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COMMUNICATIONS

Published on Web 07/28/2006

Mechanism-Based Molecular Design of Highly Selective Fluorescence Probes for Nitrative Stress

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In the last 15 years, the study of aromatic nitration has been extended to biological events because nitrated biomolecules have been implicated in various pathogenic processes, including inflammatory events, ischemia-reperfusion, and neurodegenerative disorders.[1] Nevertheless, no fluorogenic probe for monitoring nitration in biological samples has yet been developed.

In the design of novel fluorogenic probes for monitoring nitrative stress, a major obstacle must be overcome; that is, the nitro group is believed to be a strong quencher of fluorescent dyes.[2] Indeed, nitro-substituted fluorescein and BODIPY derivatives, such as **1**−**3**, are almost nonfluorescent (Figure 1A). The quenching mechanism of the nitro group is unclear, and some researchers have suggested that the nitro group has a unique action. However, we considered that the nitro group would have no unique effect, other than its electron-withdrawing effect, on an adjacent fluorophore, and that the fluorescence quantum efficiency (Φ_{fl}) can be precisely predicted.

To test our hypothesis, we first focused on the structure of nitro-substituted dyes, **1**−**3**. As previously reported, from the viewpoint of fluorescence, the structures of fluorescein and BODIPY can be divided into two parts, the xanthene (or BODIPY) moiety as a fluorophore and the benzene moiety as a fluorescence switch, which modulates the Φ_{fl} value of the fluorophore, since they are orthogonal to each other.[3] In the case of **1**, the π-electron system and transition process of the fluorophore are significantly perturbed by a directly conjugated nitro group, and as a consequence of these perturbations, **1** becomes almost nonfluorescent. On the other hand, in the cases of **2** and **3**, the nitro group is not directly conjugated to the fluorophore, but is located at a proximal position. Therefore, unlike **1**, there should be no π-electron conjugation between the fluorophore and the nitro group since the benzene moiety and the fluorophore are uncoupled, as described above. In this case, how does the adjacent nitro group significantly affect the fluorescence properties of the fluorophore?

To address this question, we next focused on the LUMO energy level of nitro-substituted aromatic rings. In general, nitro groups greatly lower the LUMO energy level of aromatic compounds due to their strong electron-withdrawing effect. Recently, we reported that an electron-deficient benzene moiety can quench the fluorescence of a fluorophore via an intramolecular photoinduced electron transfer (PeT) process from the excited fluorophore to the electron-deficient benzene moiety (donor-excited PeT; d-PeT).[4] According to this principle, in the cases of **2** and **3**, the benzene moiety should act as an electron acceptor for the excited fluorophore due to the strong electron-withdrawing effect of the nitro group, and so we hypothesized that the most plausible mechanism for the quenching of the fluorescence of **2** and **3** is the d-PeT process.

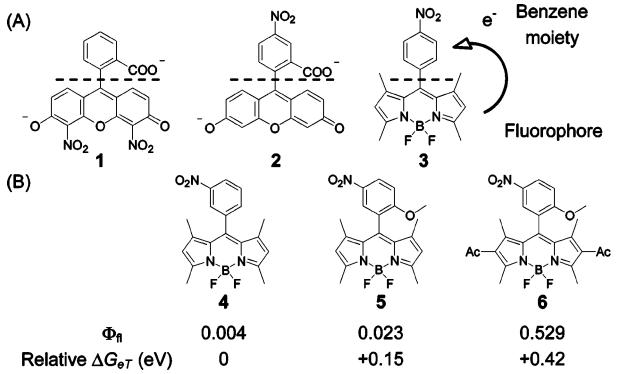

Figure 1. Structure of nitro-substituted fluorescein and BODIPY derivatives used in this study. (A) Fluorescein and BODIPY can be divided into two parts, the benzene moiety and fluorophore. (B) Φ_{fl} and relative ΔG_{eT} values of nitroBODIPY derivatives.

