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A cyclopropene electrophile that targets glutathione S-transferase omega-1 in cells

Gustav J. Wørmer^{[a],+}, Bente K. Hansen^{[a],+}, Johan Palmfeldt^[b], Thomas B. Poulsen^{[a],*}

[a] Department of Chemistry, Aarhus University, Langelandsgade 140, 8000 Aarhus C, Denmark

[b] Department of Clinical Medicine – Research Unit for Molecular Medicine, Aarhus University hospital, Palle Juul-Jensens Boulevard 82, 8200 Aarhus N, Denmark

[+] These authors contributed equally to this work

* E-mail: thpou@chem.au.dk

Abstract.

Cyclopropenes are an important new addition to the portfolio of functional groups that can be used for bioorthogonal couplings. The inert nature of these highly strained compounds in complex biological systems is almost counterintuitive given their established electrophilic properties in organic synthesis. Here we provide the first demonstration of a cyclopropene that is capable of direct conjugation to protein targets in cells and show that this compound preferentially alkylates the active site cysteine of glutathione S-transferase omega-1 (GSTO1).

Small electrophilic molecules intersect several areas of contemporary chemical biology and biomedicine. They are used as warheads in targeted covalent inhibitors,¹ enable bioconjugation to native proteins through nucleophilic amino acid residues,² and are used for activity-based³ or reactivity-based⁴ protein profiling methods. Recently, collections of small electrophiles have been used in diverse experimental settings from forward chemical genetic screens resulting in the identification – facilitated by the covalent interaction⁵ – of novel cancer targets⁶ to the mapping of proteomic targets of environmental chemicals.⁷ The identification and study of new and unusual electrophilic motifs can therefore have broad impact and is a focus-area for our lab.⁸

Strained carbocyclic ring-systems could potentially be reactive towards biological nucleophiles, but such compounds have so far found their main applications within bioorthogonal chemistry,⁹ which – paradoxically – demand them to be biologically inert. Cyclopropenes have e.g. been shown to undergo rapid coupling with tetrazines¹⁰ and with nitrile imines (1,3-dipolar photoclick reaction).¹¹ Due to the exceptionally high ring-strain in cyclopropenes (>50 kcal/mol)¹², and their established use as electrophiles in synthesis, it is rather surprising that this motif can be biochemically stable, however, both the stability and associated reactivity in tetrazine ligations are strongly modulated by the substituents present on the cyclopropene ring (Figure 1A,B). For instance, 1,2-disubstituted cyclopropenes are present in some natural products, underlining the stability of this particular substitution pattern, whereas unsubstituted cyclopropenes, with few exceptions, degrade rapidly.¹³ The majority of “bioorthogonal cyclopropenes” contain a stabilizing alkyl-substituent at C1, and to achieve fast conjugation kinetics, the linkage through C3 should minimize steric repulsion (3-monosubstituted or 3,3-spiro system) (Figure 1A,B).¹³ In synthesis, cyclopropenes are used as dichotomic electrophiles undergoing either direct addition of nucleophiles to the cyclopropene double bond (π^* -orbital) or – more rarely – fragmentation of the ring (σ^* -orbital) (Figure 1B).¹⁴ Both of these processes alleviate the ring-strain. The use of cyclopropenes as biological electrophiles has been suggested,^{15,16} but, to the best of our knowledge, never demonstrated in cellular systems. Herein, we report the discovery and characterization of such a cyclopropene electrophile that targets and inhibits glutathione S-transferase omega-1 (GSTO1) in cancer cells.

We prepared a small collection of electron-deficient cyclopropenes (**2-4**) that we hypothesized would display enhanced electrophilic reactivity compared to typical “bioorthogonal-cyclopropenes” (Figure 1A, C). For preparation of these compounds we developed highly effective catalytic conditions (0.1% $\text{Rh}_2(\text{esp})_2$, 5 equiv. TMS-propyne) for the reaction of dimethyl α -diazo-malonate with TMS-propyne to provide cyclopropene methyl ester **1** following mono-hydrolysis. (Figure 1C).

