Flavin-Based Light-Driven Fluorescent Probe for the Detection of Antioxidant Amino Acids

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We have synthesized a flavin-N(5)-oxide derivative with a p-toluenesulfonyl (**Ts-OF**) group as a "turn-on" fluorescent probe for the detection of several antioxidant amino acids and biothiols. Oxidized flavin was synthesized by using dithiothreitol as the reducing agent. **Ts-OF** showed a light-driven fluorescence enhancement in the presence of several amino acids and biothiols such as histidine (His), methionine (Met), cysteine (Cys), glutathione (GSH), and homocysteine (Hcy). The ^{1}H NMR study indicated the reductive elimination of the p-toluenesulfonyl group from **Ts-OF** in the presence of antioxidants and photo-irradiation.

It is known that several amino acids exhibit antioxidant effects.[1] Typically, sulfur-containing amino acids play crucial roles as strong antioxidants.[2] Antioxidants are closely related to the biological functions of cell proliferation, signal transduction, and tissue repair.[3] The disruption in the function of antioxidants such as methionine (Met), cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) is known to cause various diseases such as liver damage, leukocytosis, psoriasis, Alzheimer's and Parkinson's diseases, and AIDS.[4] Therefore, the detection of antioxidant biomolecules is very important. To date, various methods such as high-performance liquid chromatography,[5] electrochemical detection,[6] capillary electrophoresis,[7] optical analysis,[8] and mass spectroscopy[9] have been developed for the detection of antioxidant biomolecules. Among them, optical detection techniques have attracted considerable attention because they are convenient, simple, and precise, in addition to having low detection limits.[10] Particularly, the techniques based on "turn-on" change in fluorescence are very useful for the detection of different biomolecules in vitro and in vivo. By using the developed fluorescent probes, many scientists have researched optical sensors that can image antioxidants in cells. Specific analytes in cells could be easily visualized and detected.[11]

To investigate the fluorescence probes, we have designed a flavin-based fluorescent probe, which showed an enhancement in its fluorescence emission upon photoirradiation in the presence of several antioxidant amino acids. The synthesized

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probe (**Ts-OF**) has an aza aromatic-N-oxide group. It is very useful for photochemistry such as ring contraction, isomerization of isoxazoles to oxazoles, ring expansion, ring fragmentation, and deoxygenation.[12] By reducing it using dithiothreitol (DTT), the structure of the flavin-N(5)-oxide is converted to flavin, which has a fully conjugated backbone structure and exhibits strong fluorescence emission behavior.

The synthetic procedure of **Ts-OF** is summarized in Scheme 1. Briefly, 6-chlorohexan-1-ol was reacted with 3,4-dimethylaniline to obtain **1**. Next, 6-chlorouracil was reacted
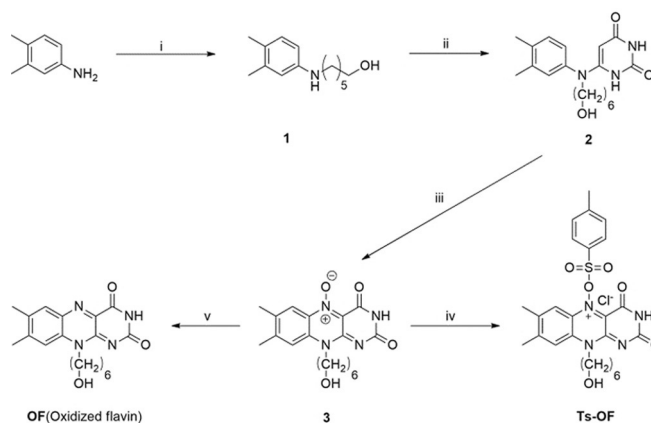

Scheme 1. Synthesis of flavin-based fluorescent probe (**Ts-OF**). Reagents and conditions: i) 6-chlorohexan-1-ol, triethylamine; ii) 6-chlorouracil, H$_2$O/dioxane; iii) NaNO$_2$, acetic acid; iv) p-toluenesulfonyl chloride, methanol; v) DTT, ethanol.

with **1** to get **2**. Subsequently, an oxidation reaction was carried out under acidic conditions with NaNO$_2$ to form the flavin derivatives **3**. Finally, the p-toluenesulfonyl group was introduced in **3** to obtain a semiquinone flavin derivative flavin-N(5)-oxide (**Ts-OF**). On the other hand, reduced flavine (**OF**) was synthesized by the reduction of **3** using DTT and used as a control molecule to compare its oxidation state with **Ts-OF**.

