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PAPER

Caged cyclopropenes for controlling bioorthogonal reactivity

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Bioorthogonal ligations have been designed and optimized to provide new experimental avenues for understanding biological systems. Generally, these optimizations have focused on improving reaction rates and orthogonality to both biology and other members of the bioorthogonal reaction repertoire. Less well explored are reactions that permit control of bioorthogonal reactivity in space and time. Here we describe a strategy that enables modular control of the cyclopropene-tetrazine ligation. We developed 3-N-substituted spirocyclopropenes that are designed to be unreactive towards 1,2,4,5-tetrazines when bulky N-protecting groups sterically prohibit the tetrazine's approach, and reactive once the groups are removed. We describe the synthesis of 3-N spirocyclopropenes with an appended electron withdrawing group to promote stability. Modification of the cyclopropene 3-N with a bulky, light-cleavable caging group was effective at stifling its reaction with tetrazine, and the caged cyclopropene was resistant to reaction with biological nucleophiles. As expected, upon removal of the light-labile group, the 3-N cyclopropene reacted with tetrazine to form the expected ligation product both in solution and on a tetrazine-modified protein. This reactivity caging strategy leverages the popular carbamate protecting group linkage, enabling the use of diverse caging groups to tailor the reaction's activation modality for specific applications.

Considerable effort has gone into the development of bioorthogonal reagents with faster kinetics or unique and mutually orthogonal reactivities[1–3]. Conversely, there have been few reagents developed for activating bioorthogonal reactivity in space and time. Such activatable bioorthogonal reagents remains dormant until activated, generally by light or an enzyme. This furnishes the ability to exert control over when and where they can react with their bioorthogonal partners. For example, a photoactivatable bioorthogonal reagent-coated surface would allow users to decide both when the reagent can be activated by illumination and where on the surface activation can occur for the reagent to participate in the bioorthogonal ligation. Examples include the tetrazole-alkene photoclick chemistry[4–6], although recent concerns of side reactions with biological nucleophiles limit its usage in systems requiring strict bioorthogonality[7]. Innovative work by Popik and coworkers exploited intrinsic photoinduced decarboxylation of cyclopropenone to create a photoactivatable cyclooctyne for Cu-free click chemistry[8,9], and Fox and coworkers created a redox-activatable tetrazine based on oxidation of dihydrotetrazine to tetrazine by exposure to the enzyme Horse Radish Peroxidase or a photosensitizer[10]. Finally, Carrico and coworkers produced a variant of the Staudinger ligation that is activated by light[11]. With the exception of the photoactivated Staudinger ligation, each activation strategy takes advantage of intrinsic properties of the reagents, making it challenging to tailor the caging strategy to a desired application. Here we describe a strategy Here we describe a strategy employing a carbamate cage that will permit control of reactivity with a wide array of carbamate-linked photocleavable protecting groups. This strategy engineers control of the ligation between 1,2,4,5-tetrazines and cyclopropenes that will enable the use of convenient fluorogenic tetrazine reagents[12] as well as a diverse set of caging groups that will permit tailoring the reaction's activation method to the desired application.

Our strategy for controlling the cyclopropene-tetrazine ligation exploits previous reports that this reaction proceeds >7000 times slower when the cyclopropene is disubstituted vs. monosubstituted at the 3-position[13,14]. This dramatic rate difference is due to unfavorable steric interactions between the C3 substituents and tetrazine in the transition state[14] (**Figure 1a**). We reasoned that a nitrogen, positioned at C3 of the cyclopropene in a bicyclic system, would adopt a pyramidal geometry that is amenable to the tetrazine's approach. Importantly, the introduction of nitrogen to C3 is likely to have a notable effect on the electronics of the cyclopropene. Moreover, the nitrogen's addition is key to the molecule's

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design and distinguishes the scaffold from known

