Bioorganic & Medicinal Chemistry Letters 18 (2008) 4380–4384

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Design and synthesis of regioisomerically pure unsymmetrical xanthene derivatives for staining live cells and their photochemical properties

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ARTICLE INFO

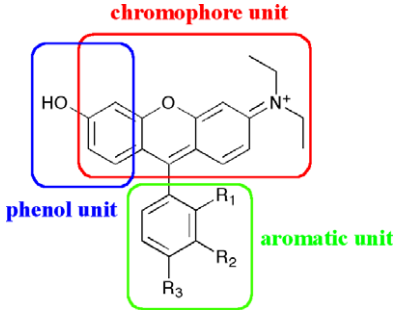
Article history:
Received 12 May 2008
Revised 14 June 2008
Accepted 18 June 2008
Available online 21 June 2008

Keywords:
Unsymmetrical xanthene derivatives
Regioisomerically pure
Photochemical properties
Chemical biology

ABSTRACT

We have demonstrated the synthesis of regioisomerically pure unsymmetrical xanthene derivatives consisting of three units which can be independently modified to control their physical properties. The photochemical properties of the synthetic unsymmetrical xanthene derivatives were investigated in solution by UV–vis absorption and fluorescence measurements, and their cell imaging properties were examined by confocal laser-scanning microscopy.

Xanthene derivatives, such as fluoresceins and rhodamines, are widely employed as molecular probes in chemical biology.[1] We have been interested in the synthesis of chromophores possessing high potential use in sensing applications. Since the first preparation of fluorescein in 1871 by von Baeyer,[2] many synthetic methods have been reported.[3] However, free modification of the xanthene skeleton as a chromophore having color diversity has been little attempted. Unsymmetrical xanthenes, such as commercially available rhodol derivatives, are typically prepared as mixtures of 5- and 6-isomers with difficulty in their separation by well-established condensation route.[4] The conjugation of these isomers to other molecules leads to differences in physical properties, including fluorescence polarity, internal fluorescence quenching, and labeling specificity.[5] To solve these problems, we assumed that xanthene derivatives can be divided into three units—chromophore, phenol, and aromatic—each of which can be independently modified. The combination of these three units would allow us to optimize the physical properties of xanthene derivatives for the desired application. We propose herein a synthetic method for a series of regioisomeric pure unsymmetrical xanthene derivatives, which involves combining three different moieties, just like pieces of a jigsaw puzzle.



Benzophenones are usually used as condensation partners of phenols for the synthesis of xanthenes.[6] This method is based on the formation of a benzophenone via the Friedel–Crafts acylation reaction of benzoyl chloride with methoxybenzene derivatives, followed by condensation with phenol derivatives to afford the desired unsymmetrical xanthenes. The photochemical properties of synthesized unsymmetrical xanthene derivatives in ethanol solution were investigated by UV–vis absorption and fluorescence measurements. We also prepared isomers of rhodol derivatives, which are hybrid compounds of rhodamine and fluorescein, and evaluated their photochemical properties by measuring absolute fluorescence quantum yield (Φ_f) and molar extinction coefficient (ε) in ethanol solution. Cell imaging properties of the rhodol derivatives were also tested using human lung carcinoma A549 cell line.

The Friedel–Crafts acylation of aromatic compounds is an important transformation reaction in organic synthesis.[7] However,