The absorption spectra of **Ts-OF** and **OF** were measured in a mixture of ethanol and H$_2$O (v/v = 1:1, 100 μm). Both **Ts-OF** and **OF** showed multiple absorption bands in the visible region. The wavelengths of the absorption maxima for **Ts-OF** and **OF** were 460 and 440 nm, respectively (Figure 1a). We could estimate that the molecular absorption coefficient of **Ts-OF** was larger than that of the oxidized flavin (**OF**). However, the fluorescence emission intensity of **Ts-OF** was significantly smaller than that of **OF**. Therefore, it was concluded that these properties would be useful for the design of turn-on fluorescence probes, because the reduction of flavin-N(5)-oxide to oxidized flavin resulted in fluorescence enhancement.
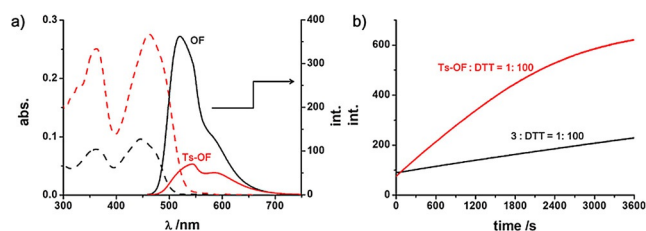
Figure 1. a) Absorption and fluorescence spectra of **OF** and **Ts-OF**, b) time-dependent fluorescence measurement of **Ts-OF** and **3** with 100 equivalents of DTT.

It is well known that flavin derivatives exhibit redox-sensitive properties.[13] Therefore, the electronic structure of flavin derivatives can be changed by electrochemical treatment and photoexcitation. Generally, oxidized flavin derivatives tend to be reduced by an intermolecular electron transfer from reducing agents.[14] The flavin derivative **3** was also reduced to **OF** upon treatment with DTT. However, the reduction of **3** with DTT required high-temperature conditions (60 °C). Although **3** responded to DTT, it was not possible to utilize it as a fluorescent probe because of the harsh reaction conditions.[15] Therefore, we introduced the *p*-toluenesulfonyl group, which is an excellent leaving group, to **3** in order to enhance the reduction reaction. As a result, **Ts-OF** (100 μM) dissolved in a mixture of ethanol and H_2O (v/v = 1:1) showed strong fluorescence enhancement upon treatment with 100 equivalents of DTT at room temperature. We also conducted the time-dependent measurement of fluorescence emission to monitor the reaction rate of **Ts-OF** and **3** with DTT. As shown in Figure 1 b, **3** shows a very slow increase in its fluorescence intensity when mixed with DTT at room temperature, whereas **Ts-OF** shows remarkable enhancement in fluorescence because the *p*-toluenesulfonyl group can be readily lost.

As the solution of **Ts-OF** and DTT showed strong fluorescence enhancement, we tested the fluorescence response of **Ts-OF** with Cys as the representative antioxidant in the biosystem. It was found that the fluorescence emission of **Ts-OF** solution (100 μM) was greatly enhanced by the addition of 1 mM Cys, and the time-dependent emission intensity changes showed complete saturation within 11 min (Figure 2 c). Also, it was faster than the treatment of DTT. Based on the time-dependent fluorescence emission measurement, continuous excitation at 450 nm was applied to the **Ts-OF** solution for the monitoring of fluorescence emission. As mentioned before, the electronic structure of flavin derivatives can be changed by electrochemical treatment and also by photoexcitation. Therefore, we expected that light irradiation would influence the rate of the reaction between **Ts-OF** and Cys. To confirm the influence of light irradiation, the **Ts-OF** solution containing Cys was incubated in the dark for 11 min. It was found that the emission of **Ts-OF** almost maintained its original intensity, indicating that the reaction is only promoted by light irradiation (Figure 2).

