Understanding the Selectivity of a Multi-Channel Fluorescent Probe for Peroxynitrite Over Hypochlorite

Quanjuan Zhang,† Na Zhang,§ Yi-Tao Long,§ Xuhong Qian†,‡ and Youjun Yang†,‡,*

† State Key Laboratory of Bioreactor Engineering, ‡ Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, § Department of Chemistry, East China University of Science and Technology, Meilong Road 130, Shanghai 200237, China

youjunyang@ecust.edu.cn

ABSTRACT: Peroxynitrite is a prominent biological reactive nitrogen species from radical combination of nitric oxide and superoxide and fundamentally involved in a broad spectrum physiological and pathological processes. Though redox-inert itself, peroxynitrite anion ($OONO^-$) attacks various biological electrophiles to generate an array of potent 2-e^- or 1-e^- oxidants, which result in cell injuries. Development of fluorescent probes for peroxynitrite, free from interferences from hypochlorite, has been an active endeavor of the chemical community. We previously reported a peroxynitrite probe (**PN600**), which could differentiate hypochlorite from peroxynitrite through a multi-channel signaling mechanism. Herein, this intriguing selectivity was accounted for through a structure-reactivity relationship study. Also, this work, together with rich literature contributions, has allowed a qualitative guideline in the use of electron-rich aromatic moieties to design probes against peroxynitrite and/or hypochlorite. The viability of this guideline was further testified by development of another list of peroxynitrite selective probes.

INTRODUCTION

Peroxynitrite ($OONO^-$) biology dates back to 1986 when superoxide ($O_2^{\bullet-}$) was shown to modulate the half-life of the endothelial derived relaxing factor (EDRF), which was identified to be nitrogen monoxide (or nitric oxide, NO) next year.[1-6] Formation of $OONO^-$ from near-diffusion controlled radical combination of NO and $O_2^{\bullet-}$ was regarded as merely a cell-detoxification mechanism of NO at the time, until Beckman suggested in 1990 that potent oxidants far more toxic than NO and "*similar to hydroxyl ($HO^{\bullet}$) in reactivity*" were effectively derived from $OONO^-$.[7-10] Much of the apparent oxidative cytotoxicity of NO, i.e. antibacterial and antimicrobial activities, dysfunction of mitochondrial respiratory chain etc., was found to be actually mediated by peroxynitrite.[11-15] Immune cells harness peroxynitrite against cancer cells or other invading pathogens for the benefit of host.[16-18] However, under pathological conditions such as autoimmune response, ischemic-reperfusion or chronic inflammation, cytotoxicity of peroxynitrite is diverted against the innocent host cells.[19-21]

Peroxynitrite unleashes its oxidative capacity and cell damages through multifaceted biochemical pathways, including two-electron (2-e^-, or polar) or one-electron (1-e^- or radical) oxidation (Figure 1).[22] Thermodynamically, $OONO^-$ is undoubtedly a strong oxidant, being 36 kCal/mol higher in free energy than its structural isomer nitrate (NO_3^-), due to the presence of a weak O-O bond.[23] However, $OONO^-$ itself is in fact quite redox-inactive and functions not as an oxidant, but as a pro-oxidant.[19,24-25] For $OONO^-$ to exhibit its oxidative capacity, a nucleophile would have to attack the terminal oxygen atom, which is negatively charged, to displace NO_2^- as the leaving group. However, a high kinetic barrier exists because the negative charge on this terminal oxygen tends to repel the incoming nucleophile. In fact, $OONO^-$ only functions as a nucleophile in biological matrices and reacts with nothing but electrophiles, including H^+, CO_2 or various metal ion centers, to yield HOONO (pK_a = 6.8), $ONOOCO_2^-$ or metal-$OONO^-$ complexes respectively.[26-36] In sharp contrast to $OONO^-$, HOONO is a potent electrophilic, readily incorporates a nucleophilic attack to break the weak O-O bond and therefore is a potent 2-e^- oxidant.[37-40] HOONO has a reduction potential of +1.4 V ($OONO^-$, $2H^+/NO_2^-$, H_2O) and efficiently oxidizes thiols, thioethers and their selenium analogs.[37] A fraction of HOONO homolyzes to $HO^{\bullet}$ and NO_2, albeit with a low rate constant.[29,41-42] Homolysis of $ONOOCO_2^-$ or $OONO^-$-metal complexes is more

efficient in generating radicals, compared to HOONO.[43-50] Depending on cell localizations, the resulting highly cytotoxic one-electron oxidants, i.e. $HO^{\bullet}$, NO_2, and $CO_3^{\bullet-}$, may be scavenged by polyenes, vitamin E, BH_4, GSH, melatonin, urate or ascorbic acid.[51-58] However, under pathological conditions when cellular redox buffering capacity is overwhelmed, lipid modification[59-61] via peroxidation/nitration, protein tyrosine nitration[62-69], DNA oxidation to nucleobase/deoxyribose,[70-72] or DNA strand breakage[73-74] builds up and cell death is often inevitable. The bio-relevance, elusive mechanism and associated pharmacological opportunities of peroxynitrite in health, disease and aging have underscored the significance of its detection.[75-76]

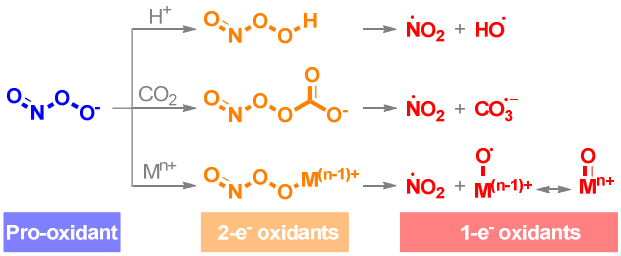

Figure 1. Focused peroxynitrite biochemistry.

Fluorescence based techniques are advantageous for biological studies due to the paramount sensitivity, instrumentational versatility and convenient implementation to complex biological systems. A number of small molecule fluorescent probes for peroxynitrite have been reported, mainly for *in vitro* applications. In accordance with the multiplicity of peroxynitrite biochemistry, the detection mechanisms of these probes are not uniform. The trifluorocarbonyl (**HKGreen1-3**),[77-80] phenyl boronic esters (**CBA**, or their free acids),[81-84] or vicinal dicarbonyl[85-86] based probes bear an electrophilic center and directly detect $OONO^-$ (Figure 2A). Hydrozine[87-88] and selenium[89-92] based probes detect HOONO (Figure 2B and 2C). The probes (**NiSPY**s) were designed to respond to the nitrative capacity of peroxynitrite (Figure 2D).[93-94] *In vivo* tyrosine nitration occurs via radical pathway[68-69], but nitration of NiSPy has not been mechanistically elucidated. Some other probes, including **DHR 123**, **APF**, **HKGreen4**, and our **PN600**, were based on an electron-rich aromatic moiety, which may be oxidized by HOONO, or alternatively by those secondary radicals derived from peroxynitrite (Figure 2E).[95-102]

