An efficient and concise method to synthesize locked GFP chromophore analogues

Soumit Chatterjee, Peter Karuso

PII: S0040-4039(16)31324-7
DOI: http://dx.doi.org/10.1016/j.tetlet.2016.10.021
Reference: TETL 48191

To appear in: *Tetrahedron Letters*

Received Date: 24 September 2016
Revised Date: 4 October 2016
Accepted Date: 7 October 2016

Please cite this article as: Chatterjee, S., Karuso, P., An efficient and concise method to synthesize locked GFP chromophore analogues, *Tetrahedron Letters* (2016), doi: http://dx.doi.org/10.1016/j.tetlet.2016.10.021

Tetrahedron Letters
journal homepage: www.elsevier.com

An efficient and concise method to synthesize locked GFP chromophore analogues

Soumit Chatterjee and Peter Karuso*

Department of Chemistry and Biomolecular Sciences. Macquarie University, Sydney, NSW 2109, Australia

ARTICLE INFO

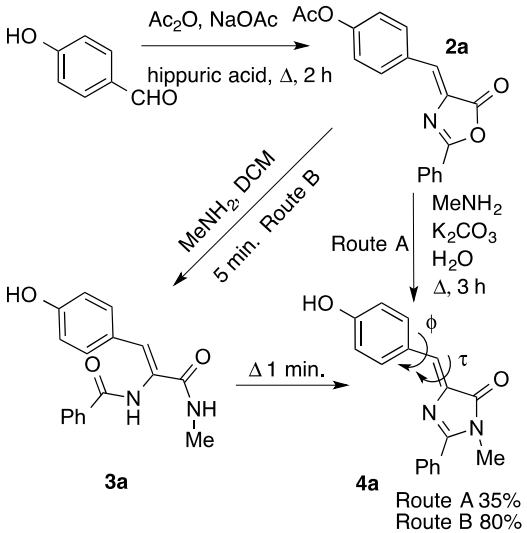
Article history:
Received
Received in revised form
Accepted
Available online

Keywords:
Green Fluorescent Protein
HBDI
fluorescence
Knovenagel condensation
oxazolinone

ABSTRACT

A series of GFP analogues, which are fluorescent in the solid state at room temperature, but weakly fluorescent in solution, have been synthesized *via* an oxazolone formation process that involves a condensation reaction in the presence of a Lewis acid following a Knoevenagel condensation. A ring opened intermediate is formed which cyclizes readily upon heating to produce the imidazolinone. This method is faster, simpler and produces higher yields than alternative methods. A few analogues represent locked GFP derivatives where the exocyclic single bond rotation has been stopped. Weak fluorescence, even after stopping single bond rotation, indicates that restriction of conformation is not effectively controlled and that the double bond rotation is solely responsible for the major non-radiative pathway.

The Green Fluorescent Protein (GFP) family is extensively used in cell biology as a genetically encoded, fluorescent marker.[1-6] In wild-type GFP, the chromophore is (*Z*)-5-(4-hydroxybenzylidene)–3,5-dihydro-4*H*-imidazol-4-one (*p*-HBDI).[7] However, the isolated chromophore is non-fluorescent ($\phi_f < 10^{-4}$) in contrast to the protein ($\phi_f = 0.8$).[8-10] This has been found to be due to non-radiative pathways (ϕ and τ rotation in the excited state) for the free fluorophore that is not available to the chromophore inside the constrained GFP β-barrel.[11-14] Although numerous structural analogues of the GFP chromophore have been prepared, only a few synthetic routes are utilized. In general, these routes start with Erlenmeyer azalactone synthesis, in which the reaction of *N*-acylglycines with arylaldehydes under the influence of sodium acetate in acetic anhydride results in the formation of 4-arylidene-5-oxazolinones (Scheme 1). Direct condensation of the oxazolinones with primary amines in the presence of a base is the default approach for synthesizing the final 4-arylidene-5-imidazolinones (Scheme 1, Route A).[9,15-17] An indirect approach proceeding *via* a cinnamide (**3a**), followed by dehydration is generally higher yielding (Scheme 1, Route B).[18-20] In our hands, route B was more time efficient with more than double the yield obtained when starting from *p*-hydroxybenzaldehyde despite the extra synthetic step. The reason for this was the quantitative conversion of **2a** to **3a,** which required no purification (other than filtration) and the second step simply requires column chromatography. However, the Erlenmeyer method (the first step in Scheme 1) fails with arylketones due to their poor reactivity compared to aldehydes. A number of variations, including catalytic lead acetate, bases and phosphorous oxychloride, were tried but to no avail.[21-23] An alternate route was explored *via* Knovenagel condensation of *N*-acylglycines.[24,25] This $TiCl_4$ mediated aldol condensation, was successfully implemented by Tepe and co-workers[26] and subsequently by Chou and co-workers to condense indanones with 2,3-dimethylimidazolin-4-one, for example going directly from compound **1f** to **4f**.[27]



