

## Full Paper

## Synthesis of Arylazide- and Diazirine-Containing CrAsH-EDT<sub>2</sub> Photoaffinity Probes

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Two photo-crosslinking biarsenical (CrAsH-EDT<sub>2</sub>)-modified probes were synthesized that are expected to be useful tools for tetracysteine-labeled proteins to facilitate the co-affinity purification of their DNA binding sequences and interacting proteins. In addition, improvements for the synthesis of CrAsH-EDT<sub>2</sub> and *N*<sup>1</sup>-(4-azido-2-nitrophenyl)hexane-1,6-diamine are reported. Both photoprobes effectively entered HeLa cells (and the nucleus) and were dependent on the tetracysteine motif in recombinant DMRT1 (doublesex and Mab3-related transcription factor) to induce fluorescence, suggesting that their crosslinking abilities can be exploited for the identification of nucleic acids and proteins associated with a protein of interest.

**Keywords:** Biarsenical probe / Fluorescence / Photoaffinity probes / Tetracysteine-tagged recombinant DMRT1

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### Introduction

The ability to identify proteins that associate with DNA or other proteins in living cells is important for the discovery of signal modulation processes. Currently, available methods often rely on the interactions between DNA sequences and proteins, which are strong enough to survive cellular disruption and DNAase protection after protein binding or immunoprecipitation [1]. The availability of new chemical probes that can be used to identify the sites of DNA–protein interactions and protein–protein interactions when the interactions are weak would expand the range of capabilities

to identify new DNA sequences and protein–protein interactions that are impossible to identify using current methods. Such tool compounds need to be able to penetrate living cells, bind to a specific protein binding site, not disrupt protein function, and form a stable covalent link to the binding partner of the protein. The method developed by the Tsien group for site specific labeling of recombinant proteins that contain appropriately spaced tetracysteines, fulfills most of these conditions, but this molecular probe forms a reversible covalent bond rather than a stable covalent link [2]. We therefore decided to develop new reagents that could covalently link proteins with DNA or protein ligands and thereby render both transient and stable interactions into a coupled system, resistant to degradation during harsh isolation and identification procedures. We reasoned that the most effective way to accomplish our goal would be to tether a photactivatable group [3, 4] to the probe developed by Tsien. The modified proteins described in the method by Tsien have the general sequence Cys-Cys-Xaa-Xaa-Cys-Cys in which Xaa is an amino acid other than cysteine. Such

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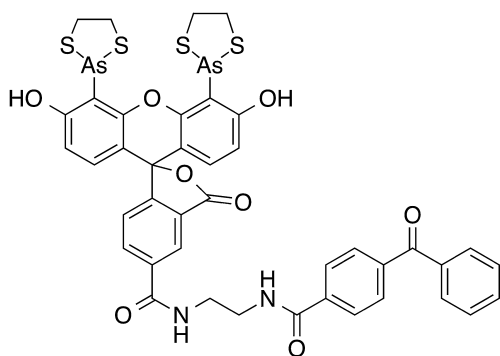
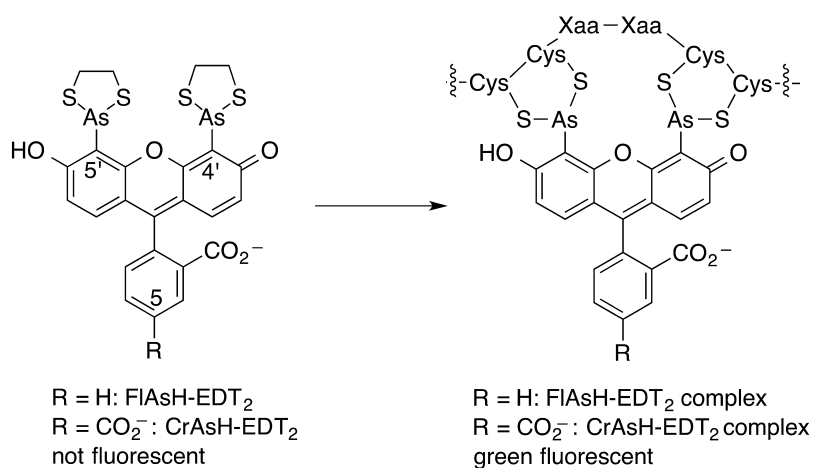
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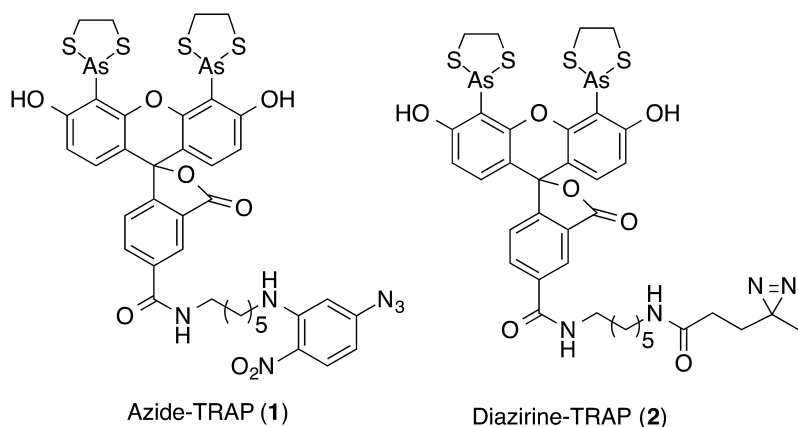
tetracysteine motifs are very rare in naturally occurring proteins, and therefore only the tagged recombinant protein is labeled with the membrane-permeable biarsenical probe, known as FIAsh-EDT<sub>2</sub> (fluorescein arsenical helix binder), which is a fluorescein containing 4',5'-di(1,3,2-dithiarsolan-2-yl) groups (Fig. 1). When FIAsh is bound to two ethane-dithiol (EDT) molecules, its fluorescence is almost completely quenched but on reaction with a tetracysteine peptide