Figure 1. (A) Examples of cyclopropenes used in bioorthogonal reactions. (B) Characteristics and reactivities of cyclopropenes and schematic of anti-bonding orbitals. (C) Syntheses of electrophilic cyclopropenes.

From **1**, we could either prepare the propargyl ester (**2**), the corresponding amide (**3**) or, *via* a Curtius rearrangement, the propargyl carbamate (**4**).¹⁷ With these compounds in hand we evaluated their potential as biological electrophiles by incubating HCT-116 colon carcinoma cells overnight with a concentration series of the compounds followed by copper-catalyzed azide-alkyne cycloaddition (CuAAC) with rhodamine-azide and in-gel fluorescence scanning to identify potential protein conjugates (Figure 2). The carbamate (**4**) afforded no labeling and although we are not aware of the use of this particular motif for bioorthogonal reactions, the lack of proteomic reactivity in this experiment suggest that such a use can be considered. Contrary to the carbamate, the mixed diester (**2**) did indeed display clear labeling of proteins, and we were intrigued by the observations that a band at approximately 27 kDa appeared to be preferentially labeled at concentrations from 4-100 μ M. Interestingly, a minor change of the propargyl ester to the corresponding amide (**3**) results in strongly reduced protein labeling. We also tested labeling by **2** in MDA-MB-231 breast cancer cells and observed a similar pattern to that observed in HCT-116 cells (Supporting Information, Figure S1). Curiously, the 27 kDa band was not observed when **2** was incubated with pre-made HCT-116 cell lysate suggesting that the observed cellular binding target is either not accessible to the probe or degraded during cell lysis (Figure S2). Moving back to intact cells, we next tested a probe with a reduced cyclopropene double bond (**5**, see Supporting Information for details on preparation) which completely abolished labeling and thereby strongly implicates the cyclopropene ring as the reactive component (Figure S3). A series of control experiments showed that probes **2-5** were stable to both acidic and neutral conditions *in vitro* (14 h) but under basic conditions (pH = 10) partial decomposition was observed, presumably due to hydrolytic cleavage of the ester groups

(Figure S4). In cell culture medium, probe **2** (500 μ M) displayed a ca. 25% reduction in concentration over 16 hours which could be at least partially attributed to albumin-conjugation (Figure S5). Finally, exposure of HCT-116 cells to probe **2** (500 μ M) resulted in a ca. 50% loss of cell viability (24 h) whereas lower concentrations of **2** and probes **3-5** (up to 500 μ M) had no effect on viability (Figure S6).

Figure 2. In-gel fluorescence scanning of lysates from HCT-116 cells treated with alkyne-tagged probes **2-4** for 14 h. Following lysis, CuAAC to rhodamine- N_3 was performed before SDS-PAGE. Gels are from a representative experiment.

To identify the 27 kDa primary binding protein of probe **2**, a larger scale experiment with 100 μ M probe and higher amounts of live HCT-116 cells was performed, followed by affinity purification by CuAAC-coupling of a biotinylated linker and pulldown using magnetic streptavidin beads (Figure 3A). A DMSO-treated sample was processed in equivalent manner (Figure 3A,B).

The dominating protein band from the SDS-PAGE was excised (approx. 25-30 kDa) and in-gel trypsin digested for detailed untargeted nanoLC-MS analysis. Database search of the identified peptides revealed glutathione S-transferase omega-1, GSTO1 (gene name *GSTO1*), as the most dominating hit with the highest number of scans and 47% sequence coverage. GSTO1 mediates deglutathionylation of various target proteins and has been subject to significant interest for inhibitor development with chloroacetamide-derivatives as a major inhibitor class targeting the active-site cysteine residue (Cys32).^{5,18,19}

Figure 3. Cyclopropene **2** alkylates GSTO1 at cysteine-32 in cells. (A) Flowchart for affinity pulldown experiment. (B) Coomassie-stained gel of eluate from streptavidin beads, # indicates BSA, * indicates streptavidin, 27 kDa band is marked with arrow. (C) MS-fragment spectrum of a GSTO1 tryptic peptide containing a modification at Cys32 derived from probe **2** indicated by a2 and b2-ions (red). Hydrolytic cleavage of the ester is likely to be a result of the conditions used for proteomic analysis, see also Figure S4. (D) siRNA knockdown of GSTO1 reduces labeling of the 27 kDa band by probe **2**.