On the basis of the above concept, it should be possible to design a highly fluorescent compound, even if it has a nitro group proximal to the fluorophore, by appropriately controlling the relative free energy change of the PeT process (ΔG_{eT}).[5] To test this hypothesis, we designed and synthesized several nitroBODIPY derivatives (Figure 1B). The Φ_{fl} values of the simplest nitroBODIPY derivatives **3** and **4**, whose benzene moiety is nitrobenzene, were very small ($\Phi_{fl} < 0.01$). To avoid the occurrence of d-PeT, we next designed **5**. Upon introducing a methoxy group into the benzene moiety, the change of the relative ΔG_{eT} value of **5**, calculated with respect to the value for **3** as a standard, was +0.15 eV. This compound **5** showed weak fluorescence ($\Phi_{fl} = 0.023$). Next, we designed **6**, which also has 4-nitroanisole as its benzene moiety, but has a significantly more electron-deficient fluorophore, by introducing acetyl groups at the C-2 and C-6 positions of BODIPY. The change of the relative ΔG_{eT} value was calculated as +0.42 eV, and indeed, the Φ_{fl} value of **6** was increased to 0.529. Therefore, we had succeeded in designing an extremely rare example of a highly fluorescent compound bearing a nitro group by utilizing a mechanism-based approach. These results show that the quenching efficiency is strongly dependent upon the ΔG_{eT} value of the d-PeT process. Now, we can understand that the nitro group simply acts as an electron-withdrawing group in this context. This is the key to the design of novel fluorescence probes for nitrative stress. With this information in hand, we designed our fluorescence probes for nitrative stress, NiSPYs (Figure 2A).

For the development of probes for nitrative stress, we made use of the PeT process from the benzene moiety to the excited fluorophore (a-PeT), that is, the opposite direction to d-PeT, to quench the fluorescence of the probe before its encounter with the target nitrative stress. On the basis of a similar strategy, we have succeeded in developing various kinds of fluorescence probes for biomolecules, such as DAMBOs for nitric oxide,[3b] whose fluores-

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10.1021/ja061972v CCC: $33.50

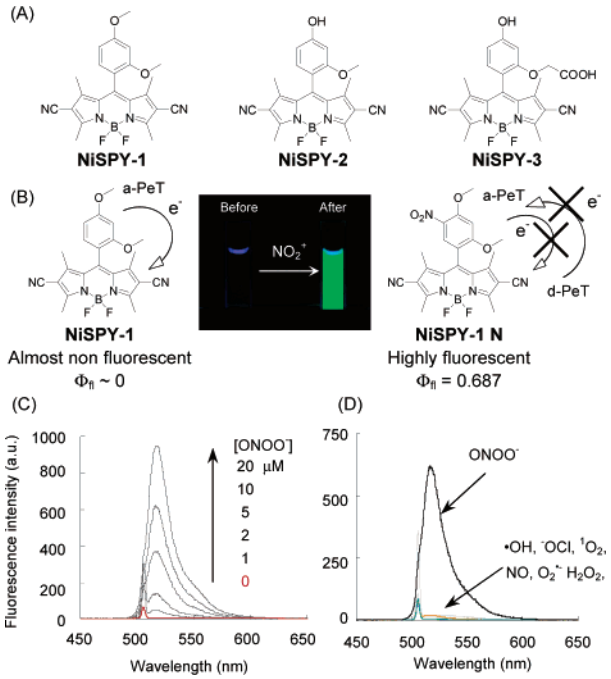

Figure 2. (A) Structures of NiSPYs. (B) Scheme and photo of NiSPY-1 before and after reaction with NO_2BF_4. (C) Fluorescence spectra of NiSPY-3 solution (10 μM NiSPY-3 in 0.1 M phosphate buffer pH 7.4 containing 0.1% DMF as a cosolvent) upon addition of peroxynitrite (final 0, 1, 2, 5, 10, 20 μM). (D) Fluorescence response of NiSPY-3 in various ROS generation systems (see Supporting Information).

