatin. All reactions were performed in 100 mM HEPES at pH 7.4. Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at –35.6 ppm.

sulfur, oxygen, and nitrogen (RSON) species (Figure 3). Both 5-fluoroisatin and 6-fluoroisatin are highly selective for $ONOO^-$ as minimal ^{19}F NMR response was observed with any other RSON species at this concentration. Even at 120 mM H_2O_2, complete conversion to the anthranilic acid was not observed (Figure S4 and S5). We measured a rate constant for the reaction of isatin with H_2O_2 of $3.3 \pm 0.4 \times 10^{-3}$ $M^{-1}s^{-1}$ at 21 ºC, indicating that H_2O_2 reacts with isatin ~10,000,000 times slower than $ONOO^-$ (Figure S6). Higher concentrations of the hydroxyl radical did not result in signal for the fluorinated anthranilic acid, although the isatin peak did disappear (Figure S4 and S5). The selectivity of 5-fluoroisatin for $ONOO^-$ was further investigated using HPLC analysis (Figure S8). Quantification of these experiments revealed that the hydroxyl radical and Angeli's salt (commonly used as a nitroxyl donor) displayed slight reactivity towards 5-fluoroisatin, producing 5-fluoroanthranilic acid in 14% and 22% yields, respectively (Figure S9). However, it should be noted that hydroxyl radical rarely diffuses far from its site of production[1] and Angeli's salt may generate $ONOO^-$ under certain conditions.[25] Taken together, these data demonstrate that the ^{19}F NMR probes 5-fluoroisatin and 6-fluoroisatin display good selectivity for $ONOO^-$ over other RSON species.

Excited by these results, we applied 5-fluoroisatin towards detecting biological $ONOO^-$. When treated with the cytokine interferon-γ (IFN-γ), A549 lung epithelial cells generate NO

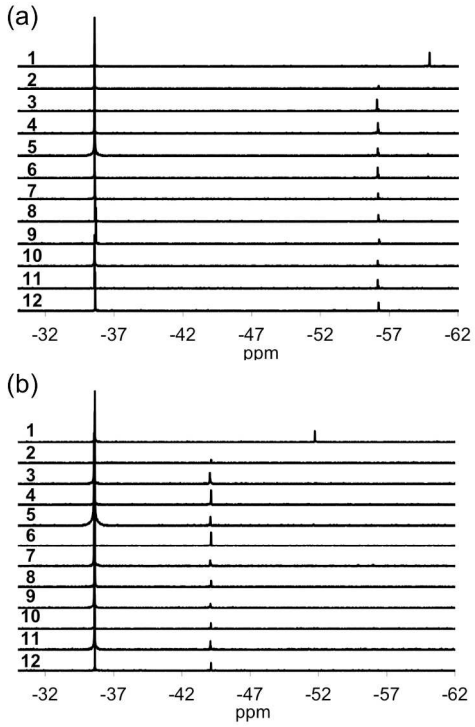


Figure 3. Selectivity of **(a)** 5-fluoroisatin and **(b)** 6-fluoroisatin versus 750 μM RSON species, except for GSH, which was reacted at 5 mM. 500 μM fluoroisatin was reacted with **1.** $ONOO^-$ **2.** •OH **3.** GSH **4.** •NO **5.** $Na_2N_2O_3$ 6. KO_2 **7.** ^tBuOOH **8.** GSNO **9.** NO_2^- **10.** ClO^- **11.** $KHSO_5$ **12.** H_2O_2. All reactions, except **2**, were performed in 100 mM HEPES at pH 7.4. Reaction **2** was performed in 100 mM PBS at pH 7.4 as described previously.[12a] Spectra were acquired with 128 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at –35.6 ppm.

