

### Bromo- and thiomaleimides as a new class of thiol-mediated fluorescence 'turn-on' reagents†

Cite this: *Org. Biomol. Chem.*, 2014, **12**, 557

Received 28th October 2013,  
Accepted 25th November 2013

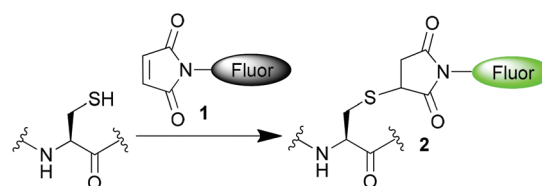
DOI: 10.1039/c3ob42141d

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Judith Youziel,<sup>a</sup> Ahmed R. Akhbar,<sup>a</sup> Qadeer Aziz,<sup>b</sup> Mark E. B. Smith,<sup>a</sup>  
Stephen Caddick,<sup>a</sup> Andrew Tinker<sup>b</sup> and James R. Baker<sup>\*a</sup>

Bromo- and thiomaleimides are shown to serve as highly effective quenchers of a covalently attached fluorophore. Reactions with thiols that lead to removal of the maleimide conjugation, or detachment of the fluorophore from the maleimide, result in 'turn-on' of the fluorescence. These reagents thus offer opportunities in thiol sensing and intracellular reporting.

Chemical reagents that undergo rapid and selective reactions with thiols are of widespread use in the construction of bio-conjugates and in the study of biological systems. They enable applications from site-selective protein modification<sup>1</sup> to the detection of small molecule thiols that play a key role in controlling the redox environment in cells.<sup>2</sup> A variety of electrophilic reagents have been developed in order to take advantage of the nucleophilic characteristics of thiols, and of these the maleimide motif **1** remains one of the most widely employed. Maleimides undergo rapid and highly selective conjugate additions with thiols, affording succinimide products **2**. Maleimides have been exploited as components in a class of 'turn-on' fluorophores for thiol detection.<sup>3</sup> In these reagents the maleimide serves to quench the attached fluorophore, attributed mechanistically to photo-induced electron transfer (PET) or intramolecular charge transfer (ICT) from the excited fluorophore to the quencher.<sup>4</sup> Once a thiol undergoes 1,4-addition to the maleimide **1** to generate the succinimide product **2** the conjugation is lost, and the product is no longer able to serve as an effective quencher, leading to a 'turn-on' of the fluorescence (Scheme 1). Such maleimide-fluorophore constructs have been employed in the detection and quantification of biological thiols such as cysteine, homocysteine and glutathione.<sup>3a</sup> In an elegant application by Keillor and co-workers dimaleimide fluorogens have been shown to require the addition of two thiols to 'turn-on' the fluorescence. They



**Scheme 1** Maleimide fluorogens which light up upon reaction with a thiol.

showed that such systems could be employed to selectively turn-on upon reaction with dithiols; and thus to selectively fluoresce upon reaction with two cysteines placed in close proximity.<sup>4b,5</sup>

We have recently reported on applications of a new class of maleimide reagents which contain a leaving group on one, or both, of the 3- and 4-positions of the maleimide.<sup>6</sup> Upon addition of a thiol these reagents undergo an addition-elimination reaction which leads to regeneration of the conjugated double bond and thus thiomaleimide products. Diverse applications of these reagents have been demonstrated; in reversible cysteine labelling, disulfide bridging, the construction of peptide and antibody conjugates, cytoplasmic cleaving protein conjugates and as reactive handles in polymers.<sup>6,7</sup>

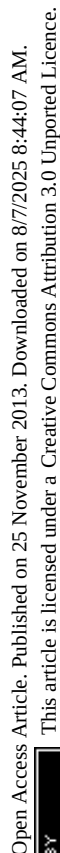
We envisaged further prospective applications of these reagents if it could be demonstrated that they quench attached fluorophores in a manner similar to the simple maleimide motif. For example bromomaleimide-fluorophore **3** would thus be 'dark' as the maleimide motif would serve to quench the attached fluorophore (Scheme 2a). Then upon addition of a single thiol the result would be a thiomaleimide **4** and thus still a dark conjugate. This conjugate would only 'turn-on' upon addition of a second thiol to afford succinimide **5**, and thus could serve as a complementary approach to that of Keillor and co-workers in fluorescently labelling dithiols in a selective manner.<sup>4b,5</sup> Alternatively the fluorophore could be present as a thioether in 'dark' thiomaleimide **6**, which upon encountering thiols would then light up following cleavage of the conjugate (Scheme 2b). If successful this method could be

