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Synthesis and incorporation of a caged tyrosine amino acid possessing a bioorthogonal handle

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ABSTRACT

Reversing a bioconjugation in a spatial and temporal fashion has widespread applications, especially toward targeted drug delivery. We report the synthesis and incorporation of an unnatural amino acid with an alkyne modified dimethoxy-*ortho*-nitrobenzyl caging group. This unnatural amino acid can be utilized in a Glaser–Hay conjugation to generate a bioconjugate, but also is able to disrupt the bioconjugate when irradiated with light. These combined features allow for the preparation of bioconjugates with a high degree of site-specificity and allow for the separation of the two components if necessary.

The ability to exogenously control protein function in a precise fashion offers a diverse and powerful tool for the manipulation of proteins. One such exogenous stimulus is light, given the ability to control both its intensity and wavelength.[1–4] Moreover, light can be utilized in both a spatial and temporal fashion that confers precise control over protein function. One mechanism to photochemically regulate protein function is through the use of photochemical caging groups.[5,6] These "caging" groups inactivate the biological function of the molecule, but are easily removed via light irradiation to restore function. One class of caging groups is derived from ortho-nitrobenzyl (ONB) moieties.[7,8] Ortho-nitrobenzyl caging groups and their derivatives have been used in a variety of applications including the inactivation of isopropyl β-D-1-thiogalactopyranoside (IPTG),[9] the photoactivation of DNA and RNA,[10–15] and the patterning of surfaces.[16] Moreover, a caged tyrosine reside (ONBY) within a protein has been utilized to control phosphorylation events,[17] modulate gene function,[18] and investigate ion channel characteristics,[19] among numerous other applications.[20]

One method of introducing this photoreactive moiety is through the site-specific incorporation of an unnatural amino acid (UAA) into proteins. An efficient mechanism has been developed that appropriates the natural machinery of the cell to genetically encode the UAA.[21,22] This is accomplished through the introduction of an orthogonal aminoacyl tRNA synthetase (aaRS)/tRNA pair that has been evolved to specifically recognize the desired UAA, and incorporate it through the suppression of the amber stop codon (TAG).[23,24] Many aaRSs have been evolved to incorporate photosensitive and photoreactive unnatural amino acids into proteins. As previously mentioned, an ONBY unnatural amino acid has been genetically incorporated to photochemically regulate β-galactosidase,[25] Cre recombinase,[18] and Taq polymerase.[26] Caged lysine (ONBK), have been utilized to photochemically control Green Fluorescent Protein (GFP) in mammalian cells.[27] An ortho-nitrobenzyl cysteine was incorporated into caspase 3 to regulate protease activity in yeast.[28] Additionally, the transcription factor Pho4 was modulated via a caged serine, enabling control over phosphorylation.[29,30] Each of these UAAs have effectively been demonstrated to regulate protein function in a site-specific fashion and with a high degree of control. We sought to translate this degree of control to the activity of bioconjugates.

In addition to the ability to control protein function, site-specific incorporation of UAAs has proven especially useful in the generation of novel bioconjugates. Bioconjugation offers a powerful tool to modify proteins through coupling with biophysical probes, therapeutic agents, or surfaces.[31,32–34] Many bioconjugation techniques use covalent linkages to bind such effector molecules to the proteins. For example, bioconjugation of drugs with polyethylene glycol (PEG) chains reduces drug degradation and immunogenicity.[35] Many of these covalent linking strategies, despite their utility, suffer from a lack of specificity, and often result in proteins conjugated to multiple partners. To achieve greater specificity, unnatural amino acids have been genetically encoded into proteins that allow for directed covalent linkages. Substantial research has been conducted employing a variety of different UAAs in several bioorthogonal reactions to prepare well-defined bioconjugates.[36–40]

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One example involves the preparation of bispecific antibodies by generating Fab fragments with a *p*-acetylphenylalanine UAA, capable of undergoing oxime ligations forming therapeutically useful bioconjugates.[39,41]