Mining of the LC-MS/MS data indeed revealed that peptides with masses corresponding to a cyclopropene-derived modification on Cys32 could be observed (Figure 3C and Figure S7), suggesting that the probe would inhibit the catalytic function of GSTO1. To validate GSTO1 as the cellular binding protein, we next silenced its expression in HCT-116 cells using siRNA (Smartpool) and exposed these cells to probe **2**. The knockdown cells maintained full viability but the labeling of the 27 kDa protein with probe **2** was reduced >70% (Figure 3D). These data demonstrate that probe **2** binds GSTO1 in cells. From the gel-based analyses, GSTO1 appeared to be the main cellular binding protein for probe **2** at concentrations up to 100 μ M (Figure 2 and Figure S1), but additional covalent targets were also observed. To provide a complete overview, we conducted an additional pulldown experiment using both 25 μ M and 100 μ M **2** followed by label-free nanoLC-MS/MS analysis on a high sensitivity mass spectrometer (Q Exactive HF-X, Thermo). More than hundred proteins were identified in these samples, and many proteins were also identified to be unspecifically bound to the beads (Supporting Information, Table S1). The ten proteins with most pronounced intensities and enrichment in probe **2** samples are depicted in Figure S8. GSTO1 was clearly the most high-intensity protein, with more than 100 times higher intensity than the median of the 37 proteins that were enriched >10 times in the comparison of the samples from 100 μ M **2** versus negative controls (DMSO). The other proteins in Figure S8 had molecular weights in the range 30-72 kDa, with 4 proteins (all members of the protein disulfide isomerase (PDI) family) in the range 47-57 kDa, which is in line with protein bands seen in Figure 2. For detailed protein data see Supporting Information, Table S1. Finally, we addressed the observation suggested from the LC-MS/MS data that **2** targeted the catalytic cysteine residue. Towards this end, we exploited the known specific activity of GSTO1 to function as a S-(phenacyl)-glutathione reductase and prepared S-(4-Nitrophenacyl)glutathione (4-NPG) whose conversion to 4-nitroacetophenone can be specifically measured by absorbance at 305 nm (Figure 4A).²⁰

Figure 4. Cyclopropene **2** inhibits the catalytic activity of GSTO1 in cells. (A) Experimental setup. (B) GSTO1 enzymatic activity was measured in lysate from HCT-116 cells treated with cyclopropene **2** or **5** for 14 h. Cyclopropene **5** is inactive. (C) siRNA knockdown of GSTO1 reduces enzymatic activity. Data is mean $\pm$ s.d. ($N=3$). Curves are from a representative experiment.

In cell lysate, GSTO1 is inactive, presumably due to disulfide formation, but addition of a reducing agent, TCEP, activates the catalytic activity (Figure 4A).²¹ We then exposed HCT-116 cells to probe **2** at different concentrations overnight, lysed the cells, added TCEP, and measured GSTO1 activity, which revealed dose-dependent inhibition of catalytic activity (Figure 4B). As expected, cyclopropane **5** was inactive in this experiment (Figure 4B) which is in accordance with the lack of labeling of GSTO1 in cells, and an optimized GSTO1 inhibitor, ML175, was active (Figure S9). In comparison, siRNA-knockdown reduced the GSTO1 activity by 75% (Figure 4C). Cyclopropene **2** could also inhibit the activity of recombinant GSTO1 (Figure S10). Collectively, these data demonstrate that cyclopropene **2** inhibits the catalytic function of GSTO1 in cells by direct alkylation of Cys32 through the cyclopropene ring.