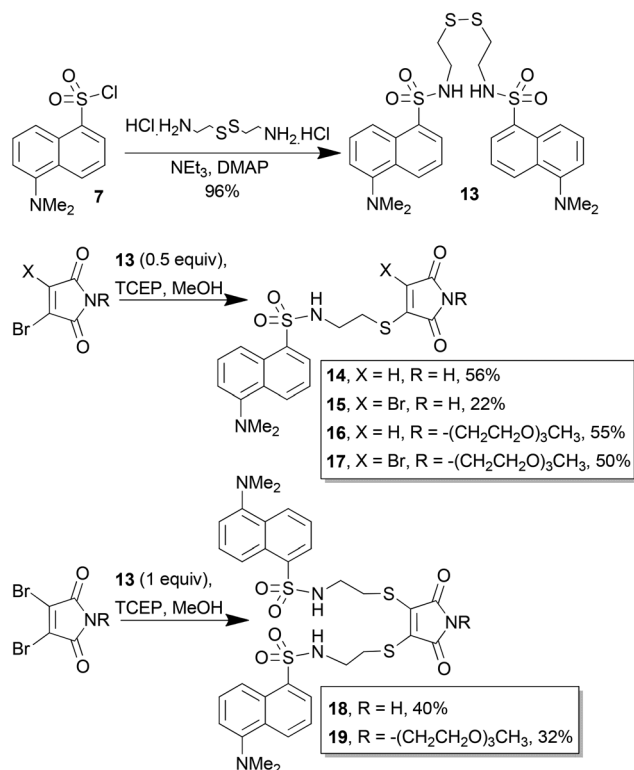
<sup>a</sup>Department of Chemistry, University College London, 20 Gordon St, London, UK.  
E-mail: j.r.baker@ucl.ac.uk; Tel: +44 (0)2076792653

<sup>b</sup>Barts and The London, Queen Mary's School of Medicine and Dentistry,  
Charterhouse Square, London, UK

† Electronic supplementary information (ESI) available: Experimental procedures and full spectroscopic data are available. See DOI: 10.1039/c3ob42141d







Scheme 4 Synthesis of dansyl-thiomaleimides 14–19.

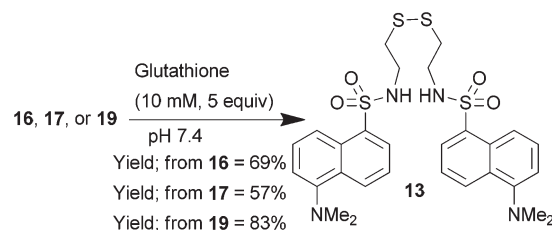
Once again the maleimide served as a highly effective quencher of the attached fluorophore (compounds 14–19, Table 2). With the dansyl group now attached *via* a thioether bond to the maleimide core, we envisaged that addition of an excess of a thiol would lead to cleavage of the fluorophore from the maleimide, *via* thiol exchange reactions. We have previously shown that thiomaleimides are sensitive to cleavage by millimolar concentrations of glutathione, a thiol that is present in such concentrations in the cytoplasm of cells.<sup>6b,h</sup> Thus we hypothesised that these quenched thiomaleimide constructs should fluoresce upon internalisation into cells.

To test whether glutathione was able to cleave these constructs it was necessary to dissolve the dansyl-thiomaleimides in an aqueous buffer; however it became apparent that compounds 14, 15 and 18 were highly insoluble. The incorporation

Table 2 Fluorescence quantum yields of compounds 13–19

| Compound    | $\epsilon_{\max}^a$ ( $M^{-1} \text{ cm}^{-1}$ ) | $\lambda_{\text{em}}$ (nm) | $\Phi_{\text{fl}}^b$ |
|-------------|--------------------------------------------------|----------------------------|----------------------|
| Dansylamide | 5546                                             | 511                        | 0.37 <sup>c</sup>    |
| 13          | 4458                                             | 516                        | 0.32                 |
| 14          | 3628                                             | 418                        | 0.021                |
| 15          | 3512                                             | 420                        | 0.033                |
| 16          | 1347                                             | 522                        | 0.037                |
| 17          | 2117                                             | 513                        | 0.030                |
| 18          | 4752                                             | 429                        | 0.016                |
| 19          | 3490                                             | 426                        | 0.026                |

<sup>a</sup> Measured at 335 nm, 25 °C. <sup>b</sup> Quantum yields were calculated according to the method shown in ref. 10. <sup>c</sup> Value taken from ref. 11.