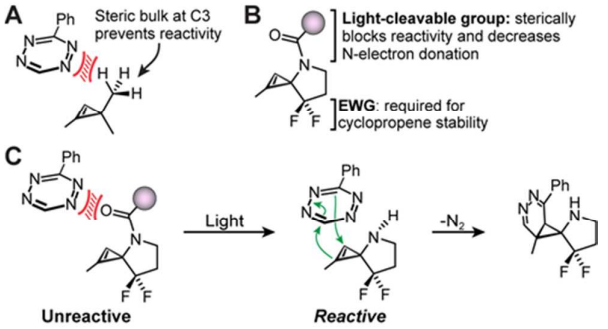
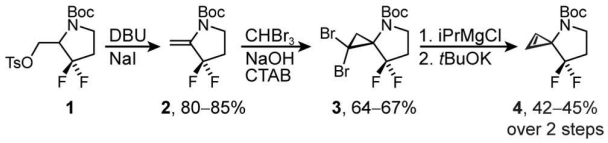


Figure 1 Leveraging sluggish reactivity between C3-disubstituted cyclopropenes and tetrazines to create an activatable ligation. (**A**) Cyclopropenes with C3 substitutions as small as a methyl group react very sluggishly with tetrazines. (**B**) Installation of a bulky light-cleavable protecting group serves as a removable inhibitor of reactivity. In molecules with this scaffold, positioning electron withdrawing groups (EWG) at the other cyclopropene C3 was necessary for stability. (**C**) Light removes a bulky protecting group, activating reactivity between cyclopropene and tetrazine to permit ligation.



Scheme 1. Synthesis of Boc-protected 3N, 3-difluoro spirocyclopropene.

cyclopropenes (*e.g.*, spiro[2.3]hex-1-ene[15]) structurally, electronically, and with respect to the molecule's synthetic strategy. Indeed, there is only one previous report of a 3N-modified cyclopropenes, the cyclopropene amino acids synthesized by Fox and coworkers[16], and in its synthesis the nitrogen was installed after formation of the cyclopropene. We hypothesized that the resulting 4-azaspiro[2,n]alkene, hereafter termed 3N-spirocyclopropene, would be unreactive when the nitrogen is modified with a bulky protecting group and reactive when the protecting group was removed (**Figure 1b–c**). As an additional level of control, we expected the increased electron withdrawing effect of a carbamate-protected vs. free nitrogen to decrease the electron density in the cyclopropene, resulting in further deceleration of the protected cyclopropene's reaction with tetrazines.

Our initial synthetic efforts focused on 3-N spirocyclopropene without electron withdrawing groups. However, in these reactions, we obtained only rearrangement products resulting from cyclopropene ring opening[17]. To mitigate ring opening, we installed a difluoro group to destabilize the partial positive charge that would be formed along the ring opening reaction coordinate. Accordingly, we began the synthesis with an elimination of Boc-protected, tosylated difluoroprolinol **1** to produce enamine **2**. A subsequent dibromocarbene addition produced dibromocyclopropane **3** in good yield. Finally, conversion to the monobromocyclopropane (**S7**, Supporting Information) and subsequent elimination afforded cyclopropene **4** in modest yield over two steps (**Scheme 1**).

Boc-protected **4** was stable for at least 6 months at -20 °C, and its free amine counterpart **5a** (**Figure 2a**) was stable for at least 6 months as a pH 1 aqueous solution at room temperature. Upon neutralization, the free amine cyclopropene 5a displayed reactivity with a 1,2,4,5-tetrazine in a mass spectrometry-based assay. However, it was prone to degradation at neutral pH over several hours, presumably via polymerization as evidenced by the generation of dimer and trimer species according to mass spectrometry analysis (**Figure S1**, Supporting Information). This result is consistent with previous reports of cyclopropene decomposition with C1 and C2 = H (**Figure 2a**, Compound **5a:** R1/R2 =H)[18,19]. Unfortunately, notable degradation on the hour timescale prohibited accurate determination of reaction rates and limited the utility of the molecule with unmodified C1/2. Thus, we sought cyclopropene modification strategies that would stabilize the cyclopropene scaffold.