To understand the fundamental reactivity of probe **2**, we conducted a series of *in vitro* reactions with thiols (Figure 5). We initially hypothesized that thiol-addition to the cyclopropene double bond would predominate and result in formation of the *trans*-cyclopropane.²² To our surprise, however, the structure formed from the reaction between 4-methylthiophenol (**6**) and **2** in the presence of DBU in CH₃CN was clearly incompatible with this scenario (Figure 5A). Rigorous purification and spectroscopic analysis instead revealed the major product (53% isolated) to be vinyl sulfide **7**, resulting from π -bond cleavage of the cyclopropene. As a minor component, we could isolate Knoevenagel product **8** which are likely formed from isomerization of **7**. Compound **7** was found to be stable under both basic and acidic conditions (Figure S4). σ -bond cleavage reactions of cyclopropenes were reported previously²³, but not uncatalyzed with thiol-nucleophiles. Reaction of probe **2** with **6** was also observed with catalytic DBU and, in accord with the labeling experiments in cells, carbamate **4** was unreactive under these conditions, whereas probe **3** also underwent σ -bond cleavage (Figure S11).

Figure 5. Reactivity of probes **2-5** with thiols *in vitro*. (A) Preparative reaction of probe **2** with thiol **6** in the presence of stoichiometric DBU. (B) Reaction of probes (7.4 mM) with GSH (7.4 mM) under buffered aqueous conditions at ambient temperature for 14 hours monitored by ¹H NMR spectroscopy. Structure of GSH-conjugate **10** was determined by NMR and LC-MS/MS (see Supporting Information for synthesis and isolation).

We also conducted experiments with N-acetylcysteamine (**9**), which is a weaker nucleophile compared to **6**, and interestingly found that σ -bond cleavage of probe **2** could be induced but demanded the presence of metal-ions such as MgCl_2 (Figure S11). Finally, we investigated the reactivity of the cyclopropenes with reduced glutathione (GSH) under different buffered aqueous conditions using ^1H NMR-spectroscopy where conversion was only observed at pH = 9.9 for **2** and **3** (+) while **4** and **5** remained intact (-) at the studied pH (Figure 5B). Subsequent purification and characterization of the glutathione-conjugate (**10**) derived from probe **2** again demonstrated that the cyclopropene ring had opened (Figure 5B). Although the specific reactivity of probe **2** involved in the labeling of cellular proteins, GSTO1 in particular, cannot be inferred from these different model reactions, we speculate that σ -bond cleavage represent the major reactivity towards cellular thiols.

In conclusion, we have reported the first example of a cyclopropene-based electrophilic probe that covalently labels cellular proteins and shown that it can be used to modify the catalytic cysteine (Cys32) in GSTO1. Our data formally suggests that cyclopropenes should not blindly be assumed as bioorthogonal although it is likely that two electron-withdrawing substituents are required for electrophilic reactivity in cells, which is e.g. demonstrated by the strongly diminished reactivity of probes **3** and **4**. The cyclopropenes join a collection of structurally unusual electrophilic probes^{8a-c} that have been discovered in recent years. We hypothesize that several opportunities exist to further tune the reactivity of these activated cyclopropenes and that these motifs may also be embedded in more complex scaffolds. Collectively, this may further broaden the use of cyclopropenes as biological electrophiles.

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References

- [1] M. Gehringer, S. A. Laufer, *J. Med. Chem.* **2019**, *62*, 5673-5724.
- [2] (a) S. R. Adusumalli, D. G. Rawale, U. Singh, P. Tripathi, R. Paul, N. Kalra, R. K. Mishra, S. Shukla, V. Rai, *J. Am. Chem. Soc.* **2018**, *140*, 15114–15123; (b) E. A. Hoyt, P. M. S. D. Cal, B. L. Oliveira, G. J. L. Bernardes, *Nat. Rev. Chem.* **2019**, *3*, 147-171.
- [3] (a) Y. Liu, M. P. Patricelli, B. F. Cravatt, *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14694–14699; (b) D. K. Nomura, M. M. Dix, B. F. Cravatt, *Nat. Rev. Cancer*, **2010**, *10*, 630-638.
- [4] E. Weerapana, C. Wang, G. M. Simon, F. Richter, S. Khare, M. B. D. Dillon, D. A. Bachovchin, K. Mowen, D. Baker, B. F. Cravatt, *Nature* **2010**, *468*, 790–797.
- [5] K. M. Backus, B. E. Correia, K. M. Lum, S. Forli, B. D. Horning, G. E. González-Páez, S. Chatterjee, B. R. Lanning, J. R. Teijaro, A. J. Olson, D. W. Wolan, B. F. Cravatt, *Nature* **2016**, *534*, 570–574.
- [6] (a) K. E. Anderson, M. To, J. A. Olzmann, D. K. Nomura DK, *ACS Chem. Biol.* **2017**, *12*, 2522-2528; (b) L. A. Bateman, T. B. Nguyen, A. M. Roberts, D. K. Miyamoto, W. M. Ku, T. R. Huffman, Y. Petri, M. J. Heslin, C. M. Contreras, C. F. Skibola, J. A. Olzmann, D. K. Nomura, *Chem. Commun.* **2017**, *53*, 7234–7237.