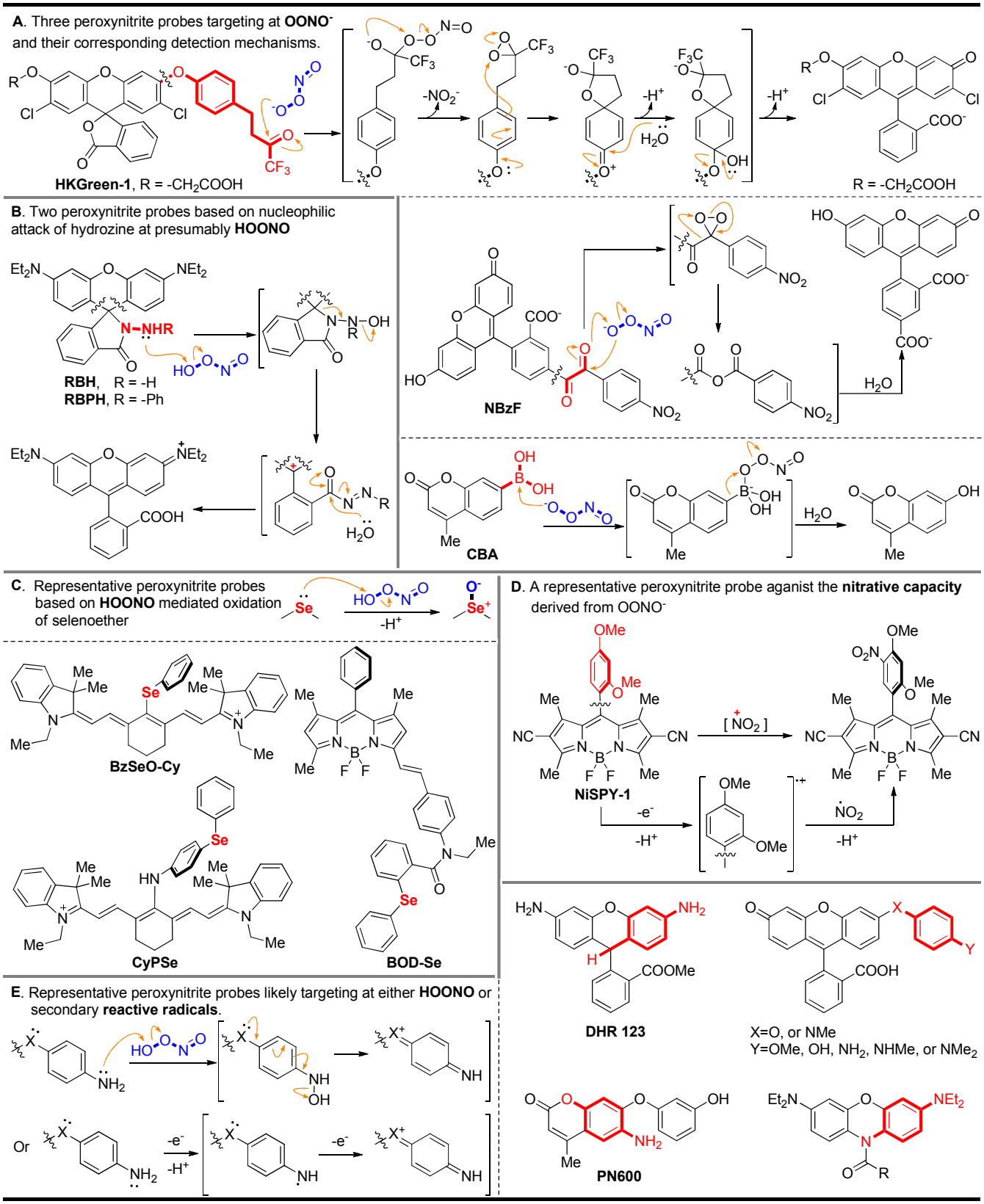

Figure 2: Classification of existing peroxynitrite probes by their detection mechanisms.

A major challenge in developing a peroxynitrite probe lies at potential cross-reactivity toward hypochlorite.[96-97,99-100,103-114] We previously discovered that **PN600** was able to differentiate peroxynitrite from hypochlorite via a facile multi-channel ratiometric signal.[101] Its differential reactivity toward hypochlorite and peroxynitrite is herein mechanistically explained. Further, a qualitative guideline was tentatively proposed regarding the use of an electron-rich benzene moiety in detection of hypochlorite and peroxynitrite.

We note that the term "peroxynitrite" has been conventionally used in a collective fashion in literature to include both the anionic $OONO^-$ and its conjugate acid, peroxynitrous acid (HOONO). Also, damages from secondary species derived from $OONO^-$, including nitrosoperoxycarbonate ($ONOOCO_2^-$), hydroxyl radical ($HO^\bullet$), nitrogen dioxide radical (NO_2), and carbonate radical anion ($CO_3^{\bullet-}$) were often generically attributed to "peroxynitrite". Throughout the discussions in this manuscript, these conventional and generic uses of "peroxynitrite" are followed. However, "$OONO^-$" is used in this manuscript when such a generic sense does not apply, as an effort to avoid ambiguity. The term "hypochlorite" is also used in a collectively sense to include both HOCl and OCl^- unless noted.

RESULTS AND DISCUSSIONS

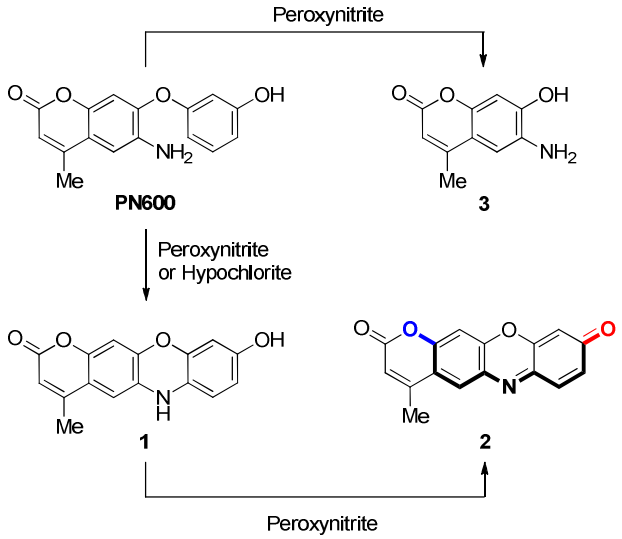

Figure 3: Overall reactivity of **PN600** toward peroxynitrite and hypochlorite.

The overall reaction scheme of PN600 toward peroxynitrite and hypochlorite. In the preceding communication, **PN600** was found to be oxidized to the red-emitting **2**, via the orange-emitting intermediate **1**, upon addition of peroxynitrite. In comparison, oxidation of **PN600** by addition of hypochlorite stopped at **1**. While investigating the oxidation mechanism of **PN600** by peroxynitrite and hypochlorite, an additional fluorescent species, with a maximum excitation wavelength at 380 nm and emission at 450 nm, was also found to have been generated upon addition of an aliquot of peroxynitrite stock solution, but not hypochlorite (*vide infra*). This fluorescent species was determined to be 6-amino-7-hydroxy-4-methylcoumarin (**3**), as its absorption and fluorescence spectra were identical with those of the independently synthesized compound **3**. Therefore, a revised-overall reactivity of **PN600** toward peroxynitrite and hypochlorite is now summarized in Figure 3.

Design and synthesis of PN600 analogs. Intuitively, the reactivity of **PN600** toward an oxidant is bestowed by the presence of its electron donating groups, which include a hydroxyl (-OH) and an amino ($-NH_2$) group. Yet, it is not obvious how the -OH and $-NH_2$ have determined the reactivity of **PN600**. We envisaged that a focused structure-reactivity relationship should provide mechanistic insights toward **PN600** oxidation and therefore synthesized an additional list of probes, i.e. **4a**, **4b**, and **4c**. Compounds (**4a**-**c**) were designed by changing the -OH of **PN600** into a less electron-donating hydrogen (-H), methoxy (-OMe) group, or a more electron-donating $-NH_2$ group respectively. Probes (**4a-c**) were conveniently synthesized in two steps (Figure 4). Ullmann type condensation between 7-fluoro-4-methyl-6-nitrocoumarin (**5**) and variously substituted phenols (**6a-c**) afforded **7a-c** smoothly without resorting to forcing conditions. Catalytic hydrogenation of **7a-c** furnished **4a**-**c**, the desired analogs of **PN600**.

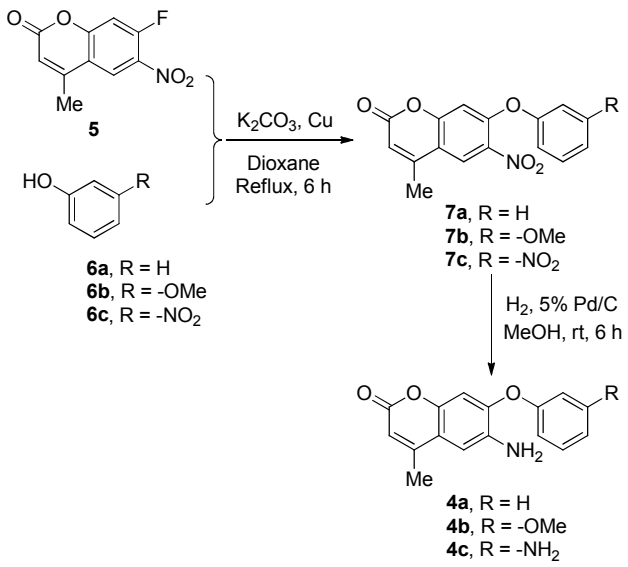

Figure 4. Structures and synthesis of **PN600** and a list of analogs **4a**-**c**.

Fluorescence titrations of 4a, 4b, PN600, 4c, by hypochlorite and peroxynitrite. An aliquot of hypochlorite or peroxynitrite stock was titrated into a solution of probe **4a**, **4b**, **PN600**, or **4c** (10 μM) respectively in phosphate buffer (50 mM at pH = 7.4) with 5% DMF as a co-solvent.