Previous reports of cyclopropene tag stability have shown that decoration of the cyclopropene C1/2 with alkyl groups can

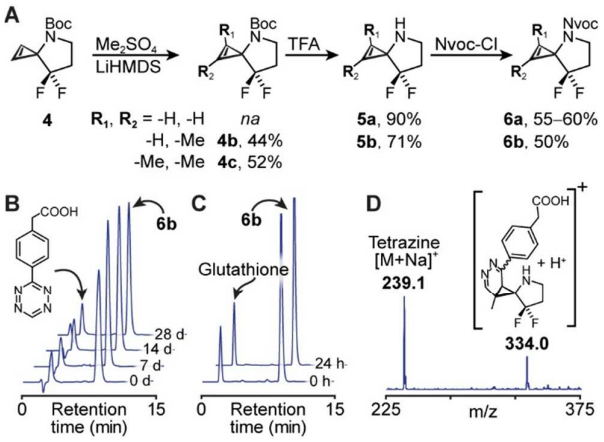


Figure 2 Synthesis and reactivity of C1/2-modified 3-N spirocyclopropenes. (**A**) Cyclopropene **4** is amenable to modifications at the C1/C2 positions and subsequent N-functionalization with light-cleavable protecting groups. (**B**–**C**) HPLC traces showing lack of reaction in 50% (v/v) MeCN in pH 7.4 PBS between (B) Nvoc-protected cyclopropene **6b** (2 mM) and tetrazine **S9** (0.5 mM) for at least 4 weeks, or (C) **6b** (2.5 mM) and glutathione (10 mM) for at least 24 h. (**D**) The reaction between 3-N deprotected cyclopropene **5b** and tetrazine **S9** showed formation of the cycloadduct in a mass spectrometry assay (m/z = 334.0).

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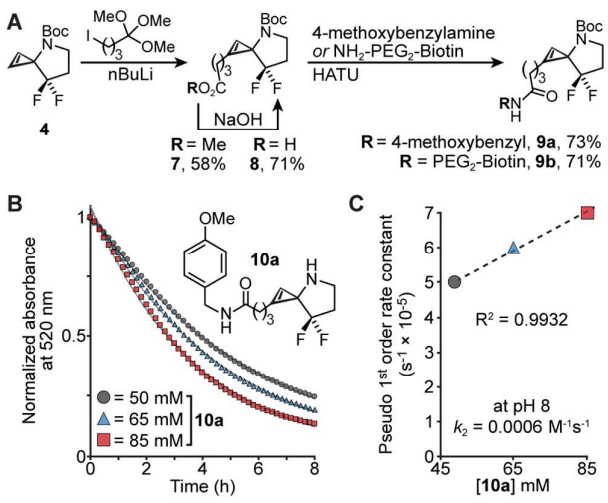
produce substantial improvements to their stability in solution[19]. Thus, to improve on the stability of **4**, we synthesized variants with substituents at the C1 and/or C2-position of the cyclopropene. Reaction of **4** with LiHMDS and dimethylsulfate produced a mixture of mono- and di-substituted cyclopropene (Compounds **4b** and **4c** in **Figure 2a**, respectively), which could be N-Boc deprotected to produce compounds **5a**/**5b** and subsequently decorated with the Nvoc light-cleavable protecting group to produce **6a**/**6b** (**Figure 2a**). We found that both the protected and free amine versions of the monomethyl cyclopropene (compounds **4b**–**6b**) were stable at neutral pH and room temperature for at least several days, enabling analysis of their reactivity with tetrazines and biological nucleophiles.

A lack of reactivity between N-protected cyclopropene **6b** and tetrazine is critical to this strategy's success as an activatable bioorthogonal ligation. Thus, we began our analysis of the molecule's properties with an evaluation of the reactivity between Nvoc-protected compound **6b** and 1,2,4,5-tetrazine **S9**. Critically, we did not observe any decomposition or ligation between **6b** and **S9** for at least 4 weeks, at which point the experiment was terminated with no observed reaction (**Figure 2b**). Additionally, we found that **6b** was stable in a 1:1 PBS:MeCN solution at 37 °C for at least 7 days (**Figure S2**, Supporting Information), and displayed no reactivity or decomposition when exposed to 10 mM L-glutathione, the



highest physiologically-relevant concentration of this biological nucleophile[20] (**Figure 2c**). Importantly, however, removal of the protecting group permitted the cyclopropene-tetrazine ligation to proceed as expected (**Figure 2d**).