- [7] D. Medina-Cleghorn, L. A. Bateman, B. Ford, A. Heslin, K. J. Fisher, E. D Dalvie, D. K. Nomura, *Chem. Biol.* **2015**, *22*, 1394-1405.
- [8] For recent examples, see e.g.: (a) Y. Qian, M. Schürmann, P. Janning, C. Hedberg, H. Waldmann, *Angew. Chem. Int. Ed.* **2016**, *55*, 7766-7771; *Angew. Chem.* **2016**, *128*, 7897-7902; (b) M. J. Matos, B. L. Oliveira, N. Martínez-Sáez, A. Guerreiro, P. M. S. D. Cal, J. Bertoldo, M. Maneiro, E. Perkins, J. Howard, M. J. Deery, J. M. Chalker, F. Corzana, G. Jiménez-Osés, G. J. L. Bernardes, *J. Am. Chem. Soc.* **2018**, *140*, 4004–4017; (c) B. Bernardim, P. M. S. D. Cal, M. J. Matos, B. L. Oliveira, N. Martínez-Sáez, I. S. Albuquerque, E. Perkins, F. Corzana, A. C.B. Burtoloso, G. Jiménez-Osés, G. J. L. Bernardes, *Nat. Commun.* **2016**, *7*, 13128 (d) B. K. Hansen, C. J. Loveridge, S. Thyssen, G. J. Wørmer, A. D. Nielsen, J. Palmfeldt, M. Johannsen, T. B. Poulsen, *Angew. Chem. Int. Ed.* **2019**, *58*, 3533-3537; *Angew. Chem.* **2019**, *131*, 3571-3575; e) L. L. Clement, M. Tsakos, E. S. Schaffert, C. Scavenius, J. J. Enghild, T. B. Poulsen, *Chem. Commun.* **2015**, *51*, 12427–12430; f) M. Tsakos, L. L. Clement, E. S. Schaffert, F. N. Olsen, S. Rupiani, R. Djurhuus, W. Yu, K. M. Jacobsen, N. L. Villadsen, T. B. Poulsen, *Angew. Chem. Int. Ed.* **2016**, *55*, 1030–1035; *Angew. Chem.* **2016**, *128*, 1042-1047; g) N. L. Villadsen, K. M. Jacobsen, U. B. Keiding, E. T. Weibel, B. Christiansen, T. Vosegaard, M. Bjerring, F. Jensen, M. Johannsen, T. Tørring, et al., *Nat. Chem.* **2017**, *9*, 264–272.
- [9] (a) N. K. Devaraj, *ACS Cent. Sci.* **2018**, *4*, 952-959; (b) D. M. Patterson, L. A. Nazarova, J. A. Prescher, *J. A. ACS Chem. Biol.* **2014**, *9*, 592-605.
- [10] (a) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, *J. Am. Chem. Soc.* **2012**, *134*, 18638-18643; (b) J. Yang, J. Ščkute, C. M. Cole, N. K. Devaraj, *Angew. Chem., Int. Ed.* **2012**, *51*, 7476-7479; *Angew. Chem.* **2012**, *124*, 7594-7597.
- [11] Z. Yu, Y. Pan, Z. Wang, J. Wang, Q. Lin, *Angew. Chem., Int. Ed.* **2012**, *51*, 10600-10604; *Angew. Chem.* **2012**, *124*, 10752-10756.
- [12] R. D. Bach, O. Dmitrenko, *J. Am. Chem. Soc.* **2004**, *126*, 4444-4452.
- [13] J. M. J. M. Ravasco, C. M. Monteiro, A. F. Trindade, *Org. Chem. Front.* **2017**, *4*, 1167-1198.
- [14] (a) A. Edwards, M. Rubina, M. Rubin, *Curr. Org. Chem.* **2016**, *20*, 1862-1877; (b) Z.-B. Zhu, Y. Wei, M. Shi, *Chem. Soc. Rev.* **2011**, *40*, 5534-5563.
- [15] (a) G. Triola, G. Fabriàs, A. Llebaria, *Angew. Chem. Int. Ed.* **2001**, *40*, 1960-1962; *Angew. Chem.* **2001**, *113*, 2014-2016; (b) J. Quintana, M. Barrot, G. Fabriàs, F. A. Camps, *Tetrahedron*, **1998**, 10187-10198; (c) M. Matsuura, Y. Saikawa, K. Inui, K. Nakae, M. Igarashi, K. Hashimoto, M. Nakata, *Nat. Chem. Biol.* **2009**, *5*, 465-467.
- [16] (a) P. N. Gurjar, A. P. Bercz, T. N. Nguyen, A. K. Vadukoot, V. A. Litosh, *Univ. J. Chem.* **2015**, *3*, 16-26; (b) N. J. Smith, K. Rohlfing, L. A. Sawicki, P. M. Kharkar, S. J. Boyd, A. M. Kloxin, J. M. Fox, *Org. Biomol. Chem.* **2018**, *16*, 2164-2169.
- [17] (a) T. N. Wheeler, J. Ray, *J. Org. Chem.* **1987**, *52*, 4875-4877; (b) V. Tarwade, O. Dmitrenko, R. D. Bach, J. M. Fox, *J. Org. Chem.* **2008**, *73*, 8189-8197; for use of Rh2(esp)2 for cyclopropenation, see: (c) B. Morandi, E. M. Carreira, *Angew. Chem. Int. Ed.* **2010**, *49*, 4294-4296; *Angew. Chem.* **2010**, *122*, 4390-4392.
- [18] see e.g.: (a) S. Piaggi, C. Raggi, A. Corti, E. Pitzalis, M. C. Mascherpa, M. Saviozzi, A. Pompella, A. F. Casini, *A. F. Carcinogenesis* **2010**, *31*, 804; (b) D. Menon, A. Innes, A. J. Oakley, J. E. Dahlstrom, L. M. Jensen, A. Brüstle, P. Tummala, M. Rooke, M. G. Casarotto, J. B. Baell, N. Nguyen, Y. Xie, M. Cuellar, J. Strasser, J. L. Dahlin, M. A. Walters, G. Burgio, L. A. J. O'Neill, P. G. Board, *Sci. Rep.* **2017**, *7*, 17832.

