ORGANIC LETTERS

2012
Vol. 14, No. 17
4406–4409

Design and Concise Synthesis of a Novel Type of Green Fluorescent Protein Chromophore Analogue

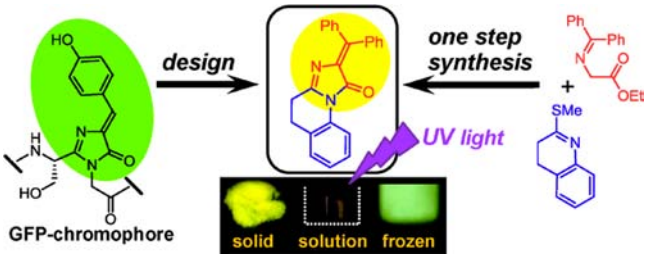
Masahiro Ikejiri,[†] Moe Tsuchino,[†] Yoshiko Chihara,[†] Takao Yamaguchi,[‡] Takeshi Imanishi,[‡] Satoshi Obika,[‡] and Kazuyuki Miyashita*,[†]

Faculty of Pharmacy, Osaka Ohtani University, Nishikiori-Kita 3-11-1, Tondabayashi, Osaka 584-8540, Japan, and Graduate School of Pharmaceutical Sciences, Osaka University, Yamadaoka 1-6, Suita, Osaka 565-0871, Japan

miyasik@osaka-ohtani.ac.jp

Received July 10, 2012

ABSTRACT



A small molecular model compound for the green fluorescent protein chromophore was readily synthesized by a novel condensation reaction of (thio)imidate with imino-ester via an aziridine intermediate. This compound showed fluorescence in the solid and frozen solution states but not in the solution state. Its fluorescent property was successfully applied in the detection of dsDNA.

A number of functional fluorescent molecules have been developed as useful imaging tools thus far, which, for instance, can help visualize various biological events through the highly sensitive detection of biologically important molecules.[1] The development of a green fluorescent protein (GFP) is one of the most remarkable milestones in this research field.[2] Despite the enormous contribution of the GFP to biological research, only a limited number of small molecular model compounds have been reported for a GFP chromophore.[3−8] This is probably because the GFP chromophore does not exhibit fluorescence when it is outside the barrel structure of the GFP. It is well-known that the conformation of the GFP chromophore is strictly restricted to a *Z*-form in the barrel structure (Figure 1), which enables the GFP to exhibit fluorescence.[9] However, without the barrel structure, fluorescent quenching of the GFP chromophore is induced by molecular motion such as the double bond isomerization of the 4-hydroxyphenylmethylene

[†] Osaka Ohtani University.
[‡] Osaka University.

(1) For a recent review, see: Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. *Chem. Rev.* **2010**, *110*, 2620–2640.

(2) GFP review:Zimmer, M. *Chem. Rev.* **2002**, *102*, 759–781.

(3) (a) He, X.; Bell, A. F.; Tonge, P. J. *Org. Lett.* **2002**, *4*, 1523–1526. (b) Petersen, M. Å.; Riber, P.; Andersen, L. H.; Nielsen, M. B. *Synthesis* **2007**, 3635–3638. (c) Lincke, K.; Sølling, T.; Andersen, L. H.; Klærke, B.; Rahbek, D. B.; Rajput, J.; Oehlenschlæger, C. B.; Petersen, M. Å.; Nielsen, M. B. *Chem. Commun.* **2010**, *46*, 734–736. (d) Kartritzky, A. R.; Yoshioka-Tarver, M.; El-Gendy, B. M.; Hall, C. *Tetrahedron Lett.* **2011**, *52*, 2224–2227.

(4) Tolbert's group reported a series of GFP chromophore analogues: (a) Tolbert, L. M.; Baldridge, A.; Kowalik, J.; Solntsev, K. M. *Acc. Chem. Res.* **2012**, *45*, 171–181and references therein. (b) Baranov, M. A.; Lukyanov, K. A.; Borissova, A. O.; Shamir, J.; Kosenkov, D.; Slipchenko, L. V.; Tolbert, L. M.; Yampolsky, I. V.; Solntsev, K. M. *J. Am. Chem. Soc.* **2012**, *134*, 6025–6032.