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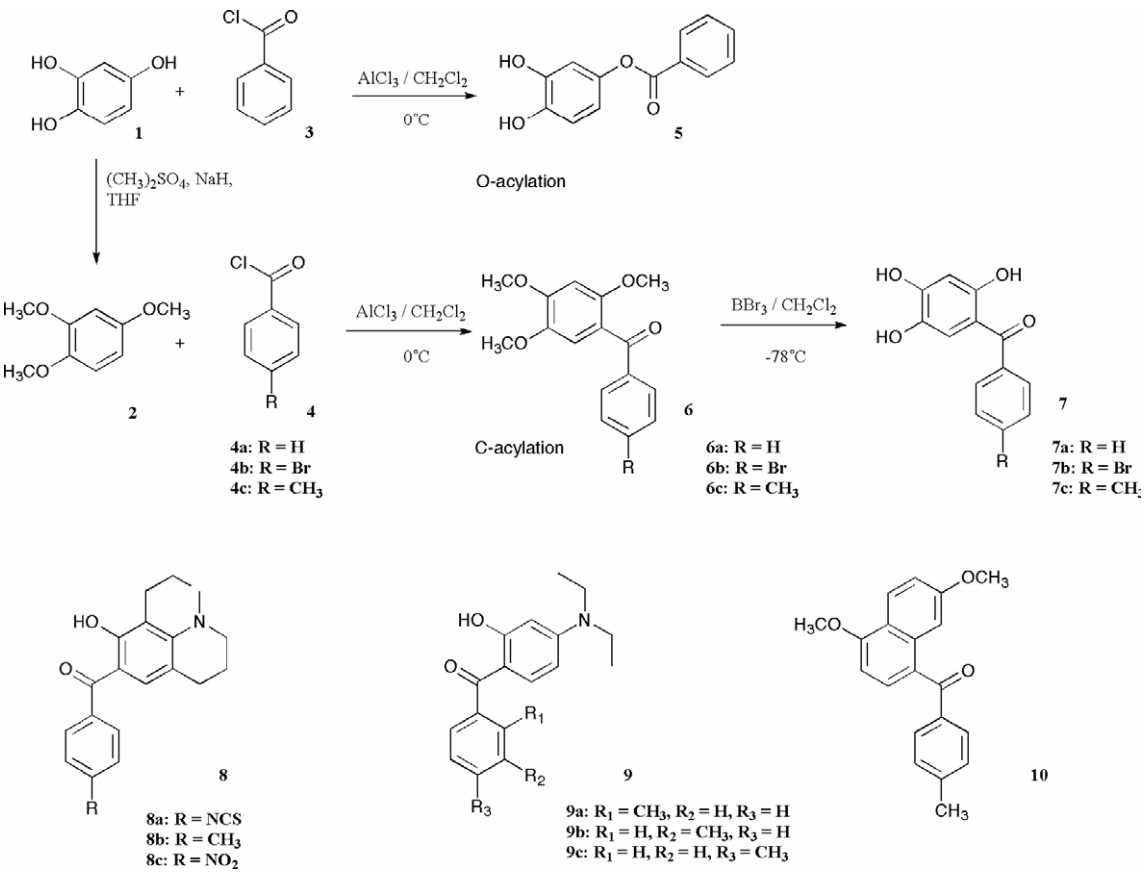
doi:10.1016/j.bmcl.2008.06.065

there are few examples of the direct acylation of phenolic compounds,[8] in particular, polyphenols, owing to severe side reactions, such as the Shotten–Baumann reaction.[9] In fact, 1,2,4-trihydroxybenzene **1** reacted with benzoyl chloride **3** with coexisting $AlCl_3$ to form 4′-*O*-monoacyl derivative **5**[10] in very high proportion without *C*-acylation. Compound **5** was similarly obtained form the reaction of acetylation compound of **1** with **3**. Methylation of **1** selectively gave 1,2,4-trimethoxybenzophenone **6a**, whose structure was supported by HSQC and HMBC analyses. Trimethoxybenzophenone derivatives (**6b** and **6c**) were similarly prepared. These benzophenone derivatives were deprotected by BBr_3 at −78 °C and trihydroxybenzophenone derivatives (**7a**–**c**) were obtained after recrystallization. Likewise, amine-conjugated benzophenones (**8** and **9**) were similarly synthesized by acylation of 8-methoxyjulolidine or 1-(diethylamino)-3-methoxybenzene with benzoyl chloride derivatives. Single crystals of **8b** and **9c** suitable for X-ray diffraction analysis were grown by slow evaporation of ethanol and H_2O solution.[11] X-ray analysis supported the finding that the acyl group was selectively introduced adjacent to the hydroxyl group. β-Diketones as benzophenones can exist in keto–enol tautomerization. Structural data indicated that both **8b** and **9c** existed in the enol form in the solid state. The acylation of 1,6-methoxynaphthalene with *p*-methylbenzoyl chloride gave 4-methylbenzoyl-1,6-methoxynaphthalene **10**[12] that was not adequate for the synthesis of xanthene derivatives. The introduction of 1,6-naphthalenediol into xanthenes is key for synthesis of long-wave fluorescent compound. Therefore, a selective synthesis involving 2-, 5-, or 7-acylation of 1,6-naphthalenediol has attracted considerable interest. The chemistry is shown in Scheme 1. These benzophenone derivatives would serve as precursors for the synthesis of regioisomerically pure unsymmetrical xanthene derivatives.

