Design and Synthesis of Intramolecular Resonance-Energy Transfer Probes for Use in Ratiometric Measurements in Aqueous Solution

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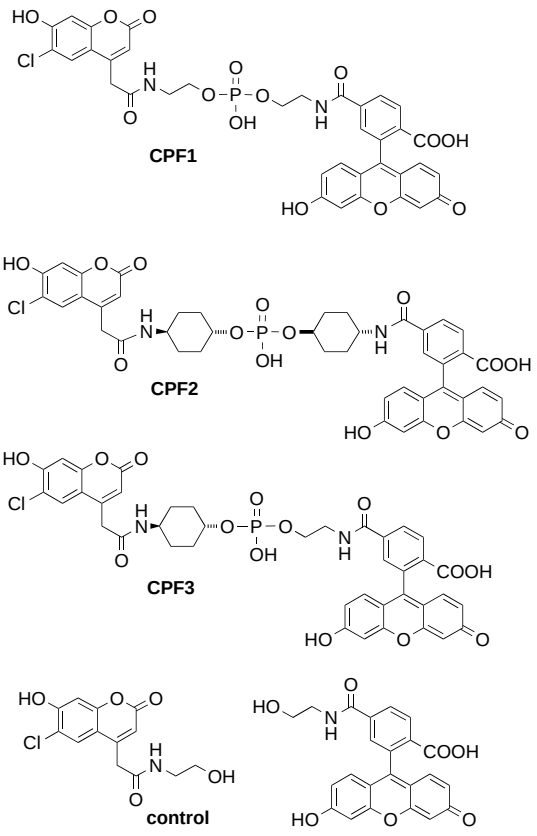
In recent years, many fluorescent probes have been developed to study biological phenomena in living cells. By using fluorescent probes, it is possible to follow a phenomenon in real time with high sensitivity. However, the fluorescence intensity of the probes is influenced by many factors, such as changes of environment around the probe (pH, polarity, temperature, and so forth), changes in the probe concentration, and changes in the excitation intensity. To reduce the influence of such factors, ratiometric measurements are utilized,[1] namely, observation of changes in the ratio of the fluorescence intensities at two wavelengths. This technique allows more precise measurement and, with some probes, quantitative detection is possible. Many probes have been developed for ratiometric measurements,[1] such as the calcium indicator Fura-2[2] and the pH indicator SNARF-1.[3] To carry out ratiometric measurements, the probe must exhibit a large shift in its emission or excitation spectrum after it reacts (or binds) with the target molecule. Resonance-energy transfer (RET) is one mechanism used to obtain a large shift in the spectral peak.

RET is a radiationless transmission of an energy quantum between two fluorophores in close proximity.[4] If the bond between two linked fluorophores, which have an overlap in their spectra, is cleaved, we observe the emission of the acceptor, due to RET, before the cleavage and the emission of the donor after. This results in a large shift in the emission peak, which can be used for ratiometric measurements.

Little work has been done on chemically synthesized intramolecular RET probes except for the probe for detecting β-lactamase activity[5] reported by Tsien and co-workers. This is because, even if two fluorophores are located in close proximity, self-quenching occurs due to the close contact of the two hydrophobic fluorophores in an aqueous solution.[6] If we could observe the emission of the acceptor in chemically synthesized RET probes, we would be able to design probes for ratiometric measurements of many enzymes, by binding two fluorophores at the ends of a peptide substrate (or another cleavable linker), which could be cleaved selectively by the enzyme.

Herein, we present a method to observe the emission of the acceptor caused by intramolecular RET, applicable for ratiometric measurements in aqueous solution. This method is based upon the idea that self-quenching between the two fluorophores can be blocked and the emission of the acceptor can be observed by restricting the flexibility of the linker between the two fluorophores, so that they can not readily come into close contact.

To investigate the relation between the flexibility of the linker and the emission spectrum, we designed and synthesized coumarin phosphodiester-linked fluoresceins (CPFs). CPFs have two fluorophores, coumarin as a donor and fluorescein as an acceptor. These two fluorophores are known to have a large overlap in their spectra and exhibit efficient emission in an aqueous solution. The phosphodiester moiety was introduced to enhance the aqueous solubility of the probe and was placed as a linker between the two fluorophores. Ethylene was chosen as a linker extension with high flexibility and cyclohexane as one with low flexibility. Three molecules with different linkers were synthesized; CPF1 with two ethylene linkers, CPF2 with two cyclohexane linkers, and CPF3 with one ethylene and one cyclohexane linker. We used a mixture of the amide form of coumarin and fluorescein as the control. CPFs were synthesized as shown in Scheme 1.