a strongly green fluorescent complex is formed. This feature is particularly useful when labeling cells expressing tetracysteine-tagged proteins.

As indicated above, identifying molecular binding partners of proteins is a challenge in a complex cellular environment. In order to improve specificity and minimize disruption or reorganization of binding partners, it would be advantageous to employ a technique that can tag one or more of the



Benzophenone photoprobe TRAP



**Figure 1.** Structures of non-fluorescent FIAsh-EDT<sub>2</sub> and CrAsH-EDT<sub>2</sub> and green fluorescent FIAsh-EDT<sub>2</sub> and CrAsH-EDT<sub>2</sub> complexed with Cys-Cys-Xaa-Xaa-Cys-Cys and structures of TRAP, azide-TRAP (1) and diazirine-TRAP (2) photo-crosslinking biarsenical probes.

partners *in situ*. Use of a CrAsH-EDT<sub>2</sub> (carboxy-FIAsH-EDT<sub>2</sub>) probe modified with photoaffinity labeling functionality spatially restricts affinity-labeled molecules to close vicinity of the tetracysteine-tagged recombinant protein. Photoaffinity crosslinking is performed in living cells and the modified protein, together with its cross-linked associates, is affinity purified, using an antibody to the modified protein. Alternatively, the photoaffinity-labeled binding partner could be retained on a tetracysteine-peptide modified resin, following exchange of the arsenical from the tetracysteine-tagged protein to the resin [5]. Captured DNA and RNA molecules can then be identified by sequence analysis to uncover transcription factor binding sites and RNA-binding protein transcripts, respectively. Captured proteins can be identified by mass spectrometry. The restricted vicinity of the crosslink and its stability will enhance specificity of the captured molecules and reveal more transient interactions. This, in turn, will improve the insight gained by such studies and facilitate understanding of important regulatory networks.

The synthesis of such a photoaffinity probe, targeted releasable affinity probe (TRAP; Fig. 1), has been reported by Yan et al. [5], in which a benzophenone moiety was linked to a bisarsenical fluorescein. The photoaffinity probe was used for capturing protein-binding partners in lysates, live bacteria and myocytes. Benzophenones are frequently used for crosslinking because they generate highly reactive diradicals that form covalent bonds in high yields. An advantage of this probe is that long wavelength can be used for irradiation, which are well tolerated by proteins. However, they do have some limitations. For example, they often require long irradiation times that can promote non-specific labeling [6], preferentially label hydrophobic amino acid residues [7], and often methionine [8]. Thus, labeling may not be optimal in all cases [9]. Therefore, it is desirable to have additional photoprobes available that act by crosslinking mechanisms that are different from benzophenone, are of different size, hydrophobicity, and linker length.