All four probes (**4a**, **4b**, **PN600** and **4c**) exhibited an emission band with a maximum at *ca*. 535 nm upon excitation at their absorption maximum of *ca*. 355 nm. Addition of hypochlorite led to a gradual decrease of their emissions (Figure 5A, 5B, 5C, 5E). It took *ca*. 6-10 equiv. of hypochlorite to consume the emission of probe **4a**, **4b, PN600** and **4c** (Figure S3). No new fluorescent species were observed to have been generated from oxidation of **4a** and **4b**. Oxidation of **PN600** by hypochlorite led to accumulation of compound **1** (Figure S4). Further oxidation of **1** to **2** by hypochlorite was not achieved as the emission of **2** at 595 nm was not observed with excitation at its maximum absorption wavelength of 550 nm (Figure 5D). Oxidation of **4c** by hypochlorite yielded a red-emitting species, with a maximum excitation at *ca*. 550 nm and emission at 605 nm (Figure 5F). The spectral properties of this red-emitting species are reminiscent of those of compound **2** and we assigned it to **9**. Compound **8** should be the intermediate between **4c** and **9**.

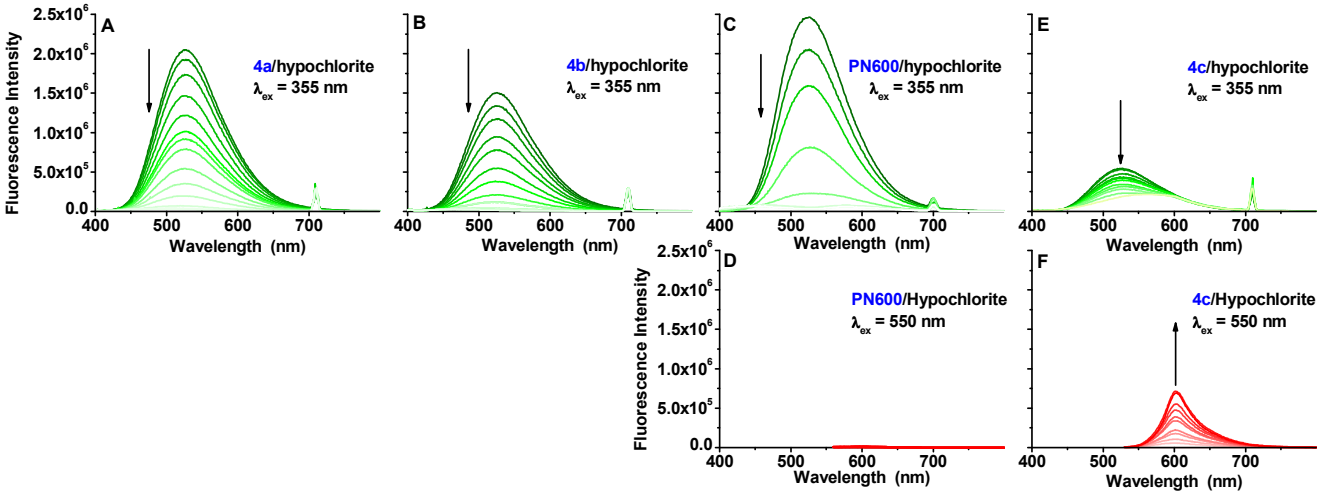

Figure 5: Fluorescence titrations of probes **4a**, **4b**, **PN600** and **4c** by hypochlorite. Excitation was set at 355 nm to monitor the decrease of the probe emission of **4a** (A), **4b** (B), **PN600** (C) and **4c** (E) upon addition of hypochlorite. Excitation was set at 550 nm to monitor the formation of **2** from **PN600** (D), and **9** from **4c** (F) upon addition of hypochlorite.

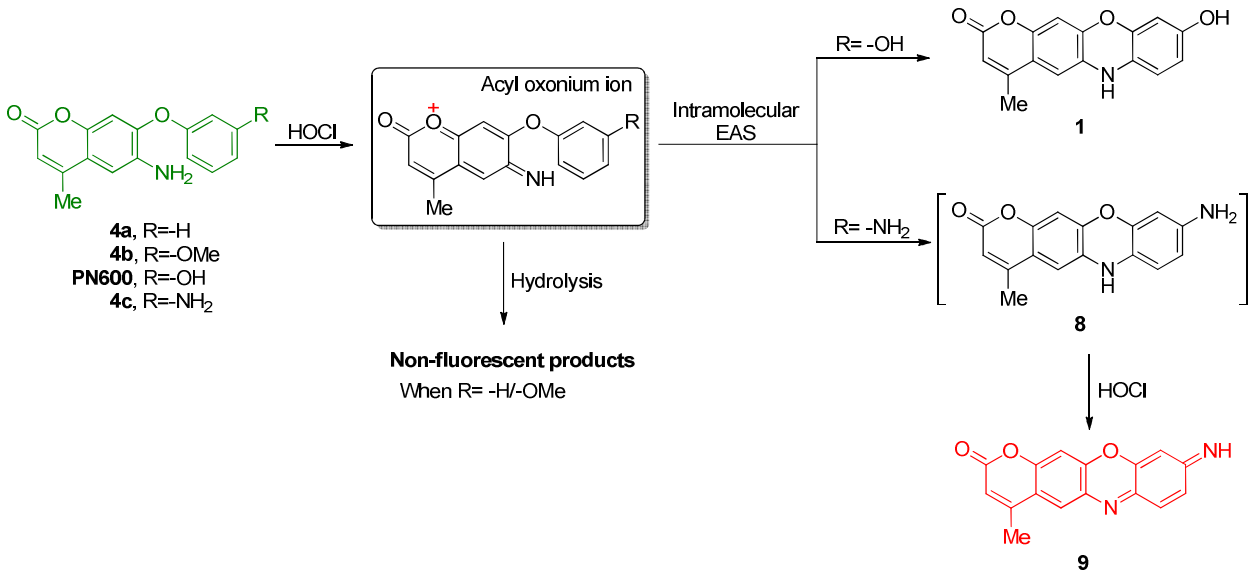

Figure 6: Proposed oxidation schemes for **4a**, **4b**, **PN600** and **4c** by hypochlorite, based on titrations shown in Figure 5.

Chemical transformations of probes **4a-b**, **PN600** and **4c** induced by addition of hypochlorite, with HOCl presumed to be the active species, are summarized in the Figure 6. The oxidative capacity of HOCl is unleashed

upon breakage of the weak oxygen-chlorine bond. Mechanistically, this occurs when a nucleophile attacks the electrophilic chlorine atom (-Cl) and displaces hydroxide (OH^-) as the leaving group. Also based on the fact that all four probes were effectively oxidized by hypochlorite with similar kinetics (Figure S5), this nucleophile is most likely -NH_2 on the coumarin moiety, which is present in all four probes. Oxidation of the coumarin moiety led to subsequent formation of an electrophilic O-acylated iminoquinone derivative (as shown in the rounded box of the Figure 6). The aroxyl moiety of **PN600** or **4c**, substituted by a strong electron donating group (i.e. –OH in **PN600** or –NH_2 in **4c**) is nucleophilic enough to furnish a cyclization to give compound **1** and **5** respectively, via an intramolecular electrophilic aromatic substitution (EAS). In case of **4a** or **4b**, whose aroxy moiety is not as nucleophilic, hydrolysis of the acyl oxonium ion presumably competed and no fluorescent products were observed to have been generated. Inability of hypochlorite to oxidize compound **1** to **2** suggests that the nucleophilicity of the diarylamino group is not potent enough to initiate an attack at HOCl. In comparison, the proposed intermediate **8** was oxidized because it has a primary aryl amino group, which is much more nucleophilic than a diarylamino group.

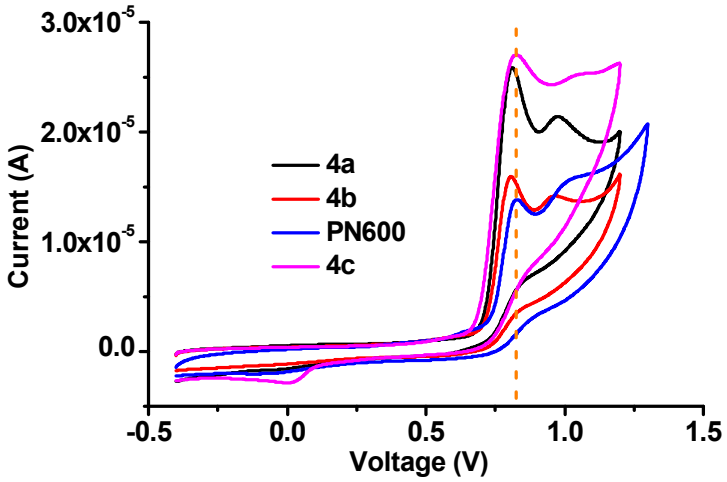

Figure 7. Cyclic voltammograms of probes **4a**, **4b**, **PN600** and **4c** in DMF.