Figure 3 Synthesis of functionalized 3-N deprotected spirocyclopropenes and kinetic evaluation of their reaction with 1,2,4,5-tetrazine **S10**. (**A**) 3-N spirocyclopropene **4** carboxylic acid functionalization and subsequent amide linkage to 4-methyoxybenzylamine or NH_2-PEG2-biotin. (**B**) Pseudo 1st order kinetic analysis of the reaction between cyclopropene **10a** and tetrazine **S10**. (**C**) 2nd order rate constant calculation of the reaction between cyclopropene **10a** and 3-phenyl-1,2,4,5-tetrazine pyrrolidinyl amide **S10**. The reactions under pseudo 1st order conditions were conducted at rt in a 96-well plate. Reaction progress was monitored by the disappearance of characteristic tetrazine absorbance at 520 nm. Each well consisted of **10a** (50/65/85 mM) and **S10** (5 mM) in 1:1 MeCN/buffer (shown here for pH 8.0 in NH_4HCO_3 buffer). Plotting the normalized absorbance *wrt* the initial tetrazine absorbance against reaction time, and then fitting using the pseudo 1st order rate equation $A = A_o.\exp(-k.[\mathbf{10a}].t)$ (A = absorbance at time t, A_o = initial absorbance, and $k.[\mathbf{10a}]$ = pseudo 1st order rate constant) provided pseudo 1st order rate constant. The 2nd order rate constants were obtained by plotting pseudo 1st order rates against the concentration of **10a**.

Next, we sought to modify **4** with carboxyl functionality to both impart stability and provide a functionalization handle to the molecule. Modification of **4** with trimethyl 4-iodoorthobutyrate in the presence of nBuLi produced the resulting methyl ester **7**, and the subsequent saponification produced the carboxylic acid **8**, both in modest yield. To this ester, we conjugated either p-methoxybenzylamine, for use in rate determination experiments, or biotin, for use in ligations to tetrazine-laden proteins, to produce **9a** and **9b** in good yield (**Figure 3a**). These were then deprotected to produce free amines **10a** and **10b**, and the biotin derivative was decorated with the light-cleavable Nvoc group to produce compound **11** (Supporting Information and **Figure 4b**).

With compound **10a** in hand, we sought to evaluate the kinetic parameters of its ligation with tetrazine. We measured pseudo 1st order rate constants at three concentrations of **10a** in buffers at pH 7.4, 8.0, and 8.8, by monitoring the disappearance of the characteristic tetrazine absorbance at 520 nm (**Figure 3b**). Analysis of these results revealed that the reaction at pH 7.4 has a 2nd order rate constant of 0.0004 $M^{-1}s^{-1}$, which increased to0.0006 $M^{-1}s^{-1}$ at pH 8.0 and 0.0009 $M^{-1}s^{-1}$ at pH 8.8 (**Figure 3c** and **Figure S3**, Supporting Information). The acceleration in the reaction rate at higher pH is likely the result of increased electron donation and/or reduced steric interactions that occur upon neutralization of a greater fraction of the nitrogen. These results were consistent with a separate HPLC-based assay evaluating pseudo 1st order kinetics (**Figure**

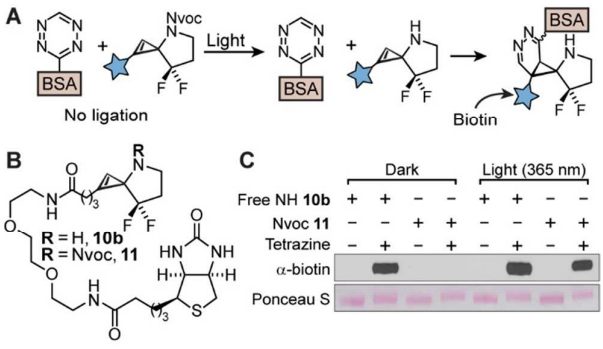


Figure 4 Light-controlled bioorthogonal reactivity of 3-N spirocyclopropenes. (**A**) Nvoc-protected cyclopropene **11** did not display any ligation to tetrazine-modified BSA. Upon exposure to 365 nm light, cyclopropene **11** generates 3N-deprotected cyclopropene **10b** which ligates to the tetrazine-modified BSA protein. (**B**) Molecular structures of biotin-containing 3-N deprotected cyclopropene **10b** and Nvoc protected 3-N cyclopropene **11**. (**C**) Chemiluminescence and Ponceau analysis of ligation between the tetrazine-modified BSA and cyclopropenes **10b** or **11**. No signal was detected for the Nvoc-caged **11** in the absence of light, whereas a strong signal was observed for the same compound after the sample was exposed to 365 nm light. Each reaction contains 5.0 μg of protein (BSA or tetrazine modified BSA) and 500 nM cyclopropene (**10b** or **11)** were either kept in dark or exposed to 365 nm light for 4 h in PBS (pH 7.4). The reactions were incubated at rt for 12–14 h and subjected to western-blot.