Benzophenones, aryl azides, and diazirines are the most commonly used probes in photoaffinity labeling studies [6, 10–12]. We therefore designed and prepared two new photoaffinity-labeled analogs, azide-TRAP (1) and diazirine-TRAP (2) of CrAsH-EDT<sub>2</sub>, which are shown in Fig. 1. Azide-TRAP (1) carries a phenylazide-based photo-crosslinker and diazirine-TRAP (2) carries a diazirin moiety at C5 of the benzoate moiety, which is remote from the biarsenical groups and should therefore not interfere with binding to the Cys-Cys-Xaa-Xaa-Cys-Cys sequence. In both probes, the linker at C5 is slightly longer than in the benzophenone-derived TRAP probe, which was designed to provide enhanced flexibility to interact with potential binding partners [13].

When exposed to UV light, the phenylazide forms a highly reactive nitrene species by loss of nitrogen [3, 4]. The nitrene can by itself react with a nucleophilic group or form a ring expanded seven-membered dehydroazepine that also can react with nucleophiles [3, 4]. Diazirine-TRAP (2) contains a

diazirine-based photo-crosslinker, which yields a reactive carbene species after irradiation as well as long-lived electrophilic diazo compounds [14, 15]. Diazirine-based photo-crosslinkers have better photostability than phenylazide-based photo-crosslinkers and are easily activated with long wave UV light and require only short irradiation times [14]. They also react with nucleophilic groups but in addition insert into C-H bonds to form a covalent bond with the target molecule [3, 4]. They are the smallest of the photoaffinity labels, which can be an advantage in labeling.

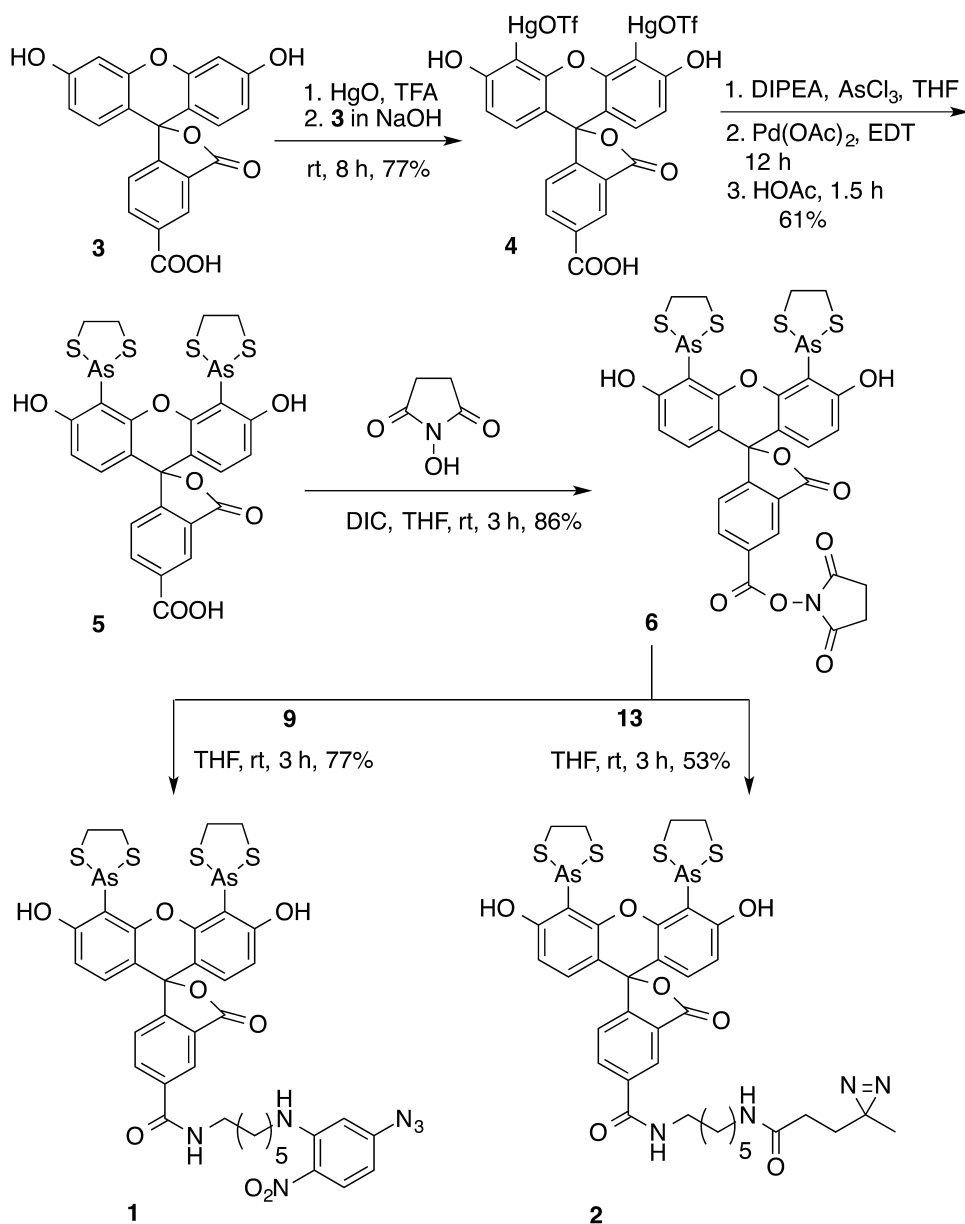
Based on their history, the photoaffinity moieties in azide-TRAP (1) and diazirine-TRAP (2) are expected to photo-crosslink DNA or proteins that bind specifically with the engineered-tagged protein of interest containing a tetracysteine tag as already demonstrated with the benzophenone-based TRAP reagent. Following photoactivation, the modified CrAsH-EDT<sub>2</sub> probes should cross-link DNA or proteins associated with the tagged protein, allowing identification of DNA regulatory sequences and protein partners. Consequently, azide-TRAP (1) and diazirine-TRAP (2) are expected to serve as additional tools for identifying protein–DNA and protein–protein interactions by eliminating non-specific noise associated with current methods and efficiently identifying novel binding partners for particular proteins of interest.