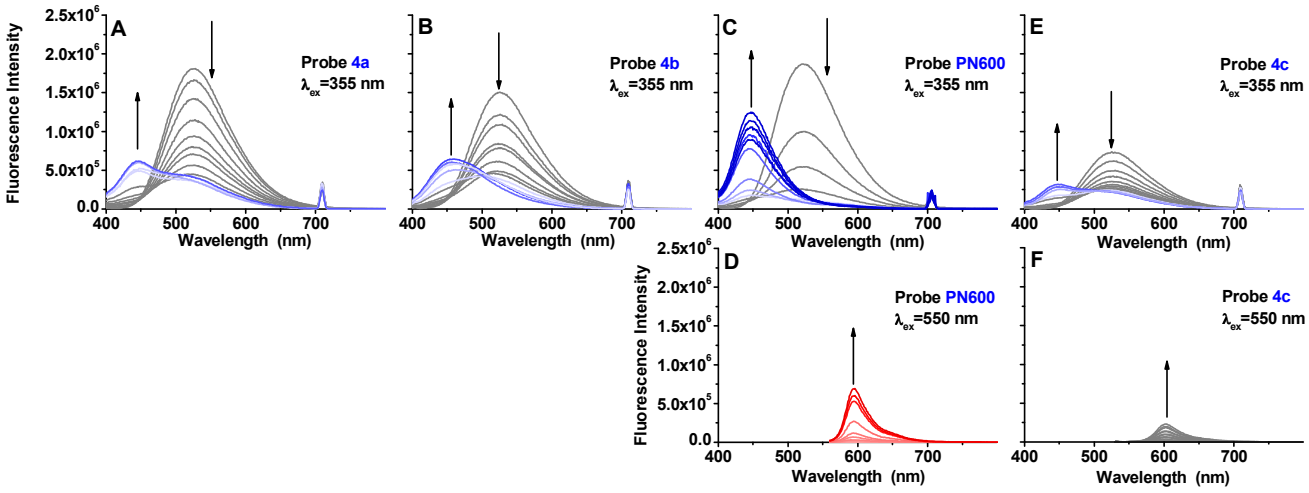

Figure 8. Fluorescence titrations of probes **4a**, **4b**, **PN600** and **4c** by peroxynitrite. Excitation was set at 355 nm to monitor the decrease of the probe emission of **4a** (A), **4b** (B), **PN600** (C) and **4c** (E) upon addition of peroxynitrite. Excitation was set at 550 nm to monitor the formation of **2** from **PN600** (D) and **9** from **4c** (F) upon addition of peroxynitrite.

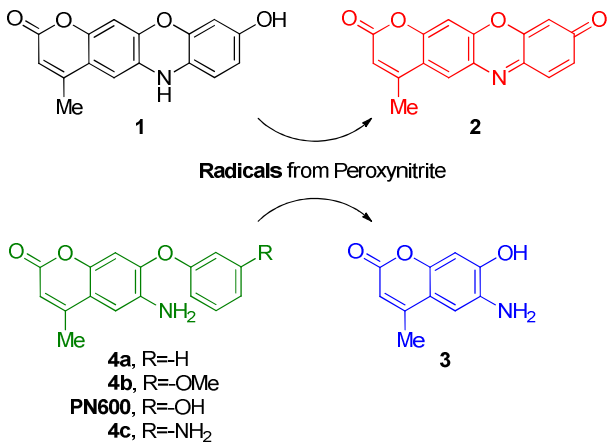

Figure 9. Oxidations of probe **4a**, **4b**, **PN600** and **4c**, which were mediated by peroxynitrite, but not hypochlorite.

The first oxidative potential of all four probes (**4a-c** and **PN600**) was essentially identical at *ca*. +0.8 V as determined by cyclic voltammetry despite the different nature of the substituent on the aroxy moiety of **4a-c** and **PN600** (Figure 7), suggesting that the coumarin moiety is where oxidation occurs most readily in each probe. Also, the first oxidative potential of **1** was measured to be -0.14 V.[101(SI)] So, probe **4a-c** or **PN600**, of a higher

oxidative potential, was oxidized by hypochlorite, while **1**, of a lower oxidative potential, was not. This interesting observation clearly indicates that oxidation by hypochlorite is not under thermodynamic control.

All the transformations of probes **4a-b PN600** and **4c** induced by hypochlorite (Figure 5) were also observed to have occurred upon addition of peroxynitrite (Figure 8, gray). This is not surprising since HOONO can presumably mediate 2-e^- oxidation, analogously to HOCl. However, we also noticed that peroxynitrite mediated some transformations, which were not seen with hypochlorite. The first such transformation was the formation of the red-emitting compound **2** from further oxidation of the intermediate **1** (Figure 8D and 9, red). Second, an emission band at 450 nm, of compound **3**, appeared upon peroxynitrite mediated oxidation of all four probes (Figure 8A, 8B, 8C, 8E and 9, blue).

Peroxynitrite can mediate 1-e^- oxidations through various secondary radicals including $OH^{\bullet}$, $CO_3^{\bullet-}$, $NO_2^{\bullet}$ etc, while hypochlorite cannot. So, these transformations unique to peroxynitrite have presumably occurred via a radical mechanism. Compound **1** contains a *para*-aminophenol moiety and such electron rich polyaromatics are known radical scavengers, i.e. to be readily oxidized by radicals.

Formation of **3** was rather unexpected because this necessitates oxidation of the less electron-rich aroxy moiety in the presence of the more electron-rich coumarin moiety. However, this seemingly unexpected observation is in fact reasonable. Secondary radicals from peroxynitrite are highly oxidative. Thus, they can indiscriminately abstract an electron from whichever oxidizable substrate they encounter first. In other words, oxidation by these radicals is kinetically controlled and steric accessibility, rather than oxidative potential, determines the profile of the reaction outcome. It happened that the coumarin moiety of probes **4a-c** and **PN600** is sterically shielded by the aroxy moiety. This explains why a kinetic oxidation of the less electron-rich aroxy moiety occurred, and ultimately resulted in the formation of **3**.

Mechanistic explanation to the overall reactivity of PN600 toward hypochlorite and peroxynitrite. With the mechanistic insights from these titrations, reactivity of **PN600** toward hypochlorite and peroxynitrite are well accounted for and summarized in the Figure 10. Nucleophilic attack commencing from its $-NH_2$ at HOCl or HOONO followed by a condensation to displace a chloride (-Cl^-) or a hydroxide (-OH^-) respectively, yields an

acyl oxonium species (**10**). Alternatively, this process can potentially take place via a radical pathway and be mediated by peroxynitrite. The resulting acyl oxonium ion (**10**) undergoes a ring closure to afford the intermediate **1**. This has explained why the addition of either hypochlorite or peroxynitrite can induce the formation of compound **1**. However, lack of a nucleophile on the scaffold of **1** strong enough to attack HOCl renders it stable toward hypochlorite. In comparison, the secondary radicals from peroxynitrite readily oxidize compound **1** to **2**. Kinetic radical oxidation to the 3-hyroxyphenoxyl moiety can occur, in a fashion similar to the step-wise radical oxidation of phenol to quinone. Ultimately, the diaryl ether linkage breaks upon hydrolysis to generate **3**.