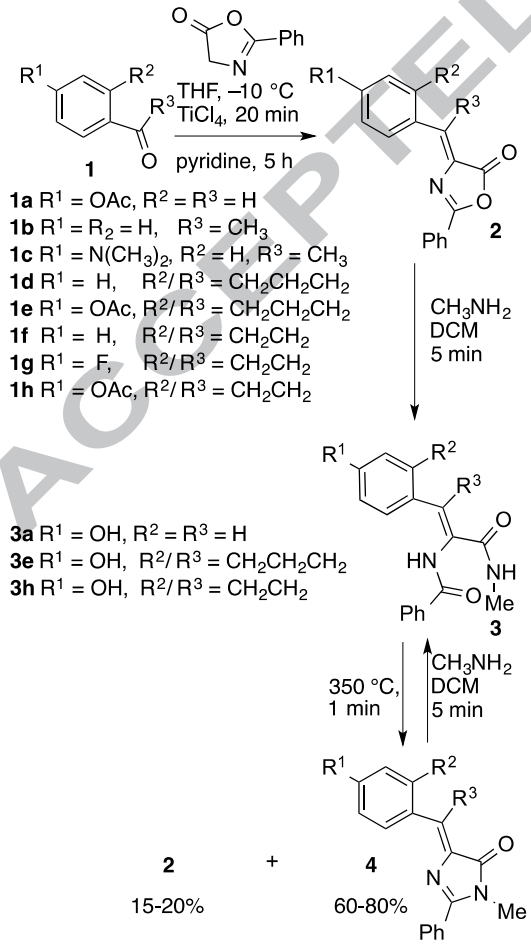
Scheme 1. Synthesis of phenyl substituted *p*-HBDI

* Corresponding author. Tel.: +61-2-9850-8290; fax: +61-2-9850-8313; e-mail: peter.karuso@mq.edu.au

However, 2,3-dimethylimidazolin-4-one is difficult to synthesize compared to 2-phenyloxazolinone. The former requires three steps starting from *N*-methylacetamide and chloroacetyl chloride[24] compared to the latter which can be synthesized on a gram scale from *N*-acylglycines (e.g. hippuric acid), in one step without purification, simply by heating in acetic anhydride for 30 minutes then pouring into ice/water.[28]

With this background, we set about to design a better synthesis of GFP chromophores that would be especially useful for generating conformationally restricted HBDI analogues *via* oxazolinone formation.[29]

A series of oxazolinones (**2**) were synthesized by the condensation of arylketones (**1a-h**) with phenyloxazolone in good yield (Scheme 2). NMR analysis showed that for a few oxazolinones (**2**), both *Z*- and *E*- isomers were formed (ESI). Keeping in mind that conversion to the final imidazolinone could lead to isomerization and that any isomers could be separated at the end, these were not separated. To our dismay, refluxing oxazolinones (**2**) in ethanol with aqueous methylamine did not form the corresponding imidazolinones (**4**)[8] but rather, a white precipitate which was shown to be the ring opened *N*-alkyl-2-acylamino-3-arylacrylamides (**3b-h**). Formation of this type of compound has previously been reported by both Chien and co-workers and Diederichsen and co-workers.[19,20] The yield of ring-opened products (**3**) was optimized by stirring the oxazolinones (**2**) in dichloromethane at room temperature and adding acetonitrile saturated with dry methylamine (2 equiv.). This minimized formation of the corresponding carboxylic acids and the reactions were generally complete within 15 minutes except for **2e** and **2h**, which required one hour due to concomitant deprotection of the phenol. The yields were generally 98-100% and 80-90% for **3e** and **3h**.