Due to the well-documented utility of both bioconjugates and photocaging, we sought to combine the two techniques into a single unnatural amino acid. The gap in the current bioorganic technology prompted our interest in developing such a bifunctional UAA. Ideally, this UAA would harbor a handle for bioconjugation and also facilitate a degree of conjugate flexibility, allowing it to disproportionate upon light irradiation.[42] These properties may be especially useful in the delivery of cytotoxic molecules via conjugation to antibody targeting agents that may alter the efficacy of the molecule due to the relative size of the antibody.[41,43] Upon delivery and endocytosis to the cell, the small molecule could be photocleaved to remove the potentially inactivating steric bulk of the antibody allowing it to achieve its full activity (Fig. 1). A purely photocleaving UAA without the capacity for bioconjugation has previously been developed and utilized to cleave the protein backbone upon light exposure.[44] Our envisioned UAA expands upon this feature to introduce a handle for a bioorthogonal conjugation reaction.

Toward this goal a novel UAA was developed containing both a dimethoxy-ortho-nitrobenzyl functionality for photoreactivity, and an alkynyl moiety for conjugation (Fig. 1). UAA **1** could be synthetically accessed beginning from commercially available vanillin (**2**) in a concise six-step sequence (Scheme 1). Vanillin was first propargylated to form **3** in order to provide the alkyne functionality for future bioconjugation. The nitro group was then installed using nitric acid to afford **4** in moderate yield and high regioselectivity. The aldehyde **4** was then reduced with sodium borohydride in 1 M NaOH to afford the alcohol **5** in 71% yield. Interestingly, reactions in ethanol, a more conventional solvent for this type of reduction, resulted in substantially lower yields even when used in large excess, necessitating the reduction in aqueous sodium hydroxide. The alcohol **5** was then brominated with PBr_3 to provide **6,** which was then alkylated with diethyl acetamidomalonate to yield the protected UAA **7**. Several conditions were examined for the conversion of **6** to **7**; however, the most efficacious reaction progressed under microwave irradiation.[45] Thus, **7** was deprotected in 6 N HCl under microwave irradiation to yield the desired UAA in an overall yield of 19%. The purity and identity of **1** was confirmed by both NMR and GC/MS analyses.

With the desired UAA in hand, it was necessary to elucidate an appropriate aaRS that could recognize and effectively incorporate **1**

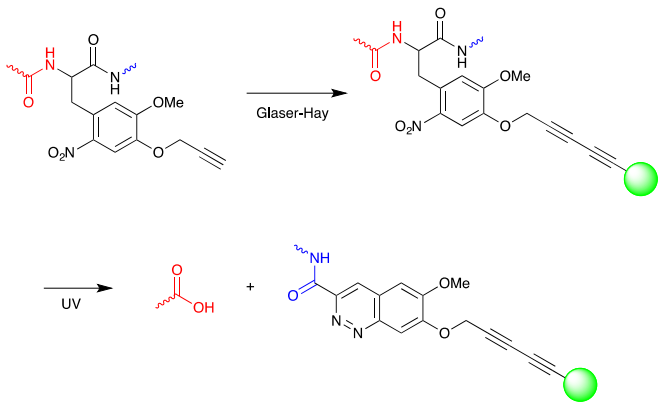
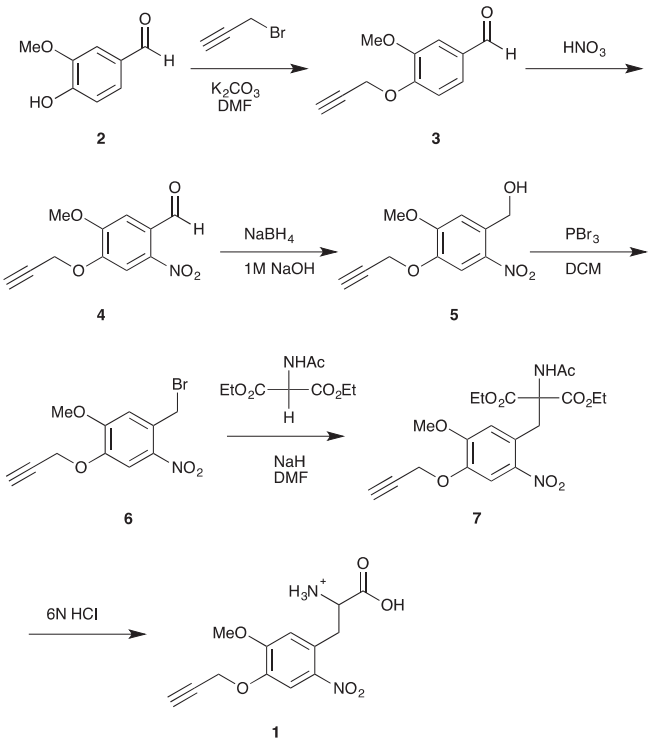