and display increased staining for nitrotyrosine residues.[26] While these observations are consistent with $ONOO^-$ generation, it has never been directly detected in this system. We therefore utilized 5-fluoroisatin to monitor $ONOO^-$ in this cellular model of lung cancer inflammation. We co-incubated ~10^6 A549 cells with 50 μM 5-fluoroisatin and 50 ng/mL IFN-γ or a vehicle control for 6–18 hours to capture live-cell $ONOO^-$ production over this interval. After incubation, a sample of the cellular media was removed and analyzed by acquiring overnight ^{19}F NMR spectra using 7300 scans (Figure 4). With this protocol, anthranilic acid concentrations as low as 1.1 μM can be detected. In the cells treated with IFN-γ, a reproducible increase in the 5-fluoroanthranilic acid peak at –60 ppm is observed, indicating increased $ONOO^-$ production (Figure 4a). Quantifying the peak intensities with reference to the TFA internal standard showed a statistically significant ($p < 0.05$) increase in the production of 5-fluoroanthranilic acid from 1.1 ± 0.3 μM (n = 5) in vehicle treated cells to 2.0 ± 0.6 μM (n = 5) for IFN-γ treated cells (Figure 4b). In order to confirm these results, we used DHR 123, a dye that responds to reactive oxygen species with moderate selectivity for $ONOO^-$,[8e] to provide an independent measure of $ONOO^-$ production in cytokine-stimulated epithelial cells (Figure S10). A clear increase in the intracellular fluorescence is observed, supporting the ability of 5-fluoroisatin to detect cellular $ONOO^-$. This ^{19}F NMR detection of $ONOO^-$ provides direct evidence for the generation of $ONOO^-$ by A549 epithelial cells in response to IFN-γ signalling, confirming and complementing the results of prior investigations.[26]

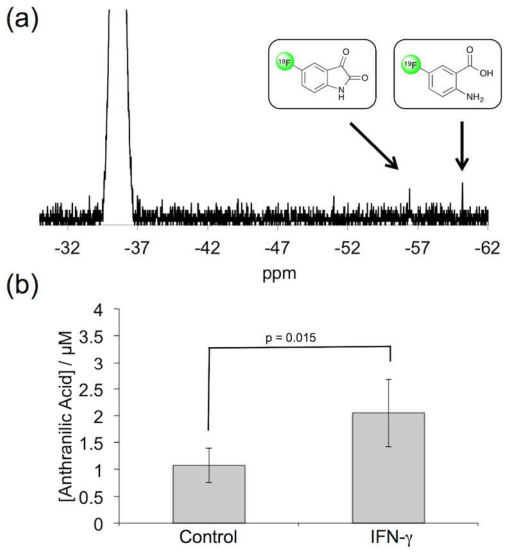


Figure 4. Detection of cellular ONOO–. **(a)** A549 cells were treated with 50 μM 5-fluoroisatin and 50 ng/mL IFN-γ for 12 hours. The media was transferred to an NMR tube and spectra were acquired with 7300 scans at 11.7 T using an internal reference peak of trifluoroacetic acid at –35.6 ppm. **(b)** Quantification of the anthranilic acid concentration determined from ^{19}F NMR peak integrations in A549 cells treated for 6–18 hours with 50 μM 5-fluoroisatin and 0.1% BSA as a vehicle control, n = 5 (Control) or 50 ng/mL IFN-γ, n= 5 (IFN-γ). Statistical analysis was performed using a two-tailed Student's t-test to give p = 0.015. Error bars are ± S.D.

To conclude, we have communicated a newly discovered isatin decarbonylation reaction that is mediated by $ONOO^-$ under physiological conditions. This rapid and biologically compatible transformation was utilized to develop two new ^{19}F NMR probes, 5-fluoroisatin and 6-fluorisatin, for the chemoselective detection of $ONOO^-$ over other RSON species. Using 5-fluoroisatin to measure $ONOO^-$ produced by living A549 lung epithelial cells in response to cytokine stimulation, we detected an increase in $ONOO^-$ over vehicle controls that matched the trend observed with the fluorescent dye DHR 123. This method non-invasively samples $ONOO^-$ in the cellular media, and we anticipate that this study will give rise to a new class of reaction-based $ONOO^-$ probes.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedure, cell culture conditions, and supplementary spectral data. See DOI: 10.1039/b000000x/

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