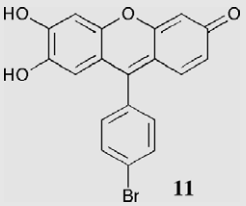
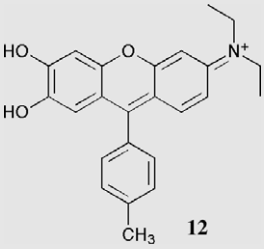
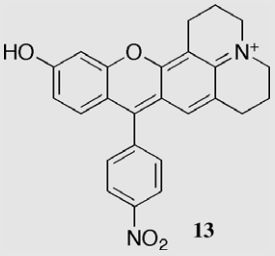
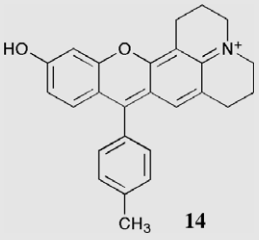
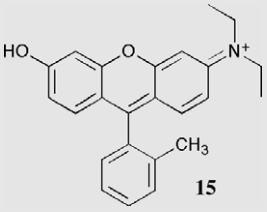
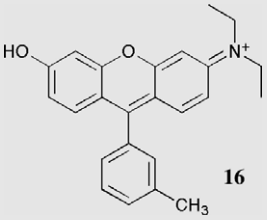
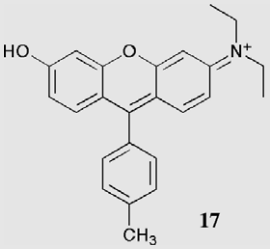
The condensation reactions of synthesized benzophenone derivatives with phenol analogues were subsequently carried out (Table 1). Although many condensing agents have been reported, CH_3SO_3H is the most popular since it acts as both condensing and oxidizing agents at low temperature.[3c] Therefore, the reactions were conducted in neat CH_3SO_3H. To synthesize unsymmetrical xanthene derivatives having pyrocathecol sites, semi-fluorescein compound **11** was prepared by condensation of **7b** with resorcinol. However, the condensation reactions of **7b**–**c** with 8-hydroxyjulolidine and/or 1-(diethylamino)-3-hydroxybenzene gave no semi-rhodamine compounds due to the formation of inseparable side products. We found that **8** and **9** are adequate for the synthesis of a series of semi-rhodamine derivatives. Condensation at 80–150°C proceeded smoothly in hot CH_3SO_3H to give the desired semi-rhodamine derivatives. All compounds except those having pyrocathecol sites **12** were simply purified by silica column chromatography using mixed CH_3OH/CH_2Cl_2 solvent. However, we were always bothered by the formation of side products. Lukhtanov and co-workers, for the first time, discussed the mechanism of the reaction of benzophenone with resorcinol by retro-Friedel–Crafts fragmentation, and developed an innovative two-step synthesis of unsymmetrical 2′-carboxyethyl-l-substituted fluoresceins to solve the problem.[6a] Using their method, it is necessary for us to characterize the side products and develop mild synthetic conditions to increase the yield.