- [19] (a) Y. Xie, J. L. Dahlin, A. J. Oakley, M. G. Casarotto, P. G. Board, J. B. Baell, *J. Med. Chem.* **2018**, *61*, 7448-7470; (b) K. Tsuboi, D. A. Bachovchin, A. E. Speers, T. P. Spicer, V. Fernandez-Vega, P. Hodder, H. Rosen, B. F. Cravatt, *J. Am. Chem. Soc.* **2011**, *133*, 16605-16616.
- [20] P. G. Board, M. Coggan, J. Cappello, H. Zhou, A. J. Oakley, M. W. Anders, *Anal. Biochem.* **2008**, *374*, 25-30.
- [21] B. Némethi, M. Poór, Z. Gregus, *Z. Anal. Biochem.* 2015, *469*, 12-18.
- [22] X. Cui, X. Xu, H. Lu, S. Zhu, L. Wojtas, X. P. Zhang, *J. Am. Chem. Soc.* **2011**, *133*, 3304-3307.
- [23] (a) Y. Liu, S. Ma, *Chem. Sci.* **2011**, *2*, 811-814; (b) R. J. Mudd, P. C. Young, J. A. Jordan-Hore, G. M. Rosair, A.-L. Lee, *J. Org. Chem.* **2012**, *77*, 7633-7639.