Figure 1. Strategy for photoregulation of protein conjugates. Once incorporated into a protein context the alkynyl handle facilitates either a Glaser–Hay reaction or a 1,3-dipolar cycloaddition to generate a bioconjugate. Irradiation with UV light then facilitates protein degradation and delivery of the bioconjugate partner. The red and blue represent the protein backbone, which upon cleavage is degraded.



Scheme 1.

into a protein. Typically, a double-sieve selection on an aaRS library is required to identify a functional aaRS; however, recent reports have found several existing aaRSs that displayed promiscuity toward the incorporation of additional UAAs.[46,47] We hoped to exploit this promiscuity to obviate the necessity of an aaRS selection, and thus initiated a screen with several existing synthetases for the incorporation of **1** using a GFP reporter (sfGFP; super-folding green fluorescent protein). The ability to incorporate the UAA would result in a fluorescent signal due to the production of functional GFP, whereas a failure to incorporate results in a non-fluorescent truncated protein due to the inability to suppress the TAG stop codon. Thus, increased fluorescence could be correlated to aaRS promiscuity toward **1**. A range of aaRSs were selected including Bipy, *p*CNF, ONBY, NapA due to either their previously reported promiscuity, their large UAA binding pocket, or their ability to incorporate structurally similar UAAs. Gratifyingly, upon screening the *p*CNF aaRS, a significant increase in fluorescence in the presence of **1** relative to the absence was observed (see Supporting Information). Consequently, the *p*CNF-aaRS was selected for expression of **1**. To confirm incorporation, cells harboring a plasmid that encodes the aaRS/tRNA pair and a plasmid encoding GFP with a TAG mutation at surface exposed residue 151 were induced with IPTG/Arabinose in the presence of **1**, and incubated 16 h at 30 °C. The cells were then lysed and GFP was purified using a commercially available Ni–NTA resin and analyzed via SDS–PAGE and fluorescence (Fig. 2). Based on both fluorescence data and gel analysis, **1** is incorporated into GFP at 68% relative to wild type GFP expression.

Following expression and purification of the GFP containing **1**, a proof-of-concept experiment was performed to ascertain the feasibility of both the bioconjugation and the photoreactivity of the UAA. Our laboratory recently reported a Glaser–Hay bioconjugation involving the coupling of terminal alkynes.[37,48] Consequently, this novel bioconjugation was employed with the new UAA based on the alkynyl handle **1**. The mutant GFP was reacted with an alkyne containing Alexafluor-488 molecule in the presence of

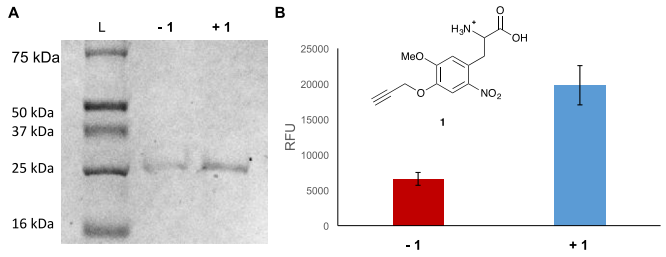


Figure 2. Protein expression with UAA **1.** (A) SDS–PAGE analysis of the GFP expression in the presence and absence of the caged tyrosine UAA. Lane 1: Molecular weight ladder; Lane 2: Expression in the absence of **1**; Lane 3: Expression in the presence of **1**. Some protein expression is visible in the – UAA lane due to the promiscuity of the *p*CNF-aaRS and its propensity to incorporate tyrosine in the absence of any UAA. (B) Fluorescence analysis of the GFP expression demonstrating a significant difference between expressions containing **1** relative to expressions in its absence.