(5) Notable phenomena have been reported, in which the encapsulation of a modified GFP chromophore induced fluorescence: (a) Baldridge, A.; Samanta, S. R.; Jayaraj, N.; Ramamurthy, V.; Tolbert, L. M. *J. Am. Chem. Soc.* **2010**, *132*, 1498–1499. (b) Cacciarini, M.; Nielsen, M. B.; de Castro, E. M.; Marinescu, L.; Bols, M. *Tetrahedron Lett* **2012**, *53*, 973–976.

(6) Wu, L.; Burgess, K. *J. Am. Chem. Soc.* **2008**, *130*, 4089–4096.

(7) Kojima, S.; Ohkawa, H.; Hirano, T.; Maki, S.; Niwa, H.; Ohashi, M.; Inouye, S.; Tsuji, F. I. *Tetrahedron Lett.* **1998**, *39*, 5239–5242.

(8) Recently, Jaffrey and co-workers reported fluorescent RNA aptamers that bind modified GFP chromophores:Paige, J. S.; Wu, K. Y.; Jaffrey, S. R. *Science* **2011**, *333*, 642–646.

(9) Niwa, H.; Inouye, S.; Hirano, T.; Matsuno, T.; Kojima, S.; Kubota, M.; Ohashi, M.; Tsuji, F. I. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 13617–13622.

10.1021/ol301901e
Published on Web 08/16/2012

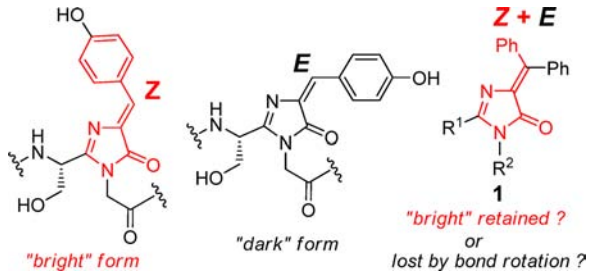


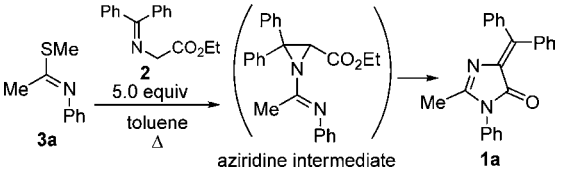
Figure 1. Structures of the GFP chromophore and our model compound **1**.

moiety,[9,10] and the resultant *E*-isomer exhibits extremely weak fluorescence.[11]

We realized that this characteristic feature of the GFP chromophore could lead to the development of a new fluorescence switch with an on/off switching mechanism.[5] On this basis, we designed a diphenylmethylene derivative **1** as a novel type of GFP chromophore model compound. This compound can be regarded as a combination of the *Z*- and *E*-forms of the benzylidene moiety, and accordingly, it can be expected to not exhibit the fluorescent quenching caused by the *E*-form isomer. Since certain types of bridged diarylmethylene groups are known to function as a shaft of light-driven molecular motors that can be rotated using UV irradiation,[12] the diphenylmethylene moiety of **1** is also expected to rotate upon exposure to UV irradiation to consume the excitation energy. We suppose that this rotation could cause fluorescence to switch off, and if it is controlled, it could turn fluorescence on. To the best of our knowledge, there has been no report on GFP chromophore model compounds that have such a diarylmethylene moiety.

For the synthesis of compound **1**, we initially attempted to apply previously reported methods that were mainly used for the syntheses of monoarylmethylene derivatives,[6,13] considering that no effective methods are known to have been reported for synthesizing diarylmethylene derivatives. However, we could not obtain **1a**. Thus, we endeavored to develop a novel synthetic method for obtaining diarylmethylene derivatives and eventually identified a suitable concise condensation reaction with imino-ester **2** and thioimidate **3a**, with the understanding that a highly reactive aziridine intermediate could transform into the desired (diphenylmethylene)imidazolinone **1a**, as shown by the reaction given in Table 1. The product, **1a**, was obtained in 55–60% yield, as expected, by mixing **2** and **3a** in hot toluene, although a long reaction time was required (Table 1, runs 1 and 2). However, contrary to expectations, the product was not obtained under refluxing toluene (run 3). As this result appeared to suggest an important role of the volatile methanethiol and/or ethanol produced in the reaction pathway, we observed the reaction in the presence of a protic additive (runs 4–7). It was found that protic additives shortened the reaction time and that acetic acid in refluxing toluene produced the best result (run 6). The use of a stronger acid (methanesulfonic acid) was found ineffective (run 7).

