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Fluorogenic Imines for Fluorescent Detection of Mannich-Type Reactions of Phenols in Water

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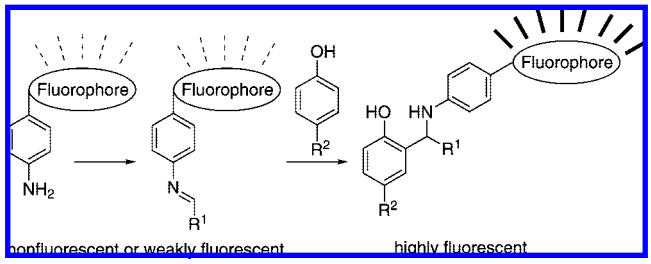

Fluorogenic imines and their precursor amines that can be used for fluorescent visualization of Mannich-type reactions of phenols in aqueous buffers have been developed. The precursor amines are aniline derivatives that are covalently conjugated to fluorophores. These amines and their imine derivatives were nonfluorescent or very weakly fluorescent. On the other hand, addition products of the imines to phenols showed more than 100-fold higher fluorescence than the imines and the precursor amines.

Fluorogenic molecules that afford fluorescent products upon bond-forming reactions allow monitoring the progress of the reactions by increase in fluorescence.[1–3] Evaluation of chemical transformations with these fluorogenic molecules accelerates characterization and screening of catalysts and/or reaction conditions.[1,2] In addition, these fluorogenic molecules enable fluorescent detection of molecules that react with the fluorogenic molecules.[1] For example, fluorogenic maleimides are nonfluorescent or very weakly fluorescent until they react with thiols; the reaction products are highly fluorescent, allowing fluorescent detection or fluorescent visualization of thiols and thiol-containing molecules.[1a–c] When intrinsically fluorescent molecules are used for bond-forming reactions, separation of product molecules from unreacted fluorescent molecules is required before analysis; an advantage of use of fluorogenic molecules is that the reaction products can be analyzed without need for a separation step.[1,2] Although there are many types of fluorogenic molecules,[1,2] fluorogenic molecules for reactions of phenols have not been reported.[4] The phenol functionality is present in tyrosine, a building block of proteins, and in many natural products.[5] Tyrosine residues on folded proteins have been used as modification sites.[4] Therefore, fluorogenic molecules that react with phenols would be useful for screening of catalysts and reaction conditions for the bond-forming reactions with phenols including phenol-bearing biomolecules and for fluorescent labeling and detection of molecules possessing accessible phenols. Here, we report the first examples of fluorogenic molecules for Mannich-type reactions of phenols in aqueous buffers.

Since imines and iminiums react with phenols through Mannich-type reactions in aqueous buffers or water-containing solvents under mild conditions,[4a,b] we chose imines as phenol-

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reacting moieties for the design of the fluorogenic molecules. We reasoned that combination of an imine moiety and a fluorophore in one molecule, in which the imine group acts as a reaction moiety and also acts a quencher that suppresses the fluorescence of the fluorophore,[1,2a,b] should provide a fluorogenic molecule for reactions with phenol derivatives. In the presence of water, imines are often hydrolyzed into amines and carbonyl compounds; therefore, consideration of fluorescence of the hydrolyzed products (i.e., amines and carbonyl compounds) was also required for the design of the imine-based fluorogenic molecules. It has been reported that an aniline amino group intramolecularly quenches fluorophore's fluorescence;[3g,6] therefore, we reasoned that molecules possessing a fluorophore and an aniline moiety should be useful precursors of fluorogenic imines. It has been suggested that presence of a free-rotating amine, which has a lone pair, of an aniline moiety is key for fluorescence quenching.[6] We hypothesized that the quenching feature of the aminophenyl moiety should differ between the amine/imine and the reaction product of the imine with phenol derivatives.