Scheme 5 Glutathione mediated cleavage of dansylthiomaleimides 16, 17, and 19.

of the OEG group on the maleimide (compounds 16, 17, and 19) was thus essential to aid solubility to enable further experiments in aqueous systems. Treatment of dansylthiomaleimide 16, dansylbromothiomaleimide 17 and didansylthiomaleimide 19 with glutathione led to release of the dansylthiol which was recovered in the form of the oxidised fluorescent disulfide 13 (Scheme 5). LCMS analysis also indicated the presence of the glutathione-maleimide by-products of this reaction. These results confirmed that glutathione is effective at cleaving dansylthiomaleimides *in vitro*.

To test whether dansyl-maleimides could potentially be employed as reporter molecules in biological applications we carried out a study on live cells, focusing our efforts on a comparison between dansyldisulfide 13 and didansylthiomaleimide 19. HEK cells were thus incubated in solutions of the dansyldisulfide 13 (Fig. 1, A) or didansylthiomaleimide 19 (Fig. 1, C) before irradiation ( $\lambda = 335 \text{ nm}$ ) and observation under a microscope. In each case a control reaction was also performed in which the cells were pre-treated with *N*-methylmaleimide<sup>12</sup> to reduce the active intracellular concentration of GSH (Fig. 1, B and D respectively). Dansyldisulfide 13 was

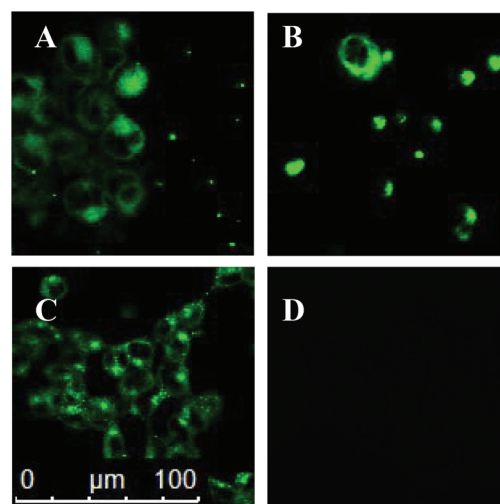


Fig. 1 Fluorescence images of HEK cells: (A) Incubated with dansyldisulfide 13 (10  $\mu\text{M}$ ) for 15 minutes at 37 °C; (B) cells pre-treated with *N*-methylmaleimide (1 mM) for 60 minutes at 37 °C, then incubated with dansyldisulfide 13 (10  $\mu\text{M}$ ) for 15 minutes at 37 °C. (C) Incubated with didansylmaleimide 19 (10  $\mu\text{M}$ ) for 15 minutes at 37 °C; (D) cells pre-treated with *N*-methylmaleimide (1 mM) for 60 minutes at 37 °C, then incubated with dansyldisulfide 19 (10  $\mu\text{M}$ ) for 15 minutes at 37 °C.



observed, as expected, to fluoresce both in the surrounding extracellular buffer as well as intracellularly. It was unaffected by pre-treatment with *N*-methylmaleimide, as it is not dependent on thiols to turn on its fluorescence. In contrast didansylthiomaleimide **19** only became fluorescent upon entering cells (no fluorescence observed extracellularly) and contacting the high concentration of free thiols. This turn-on of fluorescence was dramatically inhibited upon pretreatment with *N*-methylmaleimide. These experiments demonstrate the potential of thiomaleimides for intracellular thiol detection and as prospective reporters for the intracellular release of thiol cargos from thiomaleimide scaffolds.

## Conclusions

In conclusion we have shown that bromo- and thiomaleimide fluorogens represent a new class of reagents which are turned-on upon treatment with thiols. The fluorophore can either be connected to the nitrogen of the maleimide or *via* a cleavable thioether bond, offering a versatile platform for selective thiol sensing and reporting on intracellular delivery.

## Acknowledgements

The authors gratefully acknowledge RCUK, EPSRC, MRC, BBSRC and UCL for supporting this work, Dr Abil Aliev for NMR technical support, and Christopher Thrasivoulou for assistance with the cellular imaging.

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