Scheme 2. Efficient synthesis of restrained *p*-HBDI analogues

Dehydrative cyclization of acrylamide (**3b**) to imidazolinone (**4b**) proved to be more difficult than anticipated and standard literature procedures failed.[19,20] Fortuitously, it was discovered that drying a TLC plate with a hot-air gun turned the colorless acrylamide spot yellow. Elution of this spot separated it into two yellow bands that corresponded to **4b** and **2b** (ESI-MS). Optimization of this reaction revealed that heating the solid acrylamides (**3**) in a furnace at 350 °C for 1 min (Scheme 2) led to formation of the desired products in good to high yield (Table S4; ESI) and recovery of **2**, which was recycled, led to a cumulative yield of over 90% for all compounds. Some amount of **2** was always formed regardless of how carefully water was excluded. NMR spectroscopy indicated that only the *Z*-isomer was formed for all imidazolinones (**4**) except **4b**, where both were formed. For example an NOE was observed between H8' and H2" in **4d**. This can only arise from the Z-isomer. The steric hindrance associated with the 5- or 6-membered ring, as well as restricted rotation of the exocyclic double bond play a key role in formation of a single isomer.

Steady state absorption and emission spectra were then recorded for each compound (Table 1). Compound **4c** was non-fluorescent but **4f** was 30 times more fluorescent than *p*-HBDI and had the highest quantum yield of all the compounds synthesized.

Table 1. Steady state absorption and emission maxima and quantum yields for imidazolinones (**4**) in different solvents

Compound	Solvent	λ_{abs}^{max} (nm)	λ_{em}^{max} (nm)	ϕ_f $\times 10^{-4}$
4a	H_2O	390	480	1.9
	tert-BuOH	398	470	4.2
4b	H_2O	347	496	2.2
	tert-BuOH	357	492	1.6
4c	H_2O	436	-	-
	tert-BuOH	436	-	-
4d	H_2O	379	497	14
	tert-BuOH	379	460	19
4e	H_2O	391	552	8.7
	tert-BuOH	403	530	13
4f	H_2O	381	481	30
	tert-BuOH	381	461	25
4g	acetonitrile	377	463	5.4
4h	H_2O	400	488	6.6
	tert-BuOH	400	489	4.4

All of the conformationally restricted analogues (**4d-h**) had increased quantum yields compared to **4a-c** (Table 1). Femtosecond fluorescent transients for **4d**, **4e**, **4f** and **4h** in water and *tert*-butanol (Fig. 1B and Fig. S8; ESI) indicated a very fast relaxation process (< 5 ps) that was relatively solvent independent. This is in agreement with the observed low quantum yield (< 1%) of all the compounds (Table 1). The faster component of < 1 ps predominated for all the compounds in both solvents (Table S6; ESI).

To understand this rapid relaxation we conducted a series of DFT calculations. Scanning the τ angle (Fig. 2) revealed that the *Z*-isomer of **4e** is more stable by ~2.5 kcal/mol than the *E*-isomer, but more importantly, that the activation barrier to rotation is ~34 kcal/mol indicating that there is not isomerism in the ground state. However, in the first excited state the potential energy surface (PES) is relatively flat with a conical intersection with the ground state at a τ angle of ~ 100° (Fig. 2). The dip in energy of the S_1 state when the imidazolone and tetrahydronaphthalene rings are perpendicular is associated with concomitant flexing of the cyclohexene (R^2/R^3 bridge; Scheme 2). This would allow a facile return to the ground state (internal conversion) without emission of a photon and could explain the low quantum yields observed (Table 1) and the very fast decay recorded in upconversion experiment (Fig. 1 and S8). This indicates that restricting the ϕ angle is not sufficient for a high quantum yield

and that the τ angle also needs to be physically restrained to generate room temperature HBDI analogues.