S4, Supporting Information), which also allowed us to confirm the formation of the expected ligation product (Compound **S11**, Supporting Information). The observed 2nd order rate constant puts this spirocyclopropene in the same order of magnitude as the original, Cu-free click reactions with cyclooctyne[21]. Additionally, the acceleration in rate with increasing pH suggests that next generation reagents with lowered pKa values are an avenue for future reaction rate improvements.

Lastly, we sought to evaluate this ligation in biological contexts and to determine the success of the ligation after light activation. Towards this end, we created a solution of tetrazine-modified bovine serum albumin (BSA) and evaluated its reactivity with the biotin-conjugated free-amine or Nvoc-protected cyclopropenes **10b** and **11** in a Western blot assay (**Figure 4a–b**). As expected, incubation of 500 nM of Nvoc-protected cyclopropene **11** with tetrazine-BSA in the dark produced no observable ligation products according to Western blot analysis (**Figure 4c**), consistent with the observed long-term lack of reactivity in solution (*i.e.*, **Figure 2b**). Additionally, the Nvoc-protected cyclopropene **11** and free amine cyclopropene **10b** displayed no reactivity with unmodified BSA in light or dark exposure situations. However, upon exposure of 500 nM Nvoc-protected cyclopropene **11** to light at 365 nm we observed ligation to BSA-tetrazine, indicating that **11** can be activated *in situ* (**Figure 4c**).

In conclusion, we have developed a cyclopropene scaffold whose activity can be controlled by addition or removal of a light-cleavable protecting group. We describe the molecule's utility in the context of bioorthogonal chemistry, but the novel scaffold and ability to control the cyclopropene's reactivity could impact other areas of chemistry, such as in the creation of new polymers for materials applications (*e.g.*, in ring opening metastasis polymerizations[22]). Given the popularity of nitrogen as a target for light-removable protecting groups in biology[23,24] (*e.g.*, N-linked photocleavable protecting groups with varying properties based on coumarins[23,25,26], RuBi cage[27], nitroindonilyl[28–30], 2-nitrobenzyl[26,31], and thiochromone S,S-dioxides[32]), we expect that this activatable cyclopropene scaffold will be amenable to control via additional wavelengths of light through the application of the desired protecting group. The kinetics of this reaction with 1,2,4,5-tetrazine is sluggish relative to recent bioorthogonal ligations that have been optimized for speed, but this reagent provides control of reactivity in space and time that can be tuned to the particular application through the selection of an appropriate light-cleavable protecting group. Additionally, kinetics for these reactions are currently being optimized with analogs that lower the molecule's pKa and decrease the strength of the rate-decelerating electron withdrawing groups on the spiro ring system.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1 D. M. Patterson, L. A. Nazarova and J. A. Prescher, *ACS Chem. Biol.*, 2014, **9**, 592–605.
2 J. Li and P. R. Chen, *Nat. Chem. Biol.*, 2016, **12**, 129–137.
3 D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber and J. A. Prescher, *J. Am. Chem. Soc.*, 2012, **134**, 18638–43.
4 Z. Yu, L. Y. Ho and Q. Lin, *J. Am. Chem. Soc.*, 2011, **133**, 11912–11915.
5 C. P. Ramil and Q. Lin, *Curr. Opin. Chem. Biol.*, 2014, **21**, 89–95.
6 Z. Yu, Y. Pan, Z. Wang, J. Wang and Q. Lin, *Angew. Chem. Int. Ed. Engl.*, 2012, **51**, 10600–4.
7 Z. Li, L. Qian, L. Li, J. C. Bernhammer, H. V. Huynh, J.-S. Lee and S. Q. Yao, *Angew. Chemie Int. Ed.*, 2016, **55**, 2002–2006.
8 A. A. Poloukhtine, N. E. Mbua, M. A. Wolfert, G. J. Boons and V. V. Popik, *J. Am. Chem. Soc.*, 2009, **131**, 15769–15776.
9 S. V. Orski, A. A. Poloukhtine, S. Arumugam, L. Mao, V. V. Popik and J. Locklin, *J. Am. Chem. Soc.*, 2010, **132**, 11024–11026.
10 H. Zhang, W. S. Trout, S. Liu, G. A. Andrade, D. A. Hudson, S. L. Scinto, K. T. Dicker, Y. Li, N. Lazouski, J. Rosenthal, C. Thorpe, X. Jia and J. M. Fox, *J. Am. Chem. Soc.*, 2016, **138**, 5978–5983.
11 L. Shah, S. T. Laughlin and I. S. Carrico, *J. Am. Chem. Soc.*, 2016, **138**, 5186–5189.
12 L. G. Meimetis, J. C. T. Carlson, R. J. Giedt, R. H. Kohler and R. Weissleder, *Angew. Chemie Int. Ed.*, 2014, **53**, 7531–