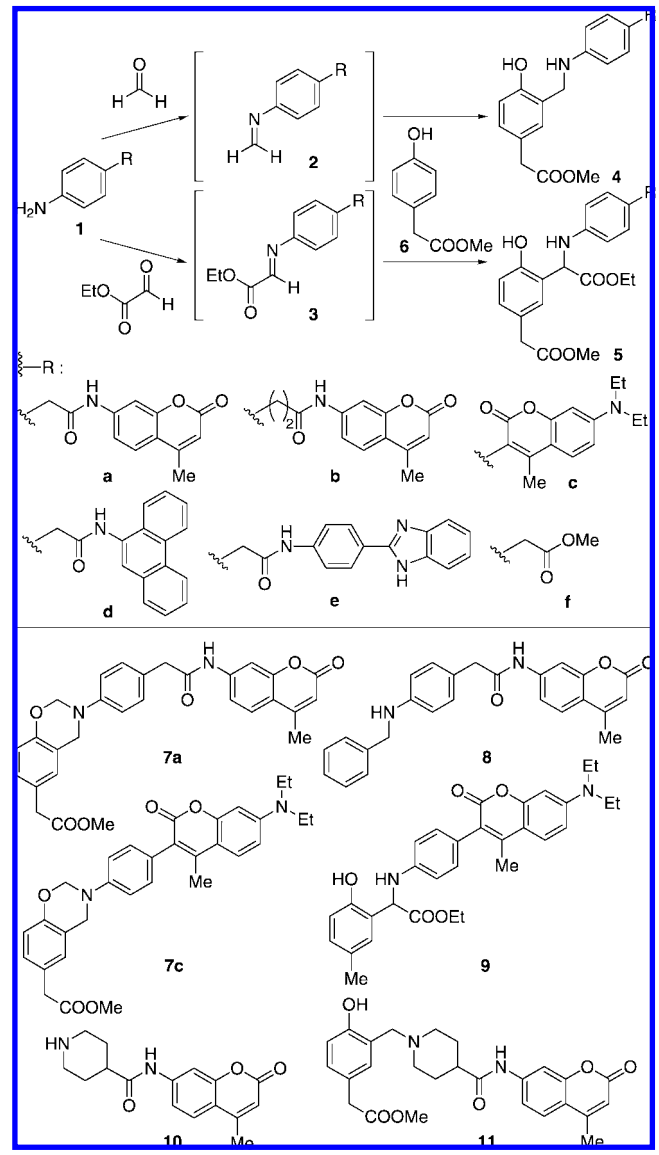
To test our hypothesis, amines, imines, addition products of phenol derivatives to the imines, and control molecules shown in Scheme 1 were designed and synthesized and the fluorescence of these compounds were analyzed in aqueous buffer (Table 1). Amines **1a**−**e** possessed fluorophores, whereas amine **1f** did not and was a control. Imines **2** and **3** were prepared by mixing amine **1** with formaldehyde or ethyl glyoxylate, respectively, in DMSO. The resulting imine was added to the buffer before the fluorescence measurements. Under the conditions of the fluorescence analysis for these imines, the corresponding amine coexisted with the imine because of partial hydrolysis of the imine.

Addition products **4** and **5** containing fluorophores were highly fluorescent (entries 3, 9, 13, 15, 18, 22, and 24).[7] On the other hand, amines and imines were nonfluorescent or were very weakly fluorescent (entries 1, 2, 8, 12, 14, 17, 21, and 23). The fluorescent Mannich-type products **4** and **5** showed more than 100-fold higher fluorescence than the corresponding amine and imine under the conditions shown in Table 1. Fluorescence emission spectra of **1c**, **3c**, and **5c** are shown in Figure 1A. Control products **4f** and **5f** (entries 10 and 11), which do not include fluorophores, and phenol derivative **6** (entries 4 and 20) were not fluorescent at the wavelengths used,[8] indicating that the fluorescence of **4a**−**e**, **5a**, and **5c** originates from the fluorophore. Compounds **7a** and **7c**, which are also possible addition products[4a] that can be formed with **4a** and **4c**, respectively, were also fluorescent (entries 5 and 16).