CuI/TMEDA for 4 h at 4 °C. The reaction was then purified by size-exclusion centrifugation and the protein was denatured to eliminate protein-derived fluorescence. The conjugation was then analyzed by SDS–PAGE (Fig. 3). As indicated on the gel, a successful conjugation was observed only in the presence of CuI, alkynyl-fluorophore, and TMEDA, further confirming the presence of **1** within the protein as the alkynyl functionality is required for a productive Glaser–Hay reaction to occur. Based on gel analysis, and absorbance measurements, the conjugation progressed almost to completion with a 93% conversion.

Upon demonstration of successful conjugation, the photolability of the UAA was assessed. Due to the mechanism of decaging, the UAA should sever the peptide backbone at the site of incorporation leading to a fragmented protein upon irradiation.[44] In order to probe the functionality of the caged amino acid, the previously prepared GFP-Alexafluor 488 conjugate was incubated in the presence and absence of non-cytotoxic UV irradiation at 365 nm for various amounts of time. The reactions were then visualized by

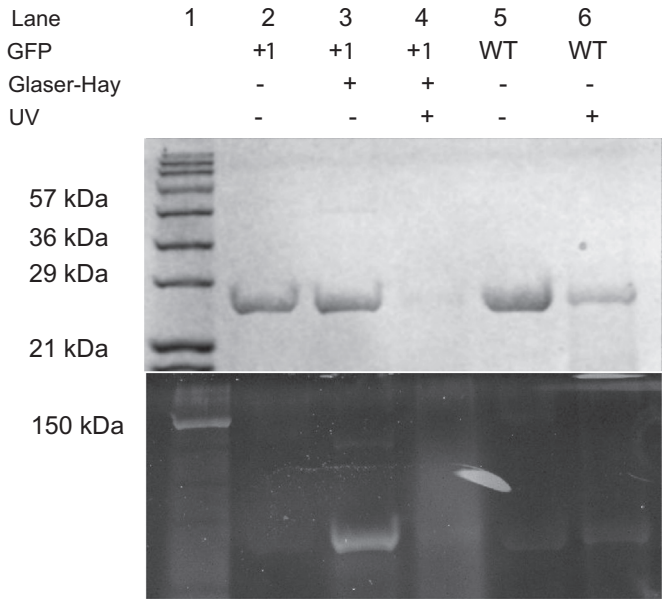


Figure 3. SDS–PAGE analysis of both the Glaser–Hay bioconjugation and the photoregulation of the conjugate. Lane 1: Protein Ladder; Lane 2: GFP containing **1** not subjected to Glaser–Hay conditions; Lane 3: GFP containing **1** coupled with AlexaFluor-488 Alkyne, but not irradiated with UV; Lane 4: GFP containing **1** coupled with AlexaFluor-488 Alkyne, and irradiated at 365 nm; Lane 5: Wild-type GFP not irradiated; Lane 6: Wild-type GFP irradiated at 365 nm.

SDS–PAGE to ascertain the ability of the UAA to perturb the GFP backbone (Fig. 3). Unfortunately, a definitive cleavage was not observed; however, protein degradation was unequivocally observed after 30 min of irradiation at 365 nm. This result suggests that the cleavage of the backbone did occur; however, under these conditions protein degradation then occurred. No reaction was observed in the conjugate that was not irradiated, or in wild-type GFP (not containing **1**), suggesting that the protein degradation is directly due to the presence of the UAA and not simply due to UV irradiation. While some minor effect of irradiation on wild-type protein may have occurred, repeating the experiment with either 20 min or 30 min of irradiation on ice resolved the loss of wild-type, while still degrading protein containing **1** (see Supporting Information). We hypothesize that the global degradation may be the result of protein instability upon cleavage. While this observed nonspecific degradation is not ideal, the decomposition would only be triggered *after* the selective uptake of the protein-small molecule conjugate into the target cell. Successful delivery of the cargo is therefore independent of a clean photocleaved protein. Current work is underway to optimize conditions to affect a more direct cleavage, as well as ascertain the source of the protein degradation.