The emission spectra of CPFs in an aqueous buffer excited at 370 nm (the excitation wavelength, λ_{ex},of the coumarin moiety) are shown in Figure 1a. Efficient RET was observed with all CPFs, as more than 90% of the emission of the coumarin donor ($\lambda_{em} = 450$ nm) was quenched. A strong emission of the fluorescein acceptor ($\lambda_{em} = 515$ nm) was observed only in CPF2. The fluorescein acceptor of CPF2 emitted 54% of the photons that excited the donor. CPF1 and CPF3 had only weak emission and showed self-quenching.

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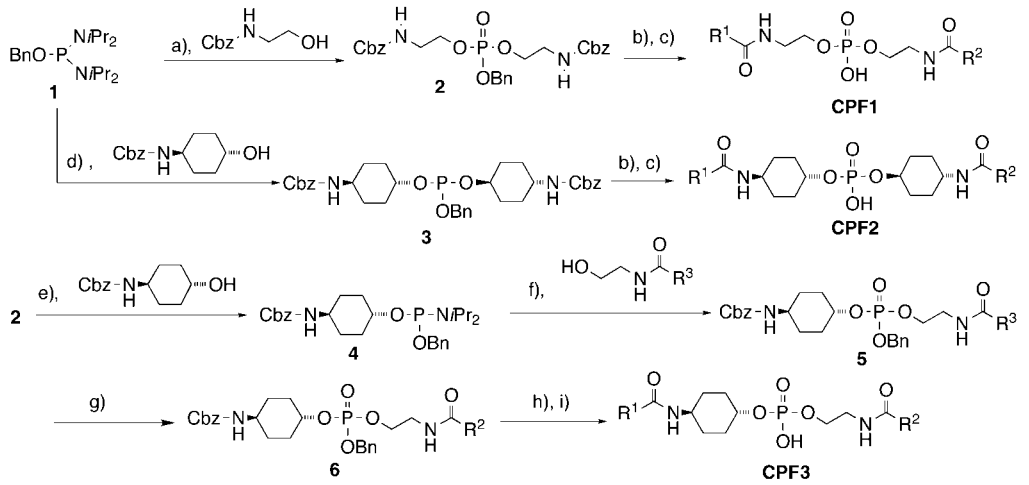

Scheme 1. Synthesis of CPFs. Compound **1** was synthesized as previously reported.[7] Flurophores: R^1 = coumarin, R^2 = fluorescein, R^3 = fluorescein dipivaloate. Synthesis: a) 1) Tetrazole, CH_2Cl_2, RT, Ar atmosphere; 2) 3-chloroperoxybenzoic acid (*m*-CPBA), −40 °C. b) 10 % Pd/C, 95 % aq. EtOH, 1 atm H_2. c) 1) Coumarin succinimide ester, MeOH, RT; 2) carboxyfluorescein succinimide ester, 100 mM aq. $NaHCO_3$, RT. d) 1) Tetrazole, CH_2Cl_2, 30 °C, Ar atmosphere; 2) *m*-CPBA, −40 °C. e) Diisopropylammonium tetrazolide, CH_2Cl_2, 30 °C. f) 1) Tetrazole, CH_2Cl_2, 0 °C, Ar atmosphere; 2) *m*-CPBA, −40 °C. g) MeOH, 2 N aq. NaOH. h) 2 % Pd/C, 95 % aq. EtOH, 1 atm H_2. i) Coumarin succinimide ester, dimethylformamide, triethylamine. All CPFs were purified by reverse-phase HPLC. Bn = benzyl; Cbz = carbobenzoxy.