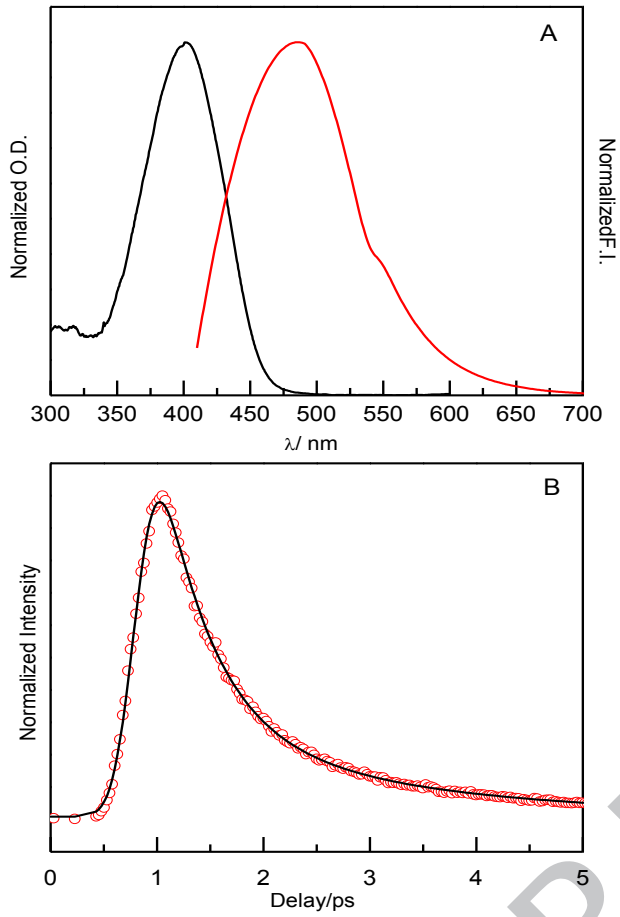


Figure 1. A) Normalized absorption (**black**) and emission spectra (**red**) and B) femtosecond fluorescence upconversion decay trace of **4h** in water. λ_{ex} = 400 nm; λ_{em} = 490 nm

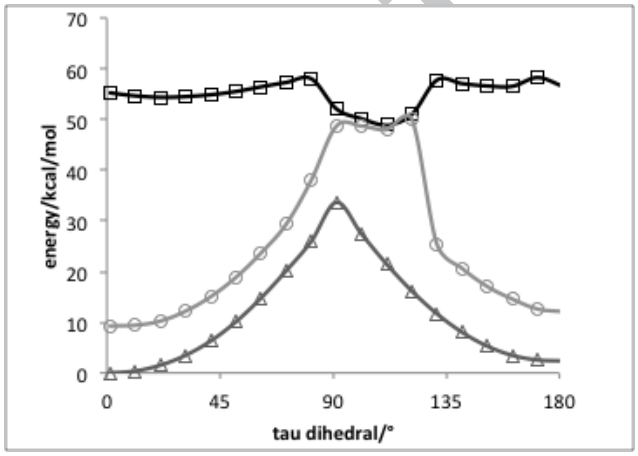


Figure 2. DFT//bp86/SV(P) calculated energies for *E*-/*Z*-isomerization (τ) for **4e**, showing the ground state (S_0; triangles), first excited state (S_1; squares) and the ground state energy at the first excited state geometry (circles)

For the phenols (**4a**, **4e** and **4h**) pH variation studies of absorption spectra were used to calculate the ground state pK_a of the respective compounds (Fig. S4-7, Table S5; ESI). The pK_a of **4e** (8.52) was an order of magnitude higher than that of the other two, suggesting that the conjugate base of **4e** is relatively unstable, perhaps due to the presence of a flexible six membered ring that can undergo facile conformational changes (chair-boat-chair), even in the ground state.

In conclusion, we have developed a new, simple, facile and high yielding route to GFP-chromophore analogues *via* oxazolinone formation. The locked analogues are brightly fluorescent in the solid state but poorly fluorescent in solution, indicating either the restriction of conformation (ϕ) is not sufficient or that other non-radiative pathways are involved. However, these studies provide valuable information on the design and synthesis of more fluorescent analogues and these will be reported in due course.

Acknowledgment

This research is supported by the Australian Research Council (DP0877999) to P.K. We gratefully acknowledge the Australian Research Council for the funding support of S.C. We thank Prof. Anindya Datta for permitting us to use absorption spectrophotometer, fluorimeter and fluorescence upconversion instrument in his laboratory.