Ultimately, the synthesis, incorporation, conjugation, and photoreaction of **1** represent a useful example for the implementation of UAAs within the context of a protein. Ideally, this work can be continued to utilize **1** in a more practically relevant protein (e.g. antibody), and probe its use in cell culture. This unique UAA represents a mechanism to both prepare and release bioconjugates and possesses a wide range of potential both in therapeutic and biomaterial applications.

In conclusion, we have both synthesized and incorporated a multi-functional unnatural amino acid, possessing both a photoreactive moiety and a bioconjugation handle. Gratifyingly, it has been employed in a proof-of-concept experiment, and was utilized in conjunction with a recently developed Glaser–Hay bioconjugation to afford a protein-small molecule conjugate. Moreover, the versatility of the alkyne handle also makes it viable for more common 1,3 cycloaddition reactions with azide partners. Furthermore, the photosensitivity of the UAA was demonstrated, as it was able to promote protein degradation upon exposure to UV irradiation. Ultimately, this useful UAA has broad utility within the field of bioconjugation and possesses innumerable potential applications.

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Supplementary data

Supplementary data (experimental procedures; characterization data; aaRS polyspecificity screens, irradiation optimization) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.09.033.

References and notes

1. Young, D.; Deiters, A. *Org. Biomol. Chem.* **2007**, *5*, 999.
2. Ellis-Davies, G. *Nat. Methods* **2007**, *4*, 619.
3. Deiters, A. *Curr. Opin. Chem. Biol.* **2009**, *13*, 678.
4. Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755.
5. Curley, K.; Lawrence, D. S. *Curr. Opin. Chem. Biol.* **1999**, *3*, 84.
6. Rothman, D. M.; Petersson, E. J.; Vázquez, M. E.; Brandt, G. S.; Dougherty, D. A.; Imperiali, B. *J. Am. Chem. Soc.* **2005**, *127*, 846.
7. Pelliccioli, A. P.; Wirz, J. *Photochem. Photobiol. Sci.* **2002**, *1*, 441.
8. Ellis-Davies, G. C.; Kaplan, J. H. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 187.
9. Young, D.; Deiters, A. *Angew. Chem. Int. Ed.* **2007**, *46*, 4290.