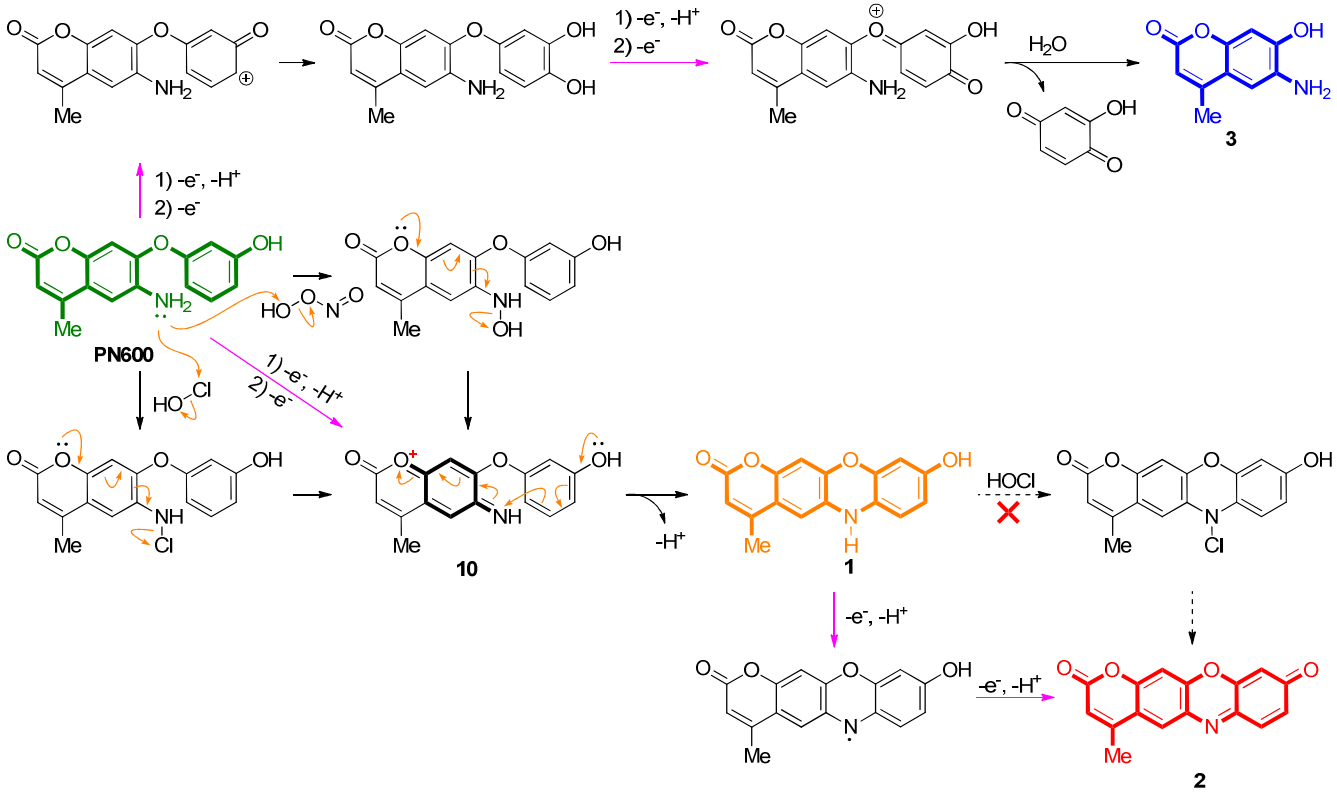

Figure 10. A proposed mechanistic explanation to the observed reactivity of **PN600** toward both hypochlorite and peroxynitrite. Note: Magenta arrows highlight the radical reactions.

An empirical guideline for development of probes targeting at peroxynitrite or hypochlorite. With the few probes of this manuscript and rich literature work, in particular those from Yang et al. and Nagano et al., we were able to categorize various electron-rich aromatic structures based on their reported reactivity toward

peroxynitrite and hypochlorite (Chart 1). Tentatively, this summary may serve as a qualitative guideline for future use of electron-rich aromatic rings as the recognition moiety of a fluorescent probe for either hypochlorite or peroxynitrite detection:

1. A phenolic hydroxyl (Ar-OH) is not nucleophilic enough to attack HOCl and initiates an oxidation.[97,101-102,115-117]
2. A diarylamino (Ar-NH-Ar') group does not react with HOCl.[101-112,115]
3. A monoarylamino ($Ar-NH_2$) group with a para-methine (-CHRR', where R and R' are alkyl or aryl) group is mildly reactive toward HOCl.[98] Removal of the *para*-methine reduces this reactivity.
4. A monoarylamino ($Ar-NH_2$) group with an oxygen or nitrogen attached to its *para* position is reactive toward HOCl.[96-97,100,102,116] A broad spectrum of substitutions to this oxygen or nitrogen atom seems to have been tolerated, including alkyl, aryl or acyl. Further substitution of this monoarylamino group by two small alkyl groups into monoaryldialkyl amino groups ($Ar-NMe_2$, or $Ar-NEt_2$) does not seem to have greatly suppressed its reactivity toward HOCl.
5. A phenolate ($Ar-O^-$) is reactive toward HOCl.[118-119]
6. All aforementioned structures are expected to exhibit reactivity toward peroxynitrite. Reaction mechanism can be 2-e^- or 1-e^- depending on the existence of a nucleophilic amino group or not.

We judiciously listed phenolate as a separate category because of **HKOCl-1**[28a], which seems not to agree with the rule 1. However, this inconsistency can be rationalized. A phenol is an acidic functional group with a pK_a of *ca*. 10. At physiological pH, more than 99% of a phenol should exist in form of its conjugate acid accordingly to Henderson-Hasselbach equation. However, the electron-withdrawing nature of the BODIPY core within the scaffold of **HKOCl-1** should also be considered. It might have lowered the pK_a of the *ortho* phenolic OH group, as a combined result of the inductive effect and long-range electronic interaction[120-121], to allow a significant portion of **HKOCl-1** stay in form of the corresponding phenolate at physiological pH. The phenolate is intuitively much more nucleophilic than the corresponding phenol and expected to be able to attack HOCl and initiate a 2-e^- oxidation. Though, the exact pK_a value of **HKOCl-1** was not reported, this analysis was supported by the recent

report, from the same authors, that *ortho*-substitution to the phenol by electron-withdrawing halogen atoms, i.e. –Cl or –F respectively, further improved the probe reactivity toward hypochlorite.[28b]

Development of hypochlorite specific probe without interferences from peroxynitrite will be challenging. An electron-rich aromatic substrate may survive HOONO, but not those secondary radicals from peroxynitrite. In fact, even not-so-electron-rich substrates do not survive these radicals, including umbelliferone, salicylate, and terephthalate.[25d] In other words, electron-rich aromatics are better suited for unspecific detection of hypochlorite and peroxynitrite, or specific detection of peroxynitrite over hypochlorite.

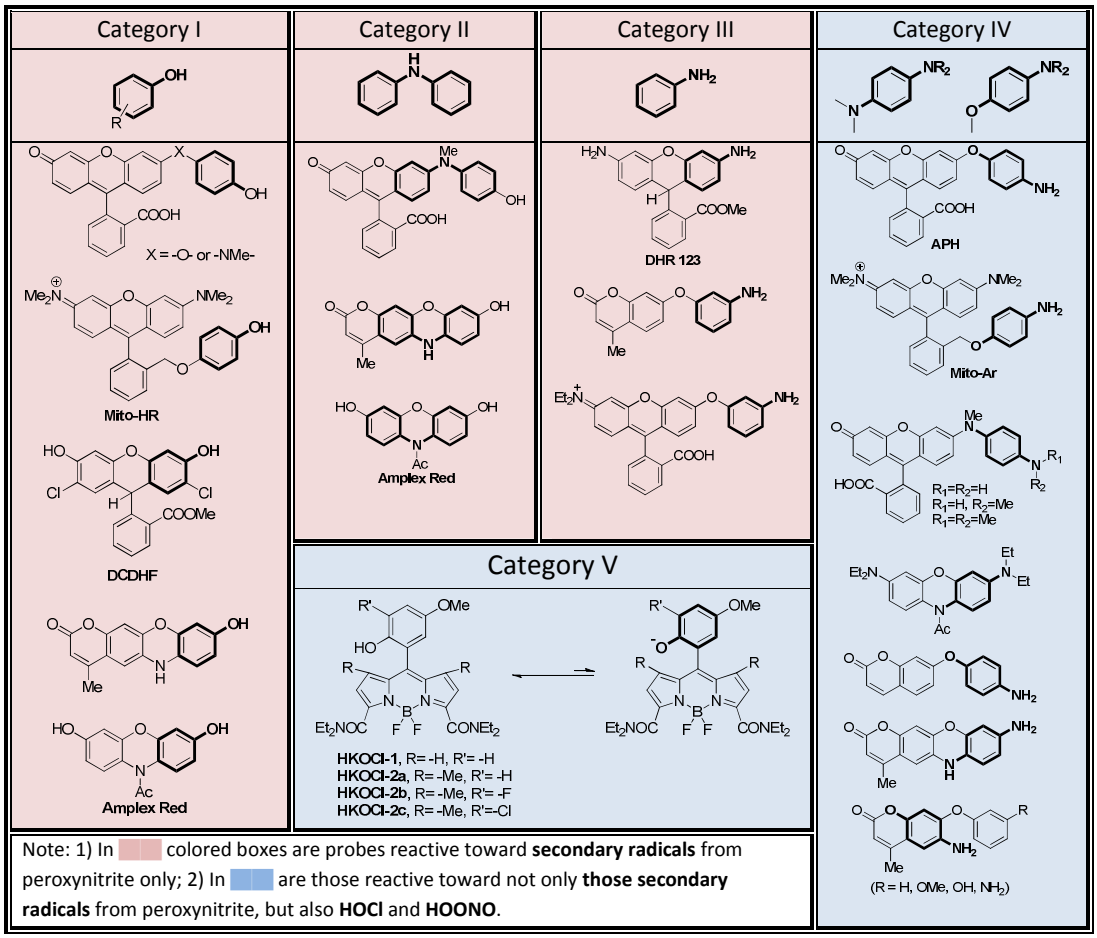

Chart 1:Classification of probes for hypochlorite and peroxynitrite based on oxidation of an electron-rich aromatic ring.