On the other hand, compound **8** (entry 6) was not fluorescent; the substituent on the nitrogen of the aniline moiety significantly affected the fluorescence. A mixture of amine **1a** and phenol derivative **6** (entry 7) was not fluorescent. These results indicate that the phenolic hydroxy group in products **4** and **5** is important for the fluorescence. Intramolecular interaction between the phenolic hydroxyl group and the amine (O---HN or OH---N) or $\pi-\pi$ stacking interactions between the aryl moieties of the phenol group and the fluorophore[2g] may be required for the fluorescence of the addition products, although other mechanisms are also possible. Addition product **9**, which was prepared by the reaction between *p*-cresol and imine **3c**, also showed fluorescence similar to that of **5c** (entry 19 versus entry 18), suggesting that addition products of other phenol derivatives to the fluorogenic imines should be fluorescent if another quenching moiety is not present in the phenol derivative. Piperidine-derived amine **10** and its addition product **11** were both highly fluorescent (entries 25 and 26); the piperidine moiety did not quench the fluorophore's fluorescence. Thus, covalent combination of an aniline moiety and a fluorophore was required for an amine to be a fluorogenic imine precursor.

In order to demonstrate the use of the fluorogenic molecules for the reaction with phenols, the reaction between **3c** and **6** was performed in aqueous buffer and the fluorescence was analyzed after 70 min (Figure 1B). The reaction mixture containing imine **3c** and phenol **6** showed significantly higher fluorescence than the solution of imine **3c** alone or of phenol **6** alone in the same buffer.[9] These results indicate that the

SCHEME 1. Amines, Imines, and Mannich-Type Reaction Products with Phenols Used for the Fluorescence Analyses



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(7) Addition products **4a**, **4c**, **5a**, and **5c** showed fluorescence in 5% DMSO/50 mM sodium phosphate, pH 6.0 or 8.0 and in 5% DMSO/50 mM Tris, pH 8.0 similar to the fluorescence observed in 5% DMSO/50 mM sodium phosphate, pH 7.0.

(8) Compounds **4**−**6** showed fluorescence characteristic of phenols.

(9) Formation of **5f** was also observed in the preparative-scale reaction of **3f** with **6** in the buffer.

TABLE 1. Fluorescence of Amines, Imines, and Addition Products[a]

entry	compound	λ_{ex} (nm)	λ_{em} (nm)	fluorescence intensity[b]
1	**1a**	350	380	<10
2	**2a**			<10
3	**4a**			1.7×10^3
4	**6**			<10
5	**7a**			1.7×10^3
6	**8**			<10
7	**1a** + **6**[c]			<10
8	**3a**			<10
9	**5a**			2.8×10^3
10	**4f**			<10
11	**5f**			<10
12	**1b**			<10
13	**4b**			1.8×10^3
14	**1c**	415	445	5
15	**4c**			1.7×10^3
16	**7c**			1.6×10^3
17	**3c**			50[d]
18	**5c**			8.0×10^3
19	**9**			7.0×10^3
20	**6**			<10
21	**1d**	350	380	<10
22	**4d**			2.5×10^3
23	**1e**	350	380	<10
24	**4e**			1.5×10^3
25	**10**	325	395	1.6×10^3
26	**11**			1.1×10^3

[a] Fluorescence was recorded on a microplate spectrophotometer using 100 μL of 5 μM solution in 5% DMSO/50 mM sodium phosphate, pH 7.0. For the imine, see the text. [b] Relative fluorescence intensity. [c] **1a** (5 μM) + **6** (5 μM). [d] The data without background correction.

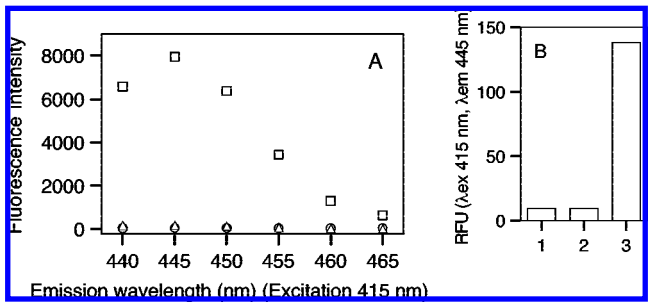

FIGURE 1. (A) Fluorescence emission spectra (λ_{ex} 415 nm) of amine **1c** (circle), imine **3c** (triangle), and addition product **5c** (square) at 5 μM in 5% DMSO/50 mM sodium phosphate, pH 7.0. (B) Fluorescence increase (λ_{ex} 415 nm, λ_{em} 445 nm) after 70 min in 5% DMSO/50 mM sodium phosphate buffer, pH 7.0: column 1, phenol derivative **6**, 2.5 mM; column 2, imine **3c**, 2.5 μM; column 3, **6**, 2.5 mM and **3c**, 2.5 μM.