10. Ceo, L. M.; Koh, J. T. *ChemBioChem* **2012**, *13*, 511.
11. Monroe, W. T.; McQuain, M. M.; Chang, M. S.; Alexander, J. S.; Haselton, F. R. *J. Biol. Chem.* **1999**, *274*, 20895.
12. Lusic, H.; Young, D.; Lively, M.; Deiters, A. *Org. Lett.* **2007**, *1903*, 9.
13. Shah, S.; Rangarajan, S.; Friedman, S. H. *Angew. Chem. Int., Ed. Engl.* **2005**, *44*, 1328.
14. Shah, S.; Jain, P. K.; Kala, A.; Karunakaran, D.; Friedman, S. H. *Nucleic Acids Res.* **2009**, *37*, 4508.
15. Rothman, D. M.; Vázquez, M. E.; Vogel, E. M.; Imperiali, B. *Org. Lett.* **2002**, *4*, 2865.
16. Lee, J. H.; Domaille, D. W.; Noh, H.; Oh, T.; Choi, C.; Jin, S.; Cha, J. N. *Langmuir* **2014**, *30*, 8452.
17. Arbely, E.; Torres-Kolbus, J.; Deiters, A.; Chin, J. W. *J. Am. Chem. Soc.* **2012**, *134*, 11912.
18. Edwards, W.; Young, D.; Deiters, A. *ACS Chem. Biol.* **2009**, *4*, 441.
19. Miller, J. C.; Silverman, S. K.; England, P. M.; Dougherty, D. A.; Lester, H. A. *Neuron* **1998**, *20*, 619.
20. Humphrey, D.; Rajfur, Z.; Vazquez, M. E.; Scheswohl, D.; Schaller, M. D.; Jacobson, K.; Imperiali, B. *J. Biol. Chem.* **2005**, *280*, 22091.
21. Xie, J.; Schultz, P. G. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 775.
22. Wang, L.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **2004**, *44*, 34.
23. Young, T. S.; Schultz, P. G. *J. Biol. Chem.* **2010**, *285*, 11039.
24. Liu, C. C.; Mack, A. V.; Tsao, M. L.; Mills, J. H.; Lee, H. S.; Choe, H.; Farzan, M.; Schultz, P. G.; Smider, V. V. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 17688.
25. Deiters, A.; Groff, D.; Ryu, Y.; Xie, J.; Schultz, P. *Angew. Chem. Int., Ed.* **2006**, *45*, 2728.
26. Chou, C.; Young, D.; Deiters, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 5950.
27. Chen, P. R.; Groff, D.; Guo, J.; Ou, W.; Cellitti, S.; Geierstanger, B. H.; Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **2009**, *48*, 4052.
28. Wu, N.; Deiters, A.; Cropp, T. A.; King, D.; Schultz, P. G. *J. Am. Chem. Soc.* **2004**, *126*, 14306.
29. Lemke, E.; Summerer, D.; Geierstanger, B.; Brittain, S.; Schultz, P. *Nat. Chem. Biol.* **2007**, *3*, 769.
30. Vázquez, M. E.; Nitz, M.; Stehn, J.; Yaffe, M. B.; Imperiali, B. *J. Am. Chem. Soc.* **2003**, *125*, 10150.
31. Stephanopoulos, N.; Francis, M. *Nat. Chem. Biol.* **2011**, *7*, 876.
32. Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: London, 1996.
33. Khare, P.; Jain, A.; Gulbake, A.; Soni, V.; Jain, N.; Jain, S. *Crit. Rev. Therap. Drug Carr. Syst.* **2009**, *26*, 119.
34. Pasut, G.; Veronese, F.; SatchiFainaro, R.; Duncan, R. *Poly Therap. I* **2006**, *192*, 95.
35. Veronese, F. M.; Pasut, G. *Drug Discovery Today* **2005**, *10*, 1451.
36. Maza, J. C.; McKenna, J. R.; Raliski, B. K.; Freedman, M. T.; Young, D. D. *Bioconjug. Chem.* **2015**.
37. Lampkowski, J. S.; Villa, J. K.; Young, T. S.; Young, D. D. *Angew. Chem., Int. Ed. Engl.* **2015**.
38. Raliski, B.; Howard, C.; Young, D. *Bioconjug. Chem.* **2014**, *1916*, 25.
39. Kim, C. H.; Axup, J. Y.; Dubrovska, A.; Kazane, S. A.; Hutchins, B. A.; Wold, E. D.; Smider, V. V.; Schultz, P. G. *J. Am. Chem. Soc.* **2012**, *134*, 9918.
40. Lee, Y. J.; Kurra, Y.; Yang, Y.; Torres-Kolbus, J.; Deiters, A.; Liu, W. R. *Chem. Commun.* **2014**, *50*, 13085.
41. Hutchins, B.; Kazane, S.; Staflin, K.; Forsyth, J.; Felding-Habermann, B.; Schultz, P.; Smider, V. *J. Mol. Biol.* **2011**, *406*, 595.
42. Li, J.; Chen, P. R. *Nat. Chem. Biol.* **2016**, *12*, 129.
43. Kazane, S. A.; Sok, D.; Cho, E. H.; Uson, M. L.; Kuhn, P.; Schultz, P. G.; Smider, V. V. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 3731.
44. Peters, F. B.; Brock, A.; Wang, J. Y.; Schultz, P. G. *Chem. Biol.* **2009**, *16*, 148.
45. Young, D.; Torres-Kolbus, J.; Deiters, A. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5478.
46. Young, D.; Young, T.; Jahnz, M.; Ahmad, I.; Spraggon, G.; Schultz, P. *Biochemistry* **2011**, *1894*, 50.
47. Young, D.; Jockush, S.; Turro, N.; Schultz, P. *Bioorg. Med. Chem. Lett.* **2011**, *21*, 7502.
48. Maza, J.; Nimmo, Z.; Young, D. *Chem. Commun.* **2016**, *52*, 88.