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7534.

13 F. Thalhammer, U. Wallfahrer and J. Sauer, *Tetrahedron Lett.*, 1990, **31**, 6851–6854.

14 D. N. Kamber, L. A. Nazarova, Y. Liang, S. A. Lopez, D. M. Patterson, H.-W. Shih, K. N. Houk and J. A. Prescher, *J. Am. Chem. Soc.*, 2013, **135**, 13680–13683.

15 Z. Yu and Q. Lin, *J. Am. Chem. Soc.*, 2014, **136**, 4153–4156.

16 F. Zhang and J. M. Fox, *Org. Lett.*, 2006, **8**, 2965–8.

17 P. Kumar, O. Zainul and S. T. Laughlin, *Org. Biomol. Chem.*, 2018, **16**, 652–656.

18 P. Dowd and A. Gold, *Tetrahedron Lett.*, 1969, **10**, 85–86.

19 J. Yang, J. Šečkutė, C. M. Cole and N. K. Devaraj, *Angew. Chemie Int. Ed.*, 2012, **51**, 7476–7479.

20 X. Jiang, J. Chen, A. Bajić, C. Zhang, X. Song, S. L. Carroll, Z.-L. Cai, M. Tang, M. Xue, N. Cheng, C. P. Schaaf, F. Li, K. R. MacKenzie, A. C. M. Ferreon, F. Xia, M. C. Wang, M. Maletić-Savatić and J. Wang, *Nat. Commun.*, 2017, **8**, 16087.

21 N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047.

22 B. R. Elling, J. K. Su and Y. Xia, *Chem. Commun.*, 2016, **52**, 9097–9100.

23 P. Klán, T. Šolomek, C. G. Bochet, A. Blanc, R. Givens, M. Rubina, V. Popik, A. Kostikov and J. Wirz, *Chem. Rev.*, 2013, **113**, 119–191.

24 J. B. Grimm, T. D. Gruber, G. Ortiz, T. A. Brown and L. D. Lavis, *Bioconjug. Chem.*, 2016, **27**, 474–480.

25 T. Furuta, S. S.-H. Wang, J. L. Dantzker, T. M. Dore, W. J. Bybee, E. M. Callaway, W. Denk and R. Y. Tsien, *Proc. Natl. Acad. Sci.*, 1999, **96**, 1193–1200.

26 G. C. R. Ellis-Davies, *Nat. Methods*, 2007, **4**, 619–628.

27 R. Cabrera, O. Filevich, B. García-Acosta, J. Athilingam, K. J. Bender, K. E. Poskanzer and R. Etchenique, *ACS Chem. Neurosci.*, 2017, **8**, 1036–1042.

28 M. Canepari, L. Nelson, G. Papageorgiou, J. E. . Corrie and D. Ogden, *J. Neurosci. Methods*, 2001, **112**, 29–42.

29 J. M. Amatrudo, J. P. Olson, H. K. Agarwal and G. C. R. Ellis-Davies, *Eur. J. Neurosci.*, 2015, **41**, 5–16.

30 S. Kantevari, S. Passlick, H.-B. Kwon, M. T. Richers, B. L. Sabatini and G. C. R. Ellis-Davies, *ChemBioChem*, 2016, **17**, 953–961.

31 A. Stutz and S. Pitsch, *Synlett*, 1999, **1999**, 930–934.

32 S. Kitani, K. Sugawara, K. Tsutsumi, T. Morimoto and K. Kakiuchi, *Chem. Commun.*, 2008, **336**, 2103.

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