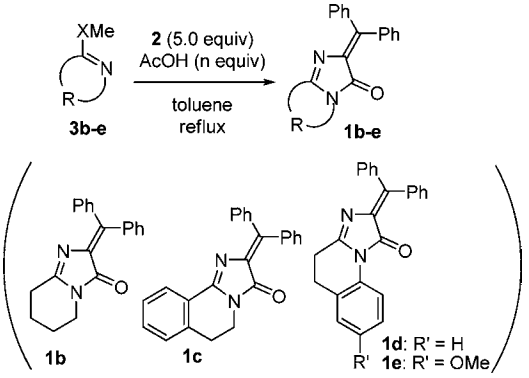
Table 1. Reaction of Thioimidate **3a** with Imino-Ester **2**



run	temp (°C)	additive (equiv)	time (days)	yield (%)
1	70	none	7.0	55
2	80	none	4.0	60
3	reflux	none	7.0	trace[a]
4	80	BuSH (1.0)	3.0	58
5	80	EtOH (5.0)	2.5	43
6	reflux	AcOH (2.0)	1.0	78
7	rt to reflux	MsOH (2.0)	1.0	0[b]

[a] Almost no reaction. [b] Complex mixture.

Table 2. Synthesis of Cyclic Imidazolinones **1b–e**



run	product **1**	X	*n*	time (days)	yield (%)
1	**b**	S	2.0	1.0	43
2	**c**	S	2.0	1.5	68
3	**d**	S	2.0	1.5	77
4	**e**	S	2.0	1.5	42
5	**e**	S	4.0	1.0	75
6	**d**	O	2.0	1.0	85

(10) Voliani, V.; Bizzarri, R.; Nifosì, R.; Abbruzzetti, S.; Grandi, E.; Viappiani, C.; Beltram, F. *J. Phys. Chem. B* **2008**, *112*, 10714–10722.
(11) Dong, J.; Abulwerdi, F.; Baldridge, A.; Kowalik, J.; Solntsev, K. M.; Tolbert, L. M. *J. Am. Chem. Soc.* **2008**, *130*, 14096–14098and references therein.
(12) Vicario, J.; Walko, M.; Meetsma, A.; Feringa, B. L. *J. Am. Chem. Soc.* **2006**, *128*, 5127–5135.
(13) Baldridge, A.; Kowalik, J.; Tolbert, L. M. *Synthesis* **2010**, 2424–2436.

Under the conditions of run 6, listed in Table 1, cyclic thioimidates **3b**–**e** (X = S) also reacted with **2** to give bicyclic imidazolinones **1b**–**e** in moderate to good yields (Table 2, runs 1–5). The reaction with imidate **3d** (X = O) was found to proceed similarly (run 6). The structure of the reaction product **1d** was confirmed by X-ray crystallographic analysis, as shown in Figure 2.

Figure 2. X-ray-based ORTEP drawings of **1d**. Ellipsoids are set at 50% probability.

Regarding the reaction mechanism (Figure 3), the attack of the imine nitrogen, and not the α-carbanion, of **2** on the thioimidate **3a** appears to be the first step, which forms the iminium intermediate **4**. The intramolecular aziridine formation of **5** and concomitant aziridine ring cleavage of **6** is expected to give product **1a**, through which the diphenylmethylene group can migrate from nitrogen to α-carbon via the aziridine intermediate **6**. Although we could not isolate the aziridine intermediate **6**, Ayyangar and co-workers reported an aziridine synthesis from the imino-ester **2**.[14]

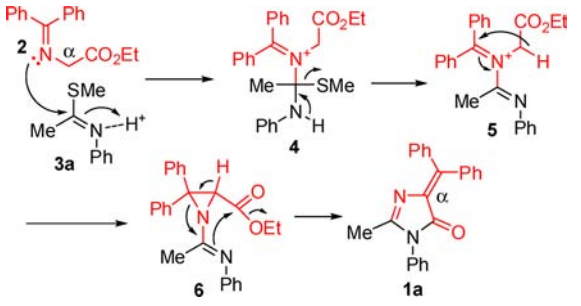


Figure 3. Possible reaction mechanism for **1a**.