The fluorescence response of **Ts-OF** to various amino acids and biothiols is shown in Figure 2. To a 100 μM solution of **Ts-OF** in 10 mM PBS (pH 7.4, 150 mM NaCl), 10 equivalents of vari-
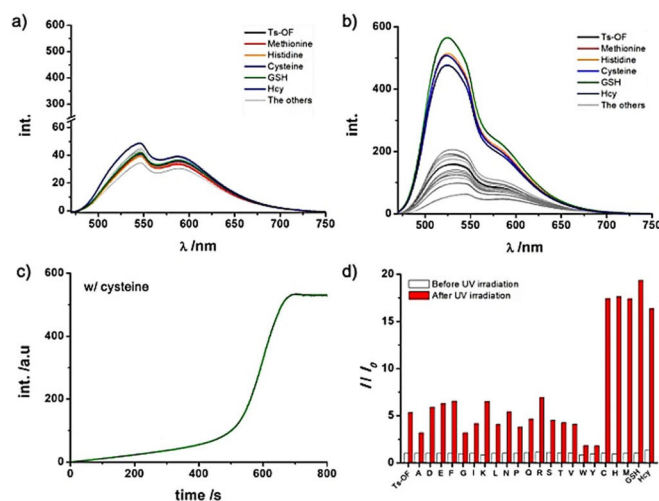

Figure 2. a) Changes in the emission intensity and fluorescence of **Ts-OF** on addition of various amino acids and b) under dark conditions; c) the time-dependent change in the emission intensity of Ts-OF monitored at 544 nm with 100 equivalents of Cys; d) emission intensity before and after UV irradiation for 11 min.

ous amino acids, GSH, and Hcy were added separately and incubated for 11 min in the dark. Although there were no spectral changes under the dark conditions, changes in the absorption and emission of these solutions of **Ts-OF** with amino acids were evident when they were subjected to irradiation by using a UV hand lamp (VILBER Lourmat). The emission intensity of the solutions containing methionine, Cys, GSH, Hcy, and histidine increased significantly upon UV irradiation (Figure 2). It is known that methionine, Cys, GSH, Hcy, and histidine have a strong antioxidant effect, because of the sulfur- and imidazole-bearing side groups. Therefore, the reaction was initiated by the photoexcitation of **Ts-OF** and reductive elimination of the *p*-toluenesulfonyl group during its interaction with antioxidant molecules. Also, we conducted the fluorescent titration with antioxidants such as Cys, GSH, Hcy, Met, and His. They required different amounts to reach the maxima of emission intensity. For example, the emission intensity of the solutions containing Cys or GSH was significantly enhanced at the amount of 10 equivalents. Met, Hcy, and His showed enhancement of the emission intensity above 3 equivalents, which is lower than Cys and GSH (Figure 3).

To confirm the reductive elimination of the *p*-toluenesulfonyl group from **Ts-OF**, ^{1}H NMR studies were carried out in D_2O. As shown in Figure 4, the aromatic proton signals of **Ts-OF** appear at 8.71 and 8.19 ppm. When an excess amount (80 equiv.) of Cys was added and the **Ts-OF** solution was irradiated with light, a new set of aromatic proton signals appeared at 8.43 and 8.16 ppm, which are consistent with the aromatic proton signals of **OF** and indicate that the *p*-toluenesulfonyl group is eliminated from **Ts-OF**. The intensity of the new peaks gradually increased with increasing exposure time to UV light, indicating a photoinduced reaction.