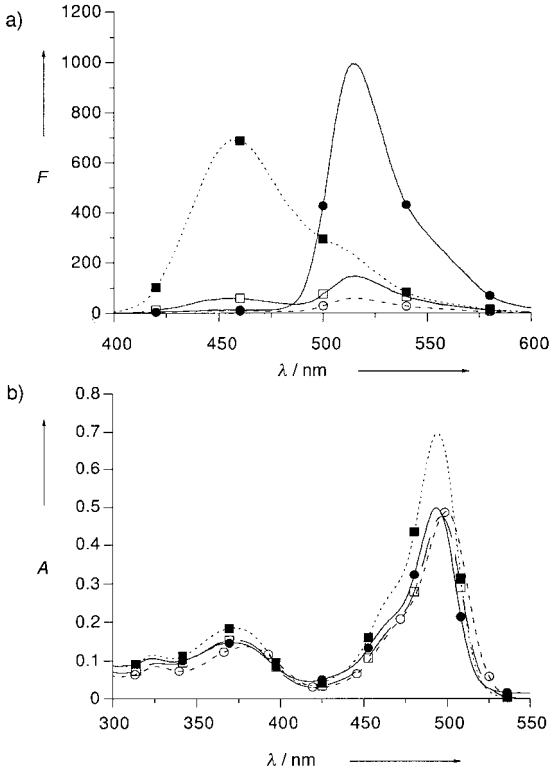

Figure 1. a) Emission (λ_{ex} = 370 nm) and b) absorption spectra of CPFs (CPF1 ○, CPF2 ●, CPF3 □, control ■; all 1 μM). These spectra were measured in 100 mM sodium phosphate buffer (pH 7.4). *F* = fluorescence intensity (in arbitrary units), *A* = absorbance.

The control mixture showed a strong emission of the coumarin acceptor, which was not quenched intermolecularly. The quantum yields of CPFs, when the fluorescein acceptor was directly excited (λ_{ex} = 492 nm, conditions as per Figure 1), were 0.10 for CPF1, 0.83 for CPF2, and 0.27 for CPF3. These results correlate well with the intensity of the emission of the fluorescein acceptor in CPFs and indicate that the emission intensity of the acceptor depends largely on the extent of self-quenching instead of the efficiency of RET.

CPF2 showed a large shift in its emission spectrum after the bond between the fluorophores was cleaved by treatment with base (Figure 2). This large shift of the emission spectrum indicates that the probe is suitable for ratiometric measurements.

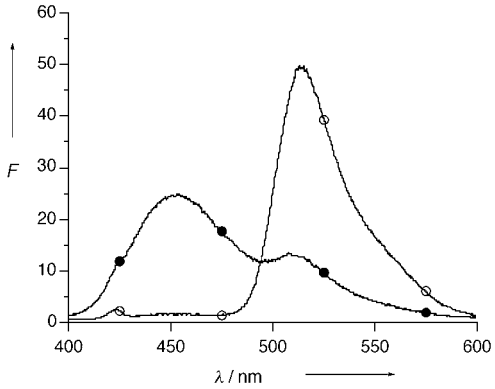

Figure 2. Emission spectra of CPF2 before and after cleavage in base (before cleavage ○, after cleavage ●). *F* = fluorescence intensity (in arbitrary units). CPF2 was cleaved in 0.1 N aq. NaOH at 50 °C. After cleavage, the solution was neutralized with 0.1 N aq. HCl and diluted with 100 mM sodium phosphate buffer (pH 7.4). The concentration of CPF2 was 0.1 μM.

The absorption spectra of CPFs in aqueous buffer are shown in Figure 1 b. We observed a large red shift in the absorption spectra of CPF1 (λ_{max} = 380 nm, 500 nm), a small shift for CPF3 (λ_{max} = 373 nm, 497 nm), and no shift for CPF2 (λ_{max} = 372 nm, 494 nm), compared to the control (λ_{max} = 373 nm, 494 nm). This shift in the absorption spectra was supposed to be due to the close contact of two fluorophores. CPF1, which had the weakest emission, had the largest shift and CPF3, which had a weak emission, had a small shift. CPF2, which had a strong emission of the acceptor, showed no shift because the two fluorophores cannot interact with each other.