We next investigated the fluorescent properties of the compounds **1** and found that **1b**–**e** exhibited fluorescence under specific conditions (Figure 4). For **1b**–**e**, although their solution state in various solvents did not produce fluorescence, their crystalline state induced fluorescence, as shown in Figure 4A.[15] In addition, although a solution of **1b**–**e** in DMSO was almost completely nonfluorescent, it

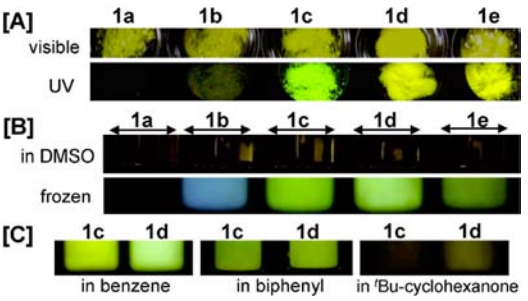


Figure 4. Photographs of fluorescence for **1a**–**e**. [A] Pictures of solid **1a**–**e** under visible light (top panel) and 365 nm UV light (bottom panel). [B] Pictures of 1.0×10^{-3} M DMSO solutions of **1a**–**e** under 365 nm UV light (top) and those of the frozen state (bottom). [C] Pictures of frozen solutions of **1c** and **d** in benzene (1.0×10^{-3} M), biphenyl (1.0×10^{-3} mol/kg), and 4-*t*-butylcyclohexanone (1.0×10^{-3} mol/kg) under 365 nm UV light. The DMSO and benzene solutions were prepared at rt and then frozen at −20 °C. The biphenyl and 4-*t*-butylcyclohexanone solutions were prepared at 90 °C and then solidified at rt.

became fluorescent when frozen (Figure 4B). Benzylidene-type model compounds are also known to exhibit fluorescence in a frozen solution state at substantially low temperatures (ethanol at 77 K);[7,9] however, **1b**–**e** showed fluorescence even at higher temperatures from around −20 °C (e.g., in DMSO and benzene) to room temperature (e.g., in biphenyl and 4-*tert*-butylcyclohexanone) (Figure 4C). From the present results, it seems that a fused six-membered ring with the imidazolinone ring is essential, and a fused benzene ring in conjugation with the imidazolinone ring expectedly increases the fluorescent intensity. A table summarizing the data of the fluorescent spectra for **1a**–**e** is given in the Supporting Information (Table S1).

The fluorescent profiles of **1a**–**e** suggest, as expected, that molecular motion, including the isomerization of the double bond, rather than the aggregation of the molecules, is related to the fluorescent switching phenomenon. The molecular motion involving double bond isomerization is not restricted in the solution state, which leads to effective and nonfluorescent energy consumption. In contrast, in a conformationally rigid system, such as in a crystalline state and a frozen solution state, the rotation of the double bond bearing large substituents such as phenyl groups is sterically prohibited, resulting in fluorescence.

An X-ray crystallographic analysis of **1d** (Figure 2) revealed that the relationship between the phenyl ring at the *Z*-position (*Z*-Ph) and the imidazolinone ring (IMN) is flatter than that between the phenyl ring at the *E*-position (*E*-Ph) and IMN. The dihedral angle between *Z*-Ph and IMN is 31.4°, whereas that between *E*-Ph and IMN is 71.4°. This steric relation between Z-Ph and IMN of **1d** is not as flat as that of the GFP chromophore in the barrel structure (the dihedral angle between the 4-hydroxyphenyl ring and the IMN of the GFP chromophore is 3.7°).[16] Even

(14) Rao, M. N.; Holkar, A. G.; Ayyangar, N. R. *Tetrahedron Lett.* **1989**, *30*, 4717–4720.

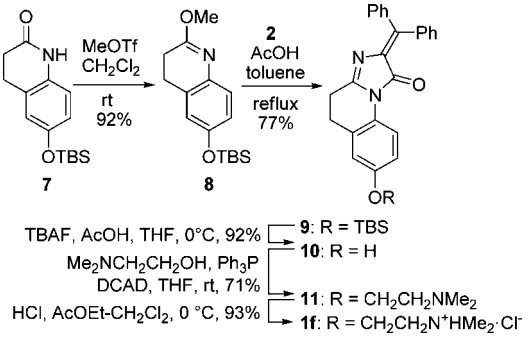
(15) Several GFP chromophore analogues are known to show fluorescence in the solid state: (a) Dong, J.; Solntsev, K. M.; Tolbert, L. M. *J. Am. Chem. Soc.* **2009**, *131*, 662–670. (b) Chuang, W. T.; Hsieh, C. C.; Lai, C. H.; Lai, C. H.; Shih, C. W.; Chen, K. Y.; Hung, W. Y.; Hsu, Y. H.; Chou, P. T. *J. Org. Chem.* **2011**, *76*, 8189–8202.