An embodiment of the guideline. Following this guideline, we designed and synthesized **11** (Figure 11) as a selective peroxynitrite probe. It falls within the category III and is expected to display good reactivity toward

peroxynitrite, but poor reactivity toward hypochlorite. A close analog (**12**, Figure 11) of an unnamed probe of category IV, which reacted well with both peroxynitrite and hypochlorite, was synthesized (Figuer 12) and titrated side-by-side with **11** (Figure 13).

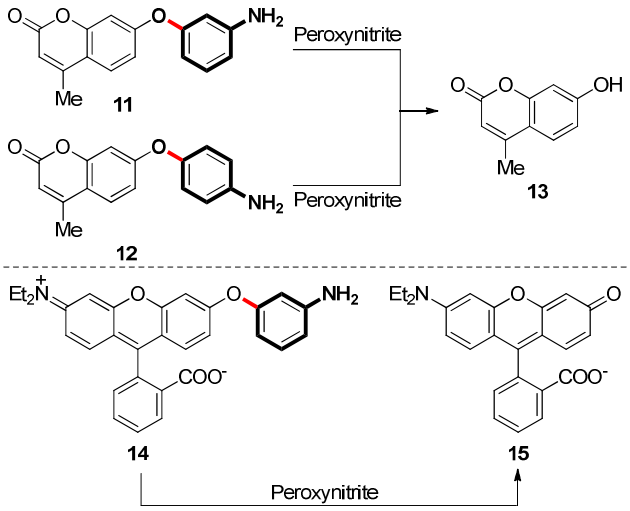

Figure 11. Oxidation pathways of probes **11**, **12** and **14** by peroxynitrite.

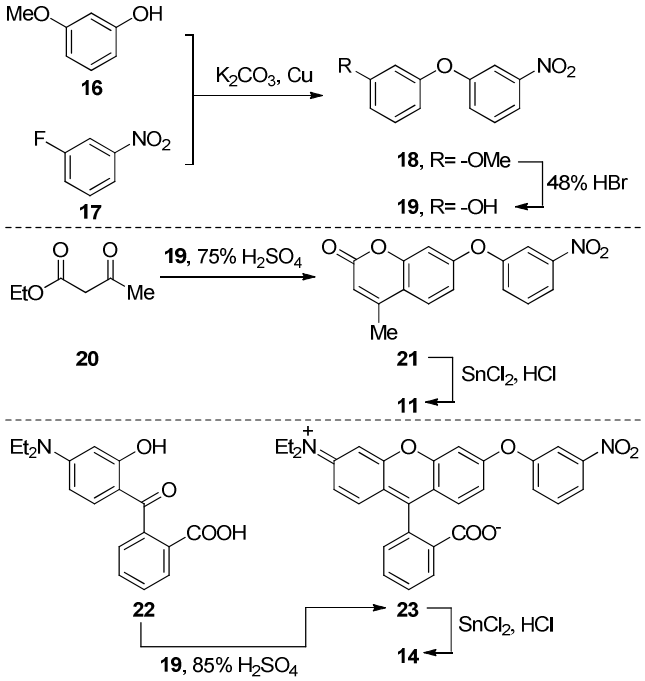

Figure 12. Synthetic Schemes of probes **11** and **14**.

Compound **11** and **12** make a good comparison as their structures differ from each other only by the position of $–NH_2$. Yet, this seemingly minor variation makes a huge difference with respect to their reactivity toward peroxynitrite and hypochlorite.

Synthesis of probes **11** and **14** started from condensation of **16** and **17** under Ullmann conditions to afford the diaryl ether **18** in a moderate yield. Demethylation of **18** in refluxing 48% HBr gave **19** nearly quantitatively. Condensation of **19** with **20** or **22** in 70% H_2SO_4 at high temperature yielded the desired coumarin (**21**) or Rhodol derivative (**23**). Reduction of the nitro group on **21** or **23** with $SnCl_2$ by refluxing in concentration HCl furnished probe **11** or **14** in good yields. Catalytic hydrogenation of **23** also partially reduced the Rhodol scaffold and therefore not suitable for preparation of **14**.

Probe **11** and **12** (10 μM) in phosphate buffer (50 mM, at pH=7.4) containing 5% DMF were titrated by hypochlorite and peroxynitrite (Figure 13A and 13B). Emission of their oxidation product, 7-hydroxy-4-methylcoumarin (**13**), with a maximum excitation at 360 nm and an emission at 450 nm, was monitored. The reported high reactivity of probe **12** toward hypochlorite was verified. Addition of *ca*. 6 equiv. of hypochlorite led to a near saturation of fluorescence enhancement. Probe **11** was not totally inert to hypochlorite. However, its signal enhancement was less than 2% of that from probe **12**. Addition of a higher dose of hypochlorite than 6 equiv. led to a gradual bleaching of fluorescence. Two probes (**11** and **12**) yielded an emission of comparable intensity upon addition of peroxynitrite. Only, it took *ca*. 10 equiv. of peroxynitrite to saturate the signal enhancement of probe **12**, while roughly twice as much of peroxynitrite was necessary to saturate the signal enhancement of probe **11**. The spectral titrations of this pair of probes exemplified the viability of the aforementioned guideline.

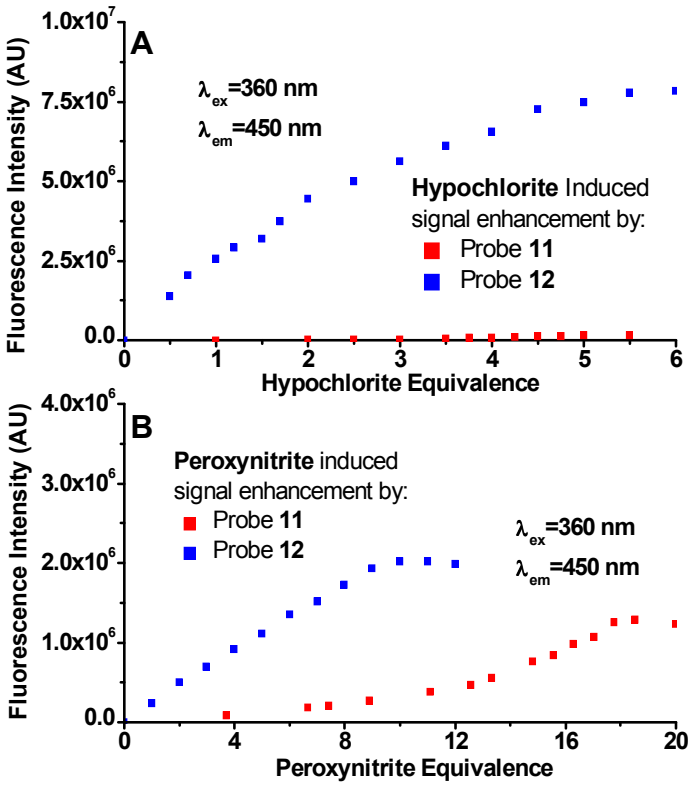

Figure 13. Dose dependent fluorescence enhancement of probes **11** and **12** by hypochlorite (A) and peroxynitrite (B).