In summary, we have synthesized a flavin-based fluorescent probe, which showed light-driven fluorescence enhancement in the presence of several amino acids and biothiols. ^{1}H NMR
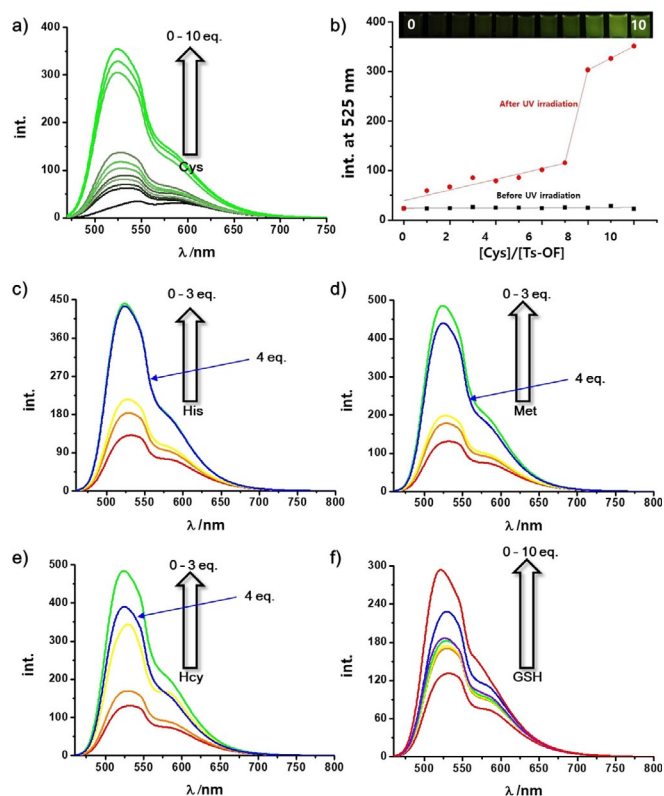
Figure 3. The spectra of fluorescence titration with various antioxidants: a) cysteine, c) histidine, d) methionine, e) homocysteine, and e) glutathione; b) the change of emission intensity according to equivalents of cysteine monitored at 525 nm.
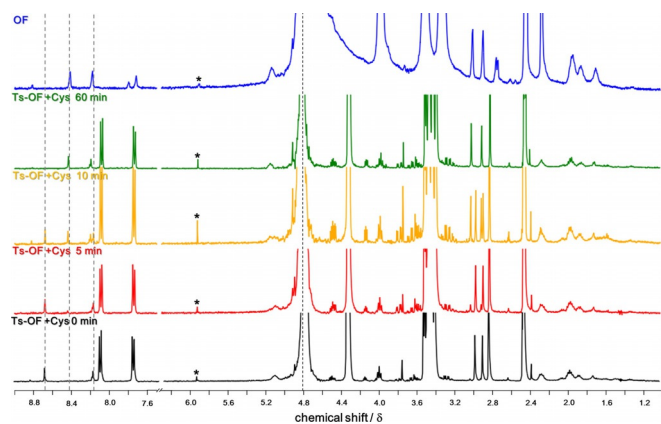
Figure 4. ^{1}H NMR experiments of Ts-OF with 80 equivalents of Cys according to exposure UV irradiation time (0, 5, 10, 60 min) in D_2O.

studies revealed that **OF** is generated from **Ts-OF** through reductive elimination of the *p*-toluenesulfonyl group. As the fluorescence response requires both chemo- and photo-stimuli, this methodology makes it possible to reduce the misreading of research results by undesirable fluorescence "turn on" processes upon the detection of specific chemicals.

Experimental Section

All commercially available chemicals were of reagent grade and used as-received without further purification. Dichloromethane (DCM) was freshly distilled before use. UV/Vis absorption spectra were recorded on a JASCO model V-660 spectrometer. Fluorescence spectra were measured by using a JASCO FP-6300 spectrophotometer. All spectral measurements were carried out in a quartz cuvette with a path length of 1 cm. ^{1}H NMR spectra were recorded on Bruker Advance PDX 250 and DPX 400 spectrometers at 25 °C in either $CDCl_3$, [D_6]DMSO, CD_3OD, or D_2O. MALDI-TOF-MS experiments were performed on a Bruker Daltonics LRF20 with dithranol (1,8,9-trihydroxyanthracene) as the matrix.

1: To a solution of 3,4-dimethylaniline (123 mmol, 15 g) in triethylamine (30 mL), 6-chlorohexanol (82.5 mmol, 11.2 g) was added and the reaction mixture was refluxed for 6 h. Subsequently, it was extracted with H_2O/CH_2Cl_2, and the organic layer was concentrated and purified by silica column chromatography using 5% MeOH/CH_2Cl_2 as an eluent to give **1** (10 g, 57%). ^{1}H NMR (400 MHz, $CDCl_3$) $\delta = 6.94–6.92$ (d, $J = 8$ Hz, 1 H), 6.44 (s, 1 H), 6.40–6.39 (d, $J = 4$ Hz, 1 H), 6.38–6.37 (d, $J = 4$ Hz, 1 H), 3.67–3.64 (t, $J = 12$ Hz, 2 H), 3.10–3.07 (t, $J = 12$ Hz, 2 H), 2.19 (s, 3 H), 2.15 (s, 3 H), 1.64–1.57 (m, 4 H), 1.48–1.40 ppm (m, 4 H).