When the emission spectra of the CPFs in methanol (10% sodium phosphate buffer) excited at 370 nm were detected, the emission of the fluorescein acceptor could be observed in all CPFs. The hydrophobic interaction between the fluorophores would be weaker in methanol than in aqueous solution and the fluorophores may not come into close contact in organic solvents. When the absorption spectra of CPFs in methanol were detected, the red shift of the absorption spectra observed in aqueous solution was not observed, indicating that the two fluorophores did not come into close contact. This result indicates that, in methanol, the hydrophobic interaction between the fluorophores is weakened and the emission of the acceptor caused by RET can be observed.

In conclusion, we have shown that in designing a RET probe for ratiometric measurements, it is possible to observe the emission of the acceptor if the structure of the probe is such as to prevent close contact of the two fluorophores.

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The Catalytic Alkylation of Aromatic Imines by Wilkinson's Complex: The Domino Reaction of Hydroacylation and *ortho*-Alkylation**

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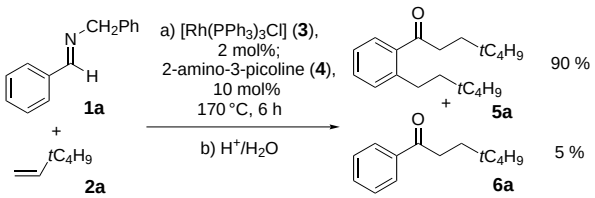
Transition metal catalyzed C–H bond activation and the subsequent coupling of the organic fragment to an olefin is a promising area in which to find a convenient method for the construction of a carbon skeleton.[1] We have studied C–H bond activation through the hydroacylation of olefins using aldehydes, alcohols, and aldimines.[2, 3] Unexpectedly during our experiments on transimination-assisted hydroacylation with aldimines,[3] *ortho*-alkylation was observed. Alkylation of aromatic ketones at the *ortho* position in a ruthenium(II)-catalyzed reaction has been reported by Murai and coworkers, this is an outstanding example of sp^2-CH/olefin coupling and a decisive breakthrough in efficiency and selectivity.[4] However, while the reaction shows a high efficiency for vinyl silane or vinyl siloxane, it exhibits limitations for other olefins, for example low reactivity for 1-alkenes bearing allylic protons, probably because of facile double bond isomerization, and no reactivity for internal olefins and α,ω-dienes.[5] Herein, we report an efficient *ortho*-alkylation of aromatic imines with various olefins by using Wilkinson's complex ($[Rh(PPh_3)_3Cl]$ (**3**)) and hydroacylation. This *ortho*-alkylation is chelation-assisted and shows generality as well as regioselectivity, and high efficiency.

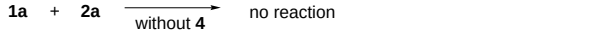
Treatment of the aldimine **1a** [Eq. (1)] with *tert*-butylethylene (**2a**) at 170 °C for 6 h with **3** (2 mol% based upon **1a**)



(1)

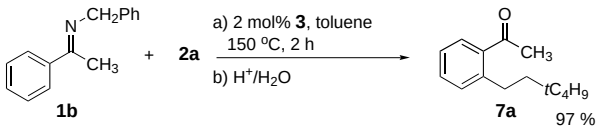
and 2-amino-3-picoline (**4**; 10 mol% based upon **1a**) as a cocatalytic system gave, after hydrolysis, compound **5a** in 90% yield along with a small amount of **6a** (5%). Compound **6a** is a hydroacylated product of **1a** and is formed by a transimination reaction, while **5a** is both a hydroacylated and an *ortho*-alkylated product. Of the various aldimines employed **1a**, prepared from benzylamine and benzaldehyde, showed the best reactivity for this simultaneous hydroacylation and *ortho*-alkylation.[3]

Compound **1a** did not react with **2a** without the cocatalyst **4** [Eq. (2)],[2] whereas, the ketimine **1b** (which is the benzylimine of acetophenone) was *ortho*-alkylated by **2a** in the



(2)

presence of **3** alone, to give **7a** in 97% yield [Eq. (3)]. These results show that the rhodium(I)-catalyzed *ortho*-alkylation takes place in ketimines, not in aldimines and that there is no *ortho*-alkylation without hydroacylation.



(3)

Ketimine **1b** was very reactive in the *ortho*-alkylation reaction with **3**; thus, various olefins were tested in reactions with this ketimine **1b** and **3** as catalyst (Table 1). In contrast to

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