Mannich product formation of the phenol with the imine was detected by increase in fluorescence. Note that imines prepared from formaldehyde and aniline derivatives have been used for labeling of proteins at tyrosine.[4a] Therefore, fluorogenic imines described here may be used for reactions with tyrosine residues of peptides and proteins.

In summary, we have developed fluorogenic imines and their precursor amines that can be used for fluorescent detection of Mannich-type reactions of phenols in aqueous buffers. These fluorogenic imines should be useful for detection of phenol-bearing molecules and for screening of catalysts and conditions for labeling reactions of phenol-bearing molecules. Our design strategy for the creation of fluorogenic imines and their precursor amines should be useful for the development of other fluorogenic molecules for bond-forming reactions.

Experimental Section

General Procedure for the Synthesis of 1. A mixture of (4-*tert*-butoxycarbonylaminophenyl)acetic acid (5 mmol), fluorophore amine (5 mmol), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (1.44 g, 7.5 mmol), and DMAP (5.0 mg, 0.04 mmol) in CH_2Cl_2 (20 mL) was stirred at room temperature for 24 h. Usual workup followed by purification by silica gel flash column chromatography afforded the amide derivative. The Boc group of this compound (3 mmol) was deprotected in trifluoroacetic acid (TFA) (10 mL)−anhydrous CH_2Cl_2 (10 mL) at room temperature, and then the solvents were removed in vacuo. Usual workup followed by purification by silica gel flash column chromatography afforded **1**.

Compound 1a. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.45 (s, 1H), 7.75 (d, J = 1.6 Hz, 1H), 7.70 (d, J = 8.4 Hz, 1H), 7.49 (dd, J = 8.4 Hz, 1.2 Hz, 1H), 6.98 (d, J = 8.0 Hz, 2H), 6.51 (d, J = 8.0 Hz, 2H), 6.25 (s, 1H), 4.94 (s, 2H), 3.47 (s, 2H), 2.39 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 170.6, 159.9, 153.5, 152.9, 147.2, 142.5, 129.4, 125.7, 122.1, 114.9, 114.7, 113.7, 112.0, 105.3, 42.6, 17.8; HRMS calcd for $C_{18}H_{17}N_2O_3$ (MH^+) 309.1234, found 309.1233.

General Procedure for the Synthesis of 4. Amine **1** (0.1 mmol) was dissolved in TFA (1 mL)−anhydrous CH_2Cl_2 (1 mL). To this solution were added (4-hydroxyphenyl)acetic acid methyl ester (19.9 mg, 0.12 mmol) and 37% formaldehyde solution in water (14.9 μL, 0.2 mmol), and the mixture was stirred for 24 h at room temperature. Usual workup followed by purification by silica gel flash column chromatography afforded **4**.

Compound 4a. ^{1}H NMR (400 MHz, CD_3OD−$CDCl_3$) δ 7.60−7.51 (m, 3H), 7.16−7.14 (m, 2H), 7.11 (d, J = 2.4 Hz, 1H), 7.05−7.03 (m, 1H), 6.79 (d, J = 8.4 Hz, 1H), 6.76−6.73 (m, 2H), 6.18 (d, J = 1.2 Hz, 1H), 4.32 (s, 2H), 3.67 (s, 3H), 3.61 (s, 2H), 3.53 (s, 2H), 2.41 (d, J = 1.2 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD−$CDCl_3$) δ 172.9, 171.1, 161.8, 154.7, 153.8, 152.9, 141.7, 129.9, 129.6, 129.1, 124.9, 124.8, 124.2, 124.1, 115.7, 115.6, 114.7, 112.7, 112.6, 106.8, 104.7, 51.8, 45.6, 43.3, 40.0, 18.2; HRMS calcd for $C_{28}H_{27}N_2O_6$ (MH^+) 487.1864, found 487.1868.

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Supporting Information Available: Additional fluorescence data, fluorescence spectra, and synthesis and characterization of compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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