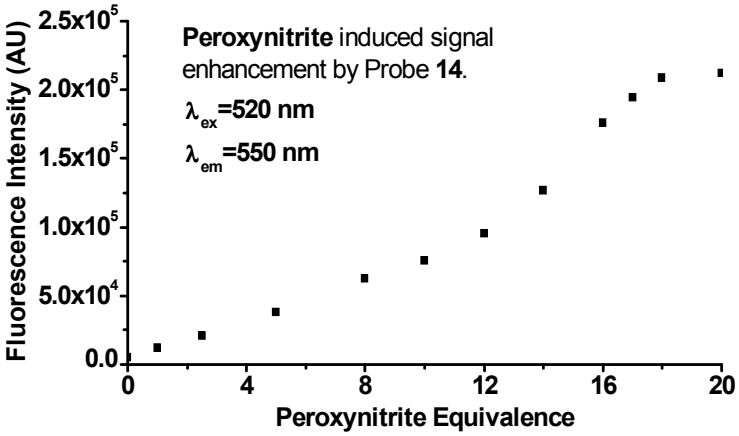

Figure 14. Dose dependent fluorescence enhancement of probes **14** by peroxynitrite.

By attaching the 3-aminophenyl group to the oxygen atom of a rhodol fluorophore (**15**), a peroxynitrite probe (**14**) of longer wavelength, was designed (Figure 11) and synthesized (Figure 12). Probe **14** inherited the

reactivity of probe **11** toward peroxynitrite (Figure 14). Probe **14** shows no reactivity toward large excess of H_2O_2, KO_2, NO, $^1O_2$ (Figure S13). Addition excess hypochlorite turned on the emission at 550 nm by *ca*. 5 fold, while excess peroxynitrite enhanced the emission at 550 nm by over 45 fold (Figure S12). This proved that probe **14** is selective toward peroxynitrite over hypochlorite.

CONCLUSION

PN600 is an interesting fluorescent probe, which yields an unusual four-channel fluorescence signal upon oxidation by peroxynitrite. It showed cross-reactivity toward hypochlorite. Oxidation by hypochlorite could be spectroscopically differentiated from oxidation by peroxynitrite. Through combined use of spectral titrations, electrochemical studies and fundamental organic arrow-pushing mechanisms, we were able to satisfyingly explain the observed reaction profile of **PN600** toward peroxynitrite and hypochlorite. With findings of this work and previous literature publications, we have proposed a qualitative guideline for use of electron-rich aromatic rings as the reactive moiety in constructing fluorescent probes for hypochlorite or peroxynitrite. Following this guideline, we further developed two peroxynitrite probes, which are indeed only minimally reactive toward hypochlorite. This supports the viability of this guideline.

EXPERIMENTAL

All chemicals and solvents were analytical grades and used without further purification. The ^{1}H-NMR and ^{13}C-NMR spectra were acquired on a Bruker AV-400 spectrometer. Chemicals shifts were referenced to the residue solvent peaks and listed in ppm. Coupling constants were given in Hz. ESI-HRMS was acquired on a TOF mass spectrometer and EI-HRMS on a Micromass GCT spectrometer. UV-Vis absorption spectra were acquired on a SHIMADZU UV-2600 UV-vis spectrophotometer. Fluorescence emission spectra were acquired on a PTI-QM4 steady-stead fluorimeter equipped with a 75 Watt Xenon arc-lamp and a R928 PMT. All excitation spectra were corrected. All emission spectra were not corrected with respect to the PMT sensitivity at different wavelengths. The excitation and emission slits were both 2 nm for all experiments. Fluorescence titrations were carried out by addition of an aliquot of the stock solution of hypochlorite, peroxynitrite or other species.

Electrochemical analyses were performed by using a computer controlled CHI 660c electrochemical station (Chenhua Co. Ltd, Shanghai, China). The electrochemical experiments were carried out in a three-electrode electrochemical cell under nitrogen atmosphere. Working electrodes were 3-mm diameter glassy carbon (GC) disks, used in conjunction with a Pt auxiliary electrode and an Ag/AgCl wire reference electrode. The concentration of each sample was 1mM, and was dissolved in N,N-Dimethylformamide (DMF). Tetrabutylammonium Perchlorate (TBAP) acted as supporting electrolyte and its concentration was 0.1 M. The data was obtained from the first cycle in cyclic voltammetry (CV), and the scan rate was 100 mV/s.

6-Amino-4-methyl-7-phenoxy-2H-chromen-2-one (**4a**). Mix **7a** (50mg, 0.17 mmol), EtOH (5 mL) and concentrated HCl (5 mL) in a 25 mL round bottom flask. Add $SnCl_2 \bullet 2H_2O$ (198 mg, 0.88 mmol) into the flask slowly with constant stirring. Heat the reaction mixture to 40 ^oC for 6 hrs before cooling to room temperature. The reaction mixture was poured in concentration $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered, passed through a silica plug to remove the colored impurities and evaporated to dryness under reduced pressure. The crude product was recrystallized in CH_2Cl_2 to afford **4a** as a yellow solid (30 mg, 67%). ^{1}H NMR(400 MHz, $CDCl_3$): δ(ppm) 7.39 (t, *J* = 8.0 Hz, 2H) , 7.20 (t, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 8.0 Hz, 2H), 6.94 (s, 1H), 6.69 (s, 1H), 6.16 (s, 1H), 3.96 (s, 2H), 2.37 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) 161.3, 155.3, 151.8, 148.3, 147.2, 134.5, 130.1, 124.7, 119.3, 115.6, 113.3, 109.2, 105.4, 18.8; EI-HRMS (*m/z*) [M^+] calcd. for C16H13NO3, 267.0895; found 267.0893.

6-Amino-7-(3-aminophenoxy)-4-methyl-2H-chromen-2-one (**4c**). Mixture of **7c** (140 mg, 0.41 mmol), 5% Pd/C (a spatula end) and 10 mL MeOH was stirred in an H_2 atmosphere (0.4 MPa) in a pressure vessel for 4 hrs at room temperature before the system was vented. The mixture was passed through a silica plug and evaporated to dryness under reduced pressure to afford a viscous residue. Upon recrystallization in a mixed solvent of CH_2Cl_2 and petroleum ether, **4c** was obtained as a light yellow solid (102 mg, 88%). ^{1}H-NMR(400 MHz, $CDCl_3$): δ(ppm) 7.16 (t, *J* = 8.0 Hz, 1H) , 6.95 (s, 1H), 6.80 (s, 1H), 6.52 (dd, *J* = 8.0,1.6 Hz, 1H), 6.45 (dd, *J* = 8.0, 1.6 Hz, 1H), 6.39 (s, 1H), 6.20 (s, 1H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) 161.4, 156.5, 151.9, 148.3, 148.2,

147.2, 134.6, 130.7, 115.6, 113.3, 111.4, 109.1, 108.9, 105.9, 105.7, 18.8; ES-HRMS (m/z): $[M]^+$ calcd. for C16H14NO3, 283.1077; found 283.1080.

4-Methyl-6-nitro-7-phenoxy-2H-chromen-2-one (**7a**). Mix **5** (50 mg, 0.22 mmol), **6** (23 mg, 0.25 mmol), K_2CO_3 (34 mg, 0.25 mmol), fresh Cu (5 mg) and 1,4-dioxane (20 mL) in a 50 mL round bottom flask. The mixture was heated to reflux overnight with stirring before cooled and filtered through a celite pad. The filtrate was dried under reduced pressure and chromatographed with petroleum ether/EtOAc (15:4, v/v) to obtain **7a** as a light yellow solid (35 mg, 53%). ^{1}H NMR(400 MHz, $CDCl_3$): δ(ppm) 8.29 (s, 1H) , 7.47 (t, *J* = 8.0 Hz, 2H), 7.31 (t, *J* = 8.0 Hz, 1H), 7.13 (d, *J* = 8.0 Hz, 2H), 6.77 (s, 1H), 6.28 (s, 1H), 2.47 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) 158.9, 156.8, 154.7, 153.9, 151.1, 136.5, 130.5, 126.2, 123.1, 120.4, 114.7, 114.5, 106.4, 18.6; EI-MS (*m/z*) $[M]^+$ calcd. for C16H11NO5, 297.0637; found 297.0638.

4-Methyl-6-nitro-7-(3-nitrophenoxy)-2H-chromen-2-one (**7c**). Mix **5** (500 mg, 2.24 mmol), **6c** (311 mg, 2.24 mmol), K_2CO_3 (310 mg, 2.25 mmol), fresh Cu powder (40 mg) and 1,4-dioxane (35 mL) in a 50 mL round bottom flask. The mixture was heated to reflux overnight with stirring before cooled and filtered through a celite pad. The filtrate was dried under reduced pressure and chromatographed with EtOAc to obtain **7c** as a yellow solid (440 mg, 57%). ^{1}H NMR(400 MHz, $CDCl_3$): δ(ppm) 8.36 (s, 1H) , 8.14 (d, *J* = 8.0 Hz, 1H), 7.90 (s, 1H), 7.64 (t, *J* = 8.4 Hz, 1H), 7.47 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.93 (s, 1H), 6.37 (s, 1H), 2.51 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ(ppm) 158.4, 157.0, 155.7, 152.1, 150.9, 149.5, 137.4, 131.2, 125.3, 123.6, 120.2, 116.5, 115.8, 114.1, 108.69, 18.7; ESI-HRMS (*m/z*): $[M+H]^+$ calcd. for $C_{16}H_{11}N_2O_7$, 343.0561; found 343.0553.