cence enhancement is based on the change of the HOMO energy level of the benzene moiety upon encountering the target molecule.[3] Considering that nitration of the aromatic ring would lower the HOMO energy level significantly, an a-PeT-based design strategy is also expected to be suitable for the development of fluorescence probes for nitrative stress. Furthermore, as shown in Figure 2B, it is important that the nitration should occur at the benzene moiety and that the resulting nitrated product should be in a fluorescence ON state. It can easily be anticipated that the nitrated product (NiSPY-1 N) would be highly fluorescent because the calculated relative ΔG_{eT} value of NiSPY-1 N is +0.79 eV, which is high enough to prevent a d-PeT process (the Φ_{fl} and relative ΔG_{eT} values of **6** were 0.529 and +0.42, respectively).

To examine whether NiSPY-1 can monitor the nitration reaction or not, we examined the reaction between NiSPY-1 and NO_2BF_4, which serves as a nitronium source.[6] Nitration reaction of the benzene moiety of NiSPY-1 proceeded rapidly in CH_3CN, and the fluorescence of NiSPY-1 was dramatically enhanced (Figure 2B). Next, we tried to detect peroxynitrite in aqueous media with NiSPY-1. Peroxynitrite is a potent nitrating agent which is generated by the reaction of superoxide with nitric oxide in biological systems.[1a] Unfortunately, NiSPY-1 could not detect peroxynitrite, due to its low reactivity and low water solubility. We therefore made some structural modifications of NiSPY-1 to generate higher reactivity and water solubility, while retaining the desired fluorescence off/on properties before and after the nitration. To provide higher reactivity, we employed a phenol derivative as the benzene moiety since it is known to be more reactive with peroxynitrite compared with anisole derivatives. Water solubility was improved by incorporating a carboxyl group in the benzene moiety, and thus we obtained the novel fluorescence probes, NiSPY-2 and NiSPY-3. As expected, the fluorescence of NiSPY-2 and NiSPY-3 was dramatically enhanced upon addition of peroxynitrite (Figure S2 and Figure 2C), and the formation of a highly fluorescent nitrated product was confirmed by LC/Fl/MS. Moreover, as shown in Figure 2D, NiSPY-3 was highly selective for peroxynitrite among various reactive oxygen species (ROS); it showed little fluorescence augmentation upon addition of other reactive oxygen species, such as hydroxyl radical ($^{\bullet}OH$), hypochlorite (^{-}OCl), singlet oxygen ($^1O_2$), nitric oxide (NO), superoxide ($O_2^{\bullet -}$), and hydrogen peroxide (H_2O_2), whereas strong fluorescence enhancement was observed upon reaction with peroxynitrite ($ONOO^-$). This is considered to be a consequence of our mechanism-based design using the nitration reaction, which is highly specific for nitrative stress, as a chemical switch for modulating the fluorescence properties of the probe. NiSPY was confirmed to be applicable for fluorescence imaging of $ONOO^-$ in living cells (Figure S7) and showed no apparent cytotoxicity under these conditions (Figure S8).

In summary, this report describes (1) a plausible mechanism of a fluorescence quenching process by the nitro group, (2) a design strategy for highly fluorescent nitroBODIPY, and (3) development of novel highly selective fluorescence probes for nitrative stress, NiSPYs. NiSPYs are the first examples of fluorogenic probes which can specifically monitor aromatic nitration. These novel fluorescence probes should be useful as tools to study the role of nitrative stress in biological samples and in pathological processes because NiSPYs enable us to confirm the generation of nitrative stress, distinguishing it from other forms of oxidative stress. Further studies on the biological applications of NiSPYs are in progress.

Acknowledgment. This study was supported in part by a grant from Hoansha Foundation to T.N. This study was supported in part by research grants (Grant Nos. 14103018, 16651106, and 16689002 to Y.U.) from the Ministry of Education, Culture, Sports, Science and Technology of the Japanese Government, and by a grant from the Kato Memorial Bioscience Foundation to Y.U.

Supporting Information Available: Synthesis, experimental details, and characterization of BODIPY derivatives. This material is available free of charge via Internet at http://pubs.acs.org.

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JA061972V