The UV–vis absorption and fluorescence emission spectra of **11**–**17** in ethanol solution were measured (see details in ESI). Φ_f



Scheme 1. Synthesis of benzophenone derivatives.

Table 1
Synthesis of unsymmetrical xanthene derivatives[a]

Reaction No.	Phenol	Benzophenone	Product	Yield (%)
1	Resorcinol	**7b**	 **11**	10
2	1,2,4-Trihydroxybenzene	**9c**	 **12**	12
3	Resorcinol	**8c**	 **13**	11
4	Resorcinol	**8b**	 **14**	52
5	Resorcinol	**9a**	 **15**	9
6	Resorcinol	**9b**	 **16**	75
7	Resorcinol	**9c**	 **17**	43

[a] Reagents and conditions: CH_3SO_3H, 80–150 °C, 2–24 h. The detailed experimental procedure is described in ESI.

Table 2
Photochemical properties of regioisomers of rhodol derivatives

Compound	Yield	Absorption maximum (nm)[a]	ε ($\times 10^4$)[a]	Emission maximum (nm)[a]	Φ_f[a,b,c]
15	9	523	8.5	555	0.706/0.708
16	75	523	8.4	557	0.592/0.602
17	43	523	9.4	556	0.599/0.613

[a] Measured in ethanol.
[b] Excited at 480 nm.
[c] Left values indicate Φ_f measured in ethanol solution and right values indicate Φ_f after excluding O_2 effect by bubbling N_2 in ethanol solution.

and ε values of regioisomers of rhodol derivatives, **15**–**17**, were measured in ethanol solution, respectively (Table 2). The purity of compound **15**–**17** was confirmed by ^{13}C NMR and analytic reversed-phase HPLC. Φ_f had no effects on singlet oxygen in ethanol solution. Φ_f values of **15** were higher than those of **16** and **17**, as shown in Table 2. Urano et al. reported that the high quantum yield of fluorescein was essential to keep the xanthene moiety and the benzene moiety orthogonal to each other with an *ortho* substituent.[12] In this study, the fluorescence properties of *ortho*-substituted rhodol were superior to those of the other isomers. One possible explanation was that the increase of radiationless transition probability due to steric rotation of the aromatic unit around the linker led to a reduction of Φ_f of *meta*- and *para*-substituted rhodols. However, drastic changes of Φ_f were not observed compared to that of conventional fluorescein.[13] Compound **15** was prepared in low yield due to steric effect. Further examination of other rhodol derivatives is required to verify the photochemical system.

Synthetic rhodol derivatives **14** and **17** were tested for their ability to diffuse into human lung carcinoma A549 cells. The purity of **14** and **17** was determined by analytic reversed-phase HPLC. Compound **14** and **17** at 1 μM were overlaid on the cells, and after incubation at 37 °C for 30 min, intracellular localization was examined by confocal laser-scanning microscopy with irradiation at two excitation wavelengths (Fig. 1). Compound **14** and **17** were easily taken up by the cells and essentially accumulated in the cytoplasm, and dual imaging colors of fluorescein (green) and rhodamine (red) were observed.

In summary, we have developed the synthesis of regioisomerically pure unsymmetrical xanthene derivatives and investigated their photochemical properties. We also observed the easy diffusion of these compounds through live cell membrane and confirmed their localization in the cytoplasm. The advantage of this method is the ease of modification of each unit and their combination for the desired application. Further efforts are ongoing to explore multifunctional tools for chemical biology by developing the three units separately and then combining them.