7-(3-Aminophenoxy)-4-methyl-2H-chromen-2-one (**11**). Mix **21** (174 mg, 0.585 mmol), EtOH (10 mL) and concentrated HCl (10mL) in a 50 mL round bottom flask. Add $SnCl_2$•$2H_2O$ (700 mg, 3.10 mmol) into the flask slowly with constant stirring. Heat the mixture to 40 ^{o}C for 6 hr before cooling to room temperature. The reaction mixture was poured in concentration $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried with MgSO4, filtered, passed through a silica plug to remove colored impurities and evaporated to dryness under reduced pressure. The crude product was recrystallized in a mixed solvent of CH2Cl2 and petroleum ether to afford **4a** as a light yellow solid (142 mg, 91 %). ^{1}H-NMR(400 MHz, CDCl3) δ(ppm) 7.52 (d, *J* = 8.4 Hz, 1H) , 7.15 (t, *J* = 8.0 Hz, 1H), 6.94 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.89 (s, 1H), 6.52 (d, *J* = 8.0 Hz, 1H), 6.44 (d, *J* = 8.0 Hz,

1H), 6.39 (s, 1H), 6.17 (s, 1H), 3.75 (s, 2H), 2.41 (s, 3H); ^{13}C NMR (100 MHz, CDCl3) δ(ppm) 161.1, 160.9, 156.2, 154.9, 152.2, 148.3, 130.7, 125.6, 115.0, 114.3, 112.8, 111.6, 109.9, 106.7, 105.4, 18.7; ESI-MS (m/z) $[M+H]^+$ calcd for C16H14NO3, 268.0968; found 268.0982.

3'-(3-Aminophenoxy)-6'-(diethylamino)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (**14**). Mix **23** (160 mg, 0.314 mmol), EtOH (10 mL) and concentrated HCl (10mL) in a 50 mL round bottom flask. Add $SnCl_2$•$2H_2O$ (350 mg, 1.55 mmol) into the flask slowly with constant stirring. Heat the mixture to 60 ^oC for 6 hr before cooling to room temperature. The reaction mixture was poured in concentration $NaHCO_3$ solution and extracted with CH_2Cl_2. The organic layer was dried with $MgSO_4$, filtered, and evaporated to dryness under reduced pressure. The crude product was chromatographed with CH_2Cl_2/MeOH (50:1, v/v) to afford **14** as a light pink solid (52 mg, 34.6%). ^{1}H NMR(400 MHz, $CDCl_3$) δ(ppm) 8.00 (d, *J* = 7.6 Hz, 1H) , 7.86 (t, *J* = 7.2 Hz, 1H), 7.60 (t, *J* = 7.6 Hz, 1H), 7.21 (d, *J* = 7.6 Hz, 1H), 7.12 (t, *J* = 8.0 Hz, 1H), 6.83 (d, *J* = 2.4 Hz, 1H), 6.65~6.71 (m, 2H), 6.57 (d, *J* = 8.8 Hz, 1H), 6.42~6.48 (m, 3H), 6.37 (t, *J* = 2.4 Hz, 1H), 6.34 (d, *J* = 2.8 Hz, 1H), 3.75 (s, 2H), 3.35 (q, *J* = 6.8 Hz, 4H), 1.16 (t, *J* = 6.8 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) 172.1, 161.9, 159.6, 155.6, 155.4, 155.3, 152.2, 150.8, 137.3, 133.1, 132.1, 131.8, 131.4, 129.9, 127.4, 126.6, 116.5, 116.4, 113.6, 112.3, 111.0, 108.9, 108.3, 107.6, 100.1, 86.8, 47.0, 15.1; EI-MS (*m/z*) [M+] calculated for C30H26N2O4, 478.1893; found 478.1898.

4-Methyl-7-(3-nitrophenoxy)-2H-chromen-2-one (**21**). Mix **19** (700 mg, 3.03 mmol) and 75% H_2SO_4 (20 mL) in a 50 mL round bottom flas. Add **20** (433 mg, 3.33 mmol) dropwise and let the reaction sit with vigorous stirring at room temperature for 24 hrs. Pour the reaction mixture into crushed ice and collect the solid via suction filtration. The crude product was chromatographed with CH_2Cl_2/Petroleum ether (5:2, v/v) to afford **21** as a light yellow solid (340 mg, 37.8%). ^{1}H NMR(400 MHz, CDCl3) δ(ppm) 8.06 (dd, *J* = 8.4, 1.2 Hz, 1H) , 7.89 (t, *J* = 2.4 Hz, 1H), 7.62 (d, *J* = 8.4 Hz, 2H), 7.58 (t, *J* = 8.4 Hz, 1H), 7.42 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.99 (dd, *J* = 8.4, 2.4 Hz, 1H), 6.96 (d, *J* = 8.4 Hz, 1H), 6.25 (d, *J* = 1.2 Hz, 1H), 2.44 (d, *J* = 1.2 Hz, 3H); 13C NMR(100 MHz, CDCl3) δ(ppm) 160.4, 158.9, 156.6, 155.0, 151.9, 130.8, 126.3, 125.5, 119.2, 116.5, 114.9, 114.3, 113.8, 106.8, 18.7; ESI-MS (m/z) $[M+H]^+$ calculated for C16H12NO5, 298.0710, found 298.0719.

3'-(Diethylamino)-6'-(3-nitrophenoxy)-3H-spiro[isobenzofuran-1,9'-xanthen]-3-one (**23**). Mix **19** (174 mg, 0.585 mmol) and **22** (235 mg, 0.585 mmol) and 85% H3PO4 (15 mL) in a 25 mL round bottom flask. Heat the flask to 100 ^{o}C with stirring for 5 hrs. Pour the mixture into saturated NaHCO3 solution and extract with CH2Cl2. The organic layer was dried with MgSO4, filtered, and evaporated to dryness under reduced pressure. The crude product was chromatographed with CH_2Cl_2:MeOH (100:1) to afford **23** as a pink solid (310 mg, 81%). ^{1}H NMR(400 MHz, $CDCl_3$) δ(ppm) 8.00~8.03 (m, 2H) , 7.90 (t, *J* = 4.0 Hz, 1H), 7.69 (t, *J* = 8.0 Hz, 1H), 7.62 (t, *J* = 8.0 Hz, 1H), 7.53 (t, *J* = 8.0 Hz, 1H), 7.39 (dd, *J* = 8.0, 4.0 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 6.88 (d, *J* = 2.4 Hz, 1H), 6.79 (d, *J* = 8.8 Hz, 1H), 6.69 (dd, *J* = 8.0, 2.4 Hz, 1H), 6.58 (d, *J* = 8.0 Hz, 1H), 6.43 (d, *J* = 2.8 Hz, 1H), 6.37 (dd, *J* = 8.8, 2.8 Hz, 1H), 3.36 (q, *J* = 7.2 Hz, 4H), 1.17 (t, *J* = 7.2 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$) δ(ppm) 169.6, 157.6, 157.4, 153.2, 153.0, 152.9, 149.8, 135.1, 130.7, 130.1, 129.8, 129.0, 127.3, 125.3, 125.1, 124.2, 118.8, 115.9, 114.5, 114.3, 108.8, 107.1, 105.0, 97.7, 44.7, 27.1, 12.6; ESI-MS (*m/z*); $[M+H]^+$ calc. for C30H25N2O6, 509.1707; found 509.1716.

Supporting Information.

Additional fluorescence spectral results, NMR and HRMS spectra of new compounds. This material is available free of charge via the Internet at http://pubs.acs.org."

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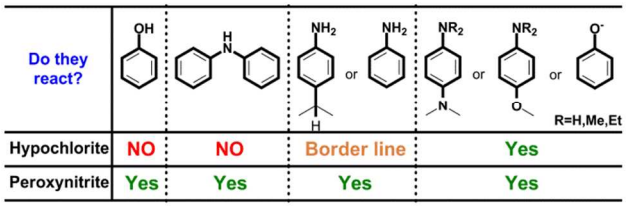
TOC graphic


Do they react?
OH
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Hypochlorite
NO
NO
Border line
Yes
Peroxynitrite
Yes
Yes
Yes
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