## Results and discussion

### Chemistry

The preparation of probes 1 and 2 was accomplished as depicted in Schemes 1–3. First, CrAsH-EDT<sub>2</sub> succinimide ester 6 was synthesized (Scheme 1) as a common precursor for the preparation of both probes 1 and 2. The synthesis of CrAsH-EDT<sub>2</sub> followed reported methods [16–21], and started with the mercuration of commercially available 5-carboxyfluorescein (3) using HgO in TFA to obtain bis-mercuric trifluoroacetate 4 in 77% yield, which was utilized for the next step without purification. Initial efforts using a palladium(II)-catalyzed transmetalation of 4 following a literature procedure [21] resulted in poor yields of intermediate 5 along with insoluble solid, presumably consisting of free arsenoxide, which is known to be prone to polymerization when concentrated. We subsequently found that the palladium(II)-catalyzed transmetalation of 4 with arsenic trichloride in THF as the solvent provided an unstable dichlorophenylarsine intermediate, which we directly converted to 5 in a one-flask procedure by adding EDT [22, 23] to furnish the CrAsH-EDT<sub>2</sub> (5) in 61% yield. Thus, the replacement of the NMP solvent with THF [24] as the solvent improved product yield (61%) compared to the previously reported yields of 20–41%. Next the carboxylic acid group in 5 was treated with *N*-hydroxy-succinimide and diisopropylcarbodiimide to provide the activated succinimide ester 6 in 86% yield.

The synthesis of phenylazide 9 was achieved in two steps from 4-fluoro-3-nitroaniline 7 (Scheme 2). Diazotization of 7



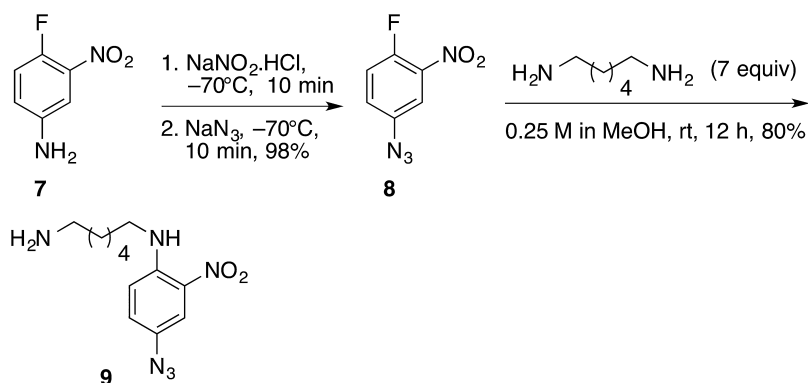
**Scheme 1.** Preparation of affinity probes azide-TRAP (**1**) and diazirine-TRAP (**2**).

followed by treatment with NaN<sub>3</sub> provided 4-azido-1-fluoro-2-nitrobenzene (**8**) in quantitative yield. Attempts to carry out a nucleophilic displacement of fluoride in **8** with hexamethylenediamine using a previously reported procedure [25] provided a mixture of **9** and bis-addition product *N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-azido-2-nitrophenyl)butane-1,4-diamine. In contrast, under dilute (0.25 M) conditions in MeOH the formation of only compound **9** was achieved in 80% yield.