Acknowledgments

We thank Ms. Mihoyo Fujitake and Dr. Katsuhiko Minoura of Osaka University of Pharmaceutical Sciences for MS and NMR measurements; Dr. Kengo Suzuki of HAMAMATSU PHOTONICS K. K. for fluorescence quantum yield determination; Dr. Seiji Komeda of Osaka University of Pharmaceutical Sciences for useful discussion; Ms. Motoko Sawatani of YAMAZEN Co. for insightful discussion; and Ms. Chiho Ikeda of Osaka University of Pharmaceutical Sciences for design of graphical abstract.

Supplementary data

Electronic Supplementary Data available: materials, synthesis, methods, spectroscopic data for novel compounds and supplementary data. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.06.065.

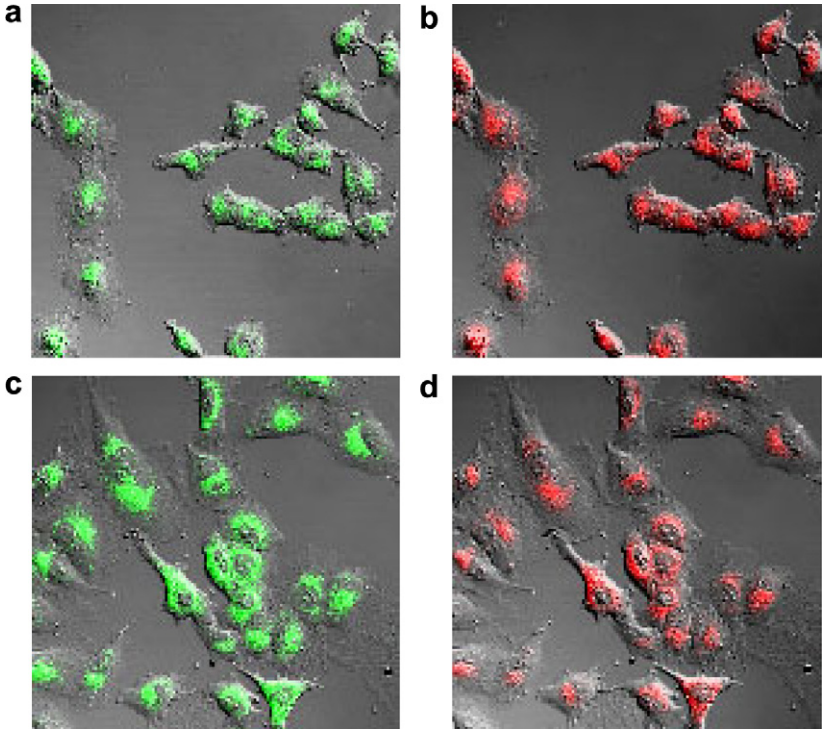


Figure 1. Top: Fluorescence images of live cells loaded with 1 μM **14**. (a) Excited at 506 nm and (b) excited at 560 nm. Bottom: Fluorescence images of live cells loaded with 1 μM **17**. (c) Excited at 488 nm and (d) excited at 543 nm.

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10. Compound **5**; ^{1}H NMR ($CDCl_3$, 500 MHz): δ 8.17 (m, 2H); 7.62 (tt, 1H, J = 7.3, 1.4 Hz); 7.49 (m, 2H); 6.87 (d, 1H, J = 8.7 Hz); 6.77 (sd, 1H, J = 2.8 Hz); 6.59 (dd, 1H, J = 8.5, 2.8 Hz). ^{13}C NMR ($CDCl_3$, 500 MHz): 165.57, 144.76, 143.76, 142.39, 133.36, 130.00. 129.64, 128.42, 115.10, 112.72, 109.29. HRMS (EI) Calcd for $C_{13}H_{10}O_4$ (M^+): 230.0579. Found: 230.0578.
11. Crystallographic data (excluding structure factors) for the structures of **8a**, **9c**, and **10** reported in this paper have been deposited with the Cambridge Crystallographic Data Center as supplementary publication Nos. CCDC-667670, CCDC-667671, and CCDC-680015, respectively. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.
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