(16) Quillin, M. L.; Anstrom, D. M.; Shu, X.; O'Leary, S.; Kallio, K.; Chudakov, D. M.; Remington, S. J. *Biochemistry* **2005**, *44*, 5774–5787.

then, this relation between *Z*-Ph and IMN of **1d** apparently reveals the existence of some conjugation between them.

Encouraged by the results, we next investigated the application of our compound **1** to the detection of double-stranded DNA (dsDNA), based on the following considerations: (1) the relatively flat structure of *Z*-Ph and IMN could intercalate into dsDNA or bind at a minor groove of dsDNA; and (2) such a phenomenon would restrict the rotation of the diphenylmethylene group, acting as a switch that would turn ON fluorescence as a consequence. On the basis of this concept, we designed and synthesized **1f**, which had a hydrophilic and interactive unit with DNA, as shown in Scheme 1. To prepare an imidate part, lactam **7**[17] was converted into **8** by methylation. The imidazolinone structure was constructed with imino-ester **2** and imidate **8** in the same manner, and the hydroxyl group of the product **9** was connected with the hydrophilic unit by a Mitsunobu reaction after a conversion into **10**. Finally, the dimethylamino group of **11** was transformed into an ammonium salt to afford **1f**.

Scheme 1



The fluorescent profile of **1f** was examined in the absence and presence of fish dsDNA, and the results are shown in Figure 5. Similar to the other compounds, **1f** did not exhibit fluorescence in a solution state. However, in the presence of dsDNA, **1f** exhibited fluorescence even in a solution state. The intensity of the fluorescence gradually increased and reached near maximum after 24 h (λ_{em}: 551 nm). A noteworthy point is that, because **1f** without DNA is almost completely nonfluorescent, the S/N value of **1f** in the presence and absence of DNA (S/N $\geq$ 500) is significantly higher than, for example, that of ethidium bromide (S/N = ~10),[18] which is generally employed in dsDNA detection.

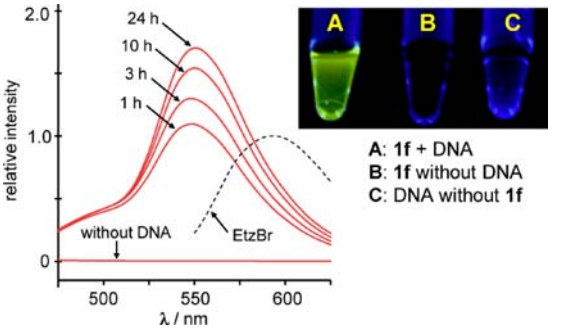


Figure 5. Fluorescent spectra and a picture of **1f** in the presence of dsDNA (0.01% of **1f** (2.1×10^{-4} M) and 0.025% of DNA in water, λ_{ex} = 327 nm). The same concentration of ethidium bromide (EtzBr) was used as a standard for the relative intensity (2.1×10^{-4} M, λ_{ex} = 330 nm, λ_{em} = 594 nm, 24 h).

In summary, we designed and synthesized fluorescent switchable GFP chromophore analogues **1** by employing a novel condensation reaction. Interestingly, this reaction accompanies an unprecedented migration of a diphenylmethylene group from nitrogen to α-carbon via an aziridine intermediate. Several compounds in **1** showed remarkable fluorescence in the crystalline and frozen solution states, whereas they did not do so in the solution state. This characteristic fluorescent behavior was successfully applied in the detection of dsDNA by employing **1f**. In the fluorescence of **1b**–**e**, dynamic double bond isomerization appears to function as an effective switch for fluorescence, which makes these compounds potentially applicable to the detection of not only dsDNA but also a variety of chemical and biological events.[8]

Supporting Information Available. Detailed experimental procedures, spectral data, and a CIF of **1d**. This material is available free of charge via the Internet at http://pubs.acs.org.

(17) Occhiato, E. G.; Ferrali, A.; Menchi, G.; Guarna, A.; Danza, G.; Comerci, A.; Mancina, R.; Serio, M.; Garotta, G.; Cavalli, A.; De Vivo, M.; Recanatini, M. *J. Med. Chem.* **2004**, *47*, 3546–3560.

(18) Olmsted, J., III; Kearns, D. R. *Biochemistry* **1977**, *16*, 3647–3654.

The authors declare no competing financial interest.