References and notes

1. Chudakov, D. M.; Matz, M. V.; Lukyanov, S.; Lukyanov, K. A. *Physiol. Rev.* **2010**, *90*, 1103-1163.
2. Tsien, R. Y. *Annu. Rev. Biochem.* **1998**, *67*, 509-544.
3. *Green Fluorescent Proteins*; Academic Press: San Diego, 1999; Vol. 58.
4. Paige, J. S.; Wu, K. Y.; Jaffrey, S. R. *Science* **2011**, *333*, 642-646.
5. Addison, K.; Heisler, I. A.; Conyard, J.; Dixon, T.; Bulman Page, P. C.; Meech, S. R. *Faraday Discuss.* **2013**, *163*, 277-296.
6. Cheng, C.-W.; Huang, G.-J.; Hsu, H.-Y.; Prabhakar, C.; Lee, Y.-P.; Diau, E. W.-G.; Yang, J.-S. *J. Phys. Chem. B* **2013**, *117*, 2705-2716.
7. Cody, C. W.; Prasher, D. C.; Westler, W. M.; Prendergast, F. G.; Ward, W. W. *Biochemistry* **1993**, *32*, 1212-1218.
8. Kojima, S.; Ohkawa, H.; Hirano, T.; Maki, S.; Niwa, H.; Ohashi, M.; Inouye, S.; Tsuji, F. I. *Tetrahedron Lett.* **1998**, *39*, 5239-5242.
9. Chen, K.-Y.; Cheng, Y.-M.; Lai, C.-H.; Hsu, C.-C.; Ho, M.-L.; Lee, G.-H.; Chou, P.-T. *J. Am. Chem. Soc.* **2007**, *129*, 4534-4535.
10. Hsieh, C.-C.; Chou, P.-T.; Shih, C.-W.; Chuang, W.-T.; Chung, M.-W.; Lee, J.; Joo, T. *J. Am. Chem. Soc.* **2011**, *133*, 2932-2943.
11. Weber, W.; Helms, V.; McCammon, J. A.; Langhoff, P. W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6177-6182.
12. Mandal, D.; Tahara, T.; Webber, N. M.; Meech, S. R. *Chem. Phys. Lett.* **2002**, *358*, 495-501.
13. Litvinenko, K. L.; Webber, N. M.; Meech, S. R. *J. Phys. Chem. A* **2003**, *107*, 2616-2623.
14. Mandal, D.; Tahara, T.; Meech, S. R. *J. Phys. Chem. B* **2004**, *108*, 1102-1108.
15. Dong, J.; Solntsev, K. M.; Poizat, O.; Tolbert, L. M. *J. Am. Chem. Soc.* **2007**, *129*, 10084-11085.
16. Dong, J.; Solntsev, K. M.; Tolbert, L. M. *J. Am. Chem. Soc.* **2006**, *128*, 12038-12039.
17. Song, W.; Strack, R. L.; Svensen, N.; Jaffrey, S. R. *J. Am. Chem. Soc.* **2014**, *136*, 1198-1201.
18. Baranov, M. S.; Lukyanov, K. A.; Ivashkin, P. E.; Yampolsky, I. V. *Synth. Commun.* **2013**, *43*, 2337-2342.
19. Lee, C.-Y.; Chen, Y.-C.; Lin, H.-C.; Jhong, Y.; Chang, C.-W.; Tsai, C.-H.; Kao, C.-L.; Chien, T.-C. *Tetrahedron* **2012**, *68*, 5898-5907.
20. Stafforst, T.; Diederichsen, U. *Eur. J. Org. Chem.* **2007**, 899-911.
21. Cativiela, C.; Diaz De Villegas, M. D.; Meléndez, E. J. *Heterocycl. Chem.* **1985**, *22*, 1655-1657.
22. Alías, M.; López, M. P.; Cativiela, C. *Tetrahedron* **2004**, *60*, 885-891.
23. Balakrishna, M. S.; Kaboudin, B. *Tetrahedron. Lett.* **2001**, *42*, 1127-1129.
24. Wu, L.; Burgess, K. *J. Am. Chem. Soc.* **2008**, *130*, 4089-4096.
25. Baldridge, A.; Kowalik, J.; Tolbert, L. M. *Synthesis* **2010**, *14*, 2424-2436.
26. Sharma, V.; Lansdell, T. A.; Jin, G.; Tepe, J. J. *J. Med. Chem.* **2004**, *47*, 3700-3703.
27. Hsu, Y.-H.; Chen, Y.-A.; Tseng, H.-W.; Zhang, Z.; Shen, J.-Y.; Chuang, W.-T.; Lin, T.-C.; Lee, C.-S.; Hung, W.-Y.; Hong, B.-C.; Liu, S.-H.; Chou, P.-T. *J. Am. Chem. Soc.* **2014**, *136*, 11805-11812.
28. Conway, P. A.; Devine, K.; Paradisi, F. *Tetrahedron* **2009**, *65*, 2935-2938.
29. Kang, J.; Zhao, G.; Xu, J.; Yang, W. *Chem. Commun.* **2010**, *46*, 2868-2870.

Supplementary Material

Synthesis of all the compounds, 1D NMR spectra, steady state absorption and emission spectra and femtosecond fluorescence decay traces.

Highlights

- Synthesis of a series of conformationally locked GFP fluorophores was achieved
- A new faster, simpler and higher yielding synthesis of GFP fluorophores is reported
- Double bond rotation is the major non-radiative pathway in these analogues

Graphical Abstract

To create your abstract, type over the instructions in the template box below.

An Efficient and Concise Method to Synthesize Locked GFP Chromophore Analogues

Soumit Chatterjee, and Peter Karuso

Leave this area blank for abstract info.

Fonts or abstract dimensions should not be changed or altered.