2: To a solution of **1** (22 mmol, 5.0 g) in a mixture of H_2O and 1,4-dioxane (v/v = 50%, 30 mL), 6-chlorouracil (7.5 mmol, 1.1 g) was added and the reaction mixture was refluxed for 15 h. Subsequently, the solvents were removed under reduced pressure using a rotary evaporator and the residue was purified by silica column chromatography using 6% MeOH/CH_2Cl_2 as an eluent to give **2** (2.35 g, 94%). ^{1}H NMR (250 MHz, [D_6]DMSO) $\delta = 10.36$ (s, 1 H), 10.15 (s, 1 H), 7.23–7.20 (d, $J = 7.5$ Hz, 1 H), 7.03 (s, 1 H), 6.98–6.94 (m, 1 H), 4.40–4.36 (t, $J = 10$ Hz, 1 H), 4.06–4.05 (d, $J = 2.5$ Hz, 1 H), 3.60–3.54 (t, $J = 15$ Hz, 2 H), 2.23 (s, 6 H), 2.08 (s, 3 H), 1.48–1.20 ppm (m, 7 H)

3: To a solution of **2** (0.31 mmol, 0.1 g) in glacial acetic acid (1.6 mL), sodium nitrite (1.47 mmol, 0.1 g) was added and the reaction mixture was further stirred for 3 h in the dark. Subsequently, H_2O was added and the solvents of the reaction mixture were removed. The residue was purified by silica column chromatography using a mixture of MeOH, ethylacetate, and CH_2Cl_2 (MeOH:EtOAc:$CH_2Cl_2 = 5:15:75$) as an eluent to give **3** (0.1 g, 97%). ^{1}H NMR (400 MHz, [D_6]DMSO) $\delta = 9.5$ (s, 1 H), 8.09 (s, 1 H), 7.80 (s, 1 H), 4.54–4.42 (m, 4 H), 2.47 (s, 3 H), 2.39 (s, 3 H), 1.72–1.68 (m, 4 H), 1.47–1.48 ppm (m, 4 H); MALDI-TOF-MS *m/z*: cald. for $C_{18}H_{22}N_4O_4$: 358.16 [M$^+$]; found: 357.88.

OF: To a solution of **3** (0.03 mmol, 0.01 g) in ethanol (20 mL), DTT (0.014 mmol, 0.02 g) was added under an inert N_2 atmosphere and refluxed for 4 h. Subsequently, the solvent was removed and the residue was recrystallized with ethanol to give **OF** (8 g, 79%). ^{1}H NMR (250 MHz, CD_3OD) $\delta = 7.97$ (s 1 H), 7.77 (s, 1 H), 3.61–3.57 (t, $J = 10$ Hz, 2 H), 2.59 (s, 3 H), 2.47 (s, 3 H), 1.94–1.75 (m, 4 H), 1.67–1.52 ppm (m, 6 H); MALDI-TOF-MS *m/z*: cald. for $C_{18}H_{22}N_4O_3$: 342.17 [M$^+$]; found: 343.83.

Ts-OF: To a solution of **3** (0.09 mmol, 0.03 g) in CH_2Cl_2, *p*-toluenesulfonyl chloride (0.13 mmol, 24.8 mg) was added and the mixture solution was stirred for 12 h at room temperature. Subsequently, the solvent was removed and the residue was purified by silica column chromatography with 25% MeOH/CH_2Cl_2 as an eluent to give **Ts-OF** (3 mg, 7%). ^{1}H NMR (400 MHz, D_2O) $\delta = 8.71$ (s, 1 H), 8.19 (s, 1 H), 8.12–8.10 (d, $J = 8$ Hz, 2 H), 7.78–7.75 (d, $J = 8$ Hz, 2 H), 4.02–3.99 (t, $J = 12$ Hz, 2 H), 2.99 (s, 3 H), 2.91 (s. 3 H), 2.84 (s, 3 H),

2.30–2.26 (m, 3H), 2.02–1.86 ppm (m, 7H); MALDI-TOF-MS m/z: cald. for $C_{25}H_{29}N_4O_6S$: 513.18 [M$^+$]; found: 514.89.

Acknowledgements

Conflict of Interest

The authors declare no conflict of interest.

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