The diazirine-based photo-crosslinker **13** (Scheme 3) was accessed from *N*-Boc hexamethylenediamine (**10**), which was

treated with commercially available diazirine *N*-hydroxy-succinimide ester **11** to furnish amide **12** in 97% yield. Exposure of **12** to TFA in CH<sub>2</sub>Cl<sub>2</sub> resulted in Boc deprotection to afford **13** in excellent yield, which was used without further purification for the synthesis of probe **2**.

Having assembled the required precursor CrAsH-EDT<sub>2</sub> succinimide ester **6** and two photo-crosslinker building blocks **9** and **13**, we accomplished the formation of the peptide bond between **6** and **9**, and **6** and **13** to prepare azide-TRAP (**1**) and diazirine-TRAP (**2**) in 77 and 53% yields, respectively (Scheme 1).



Scheme 2. Preparation of phenylazide 9.

## Biological activity

A transient transfection assay was used to determine if the new compounds could effectively enter the cell and specifically bind to proteins with the tetracysteine motif. The assay employed as a model, the transcription factor, doublesex, and Mab3-related transcription factor (DMRT1), modified at its amino terminus with an optimized biarsenical binding tetracysteine motif, FLNCCPGCCMEP (FLN-DMRT1) [26]. DMRT1 is a testis-specific transcription factor and member of a highly conserved family of proteins that contain a unique, signature DNA-binding motif, the DM domain, and often function in reproductive-related areas, such as sexual differentiation, gonad development, and sexual dimorphism [27]. The modified DMRT1 was cloned into the mammalian expression vector pcDNA3 (pc5'FLN-DMRT1) and transfected into HeLa cells, which were subsequently treated with FIAsh-EDT<sub>2</sub>, 1, or 2 and visualized for fluorescence. Treatment of pc5'FLN-DMRT1-transfected HeLa cells with FIAsh-EDT<sub>2</sub> showed many cells with strong fluorescence localized to the nucleus (Fig. 2A and D). Similarly, fluorescent nuclei were also observed in 5'FLN-DMRT1-transfected HeLa cells that were treated with 1 (Fig. 2B and E) or 2 (Fig. 2C and F). Cells were then incubated with either 250 mL of 0.45 mM FIAsh reagent,

0.45 mM probe 1, or 0.45 mM probe 2 in HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate and 3 mM 2-mercaptoethanol, BME for 30 min at 37°C in a cell culture incubator. Importantly, no fluorescence was observed in cells transfected with a vector expressing unmodified DMRT1 and treated with either 1 or 2. Thus, both 1 and 2 effectively enter the cell (and nucleus) and are dependent on the tetracysteine motif to induce fluorescence. The data suggest that the compound's crosslinking abilities can be exploited to facilitate identification of proteins and nucleic acids associated with a protein of interest.

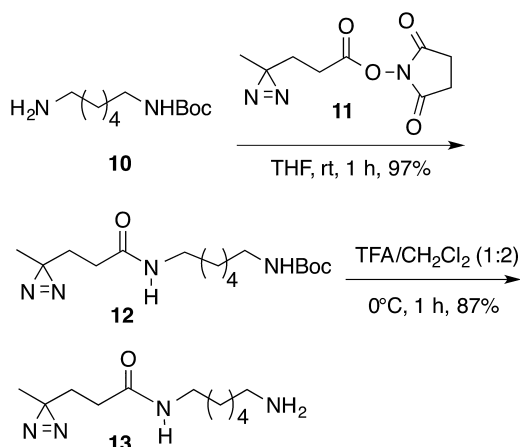
## Conclusion

We have reported the synthesis of two new photo-crosslinking biarsenical (CrAsH-EDT<sub>2</sub>) probes, one carries an aryl azide group and the other one a diazirine moiety. During their synthesis we were able to increase the yield of previously reported CrAsH-EDT<sub>2</sub> (5) to 61% by using NMP as the solvent instead of THF. This is a significant improvement over reported yields of 20–41%. Furthermore, we were able to increase the yield for known intermediate arylazide 9 to 80% yield under dilute reaction conditions, whereas the reaction under reported conditions resulted in a mixture of compound 9 and bis-addition product *N*<sup>1</sup>,*N*<sup>4</sup>-bis(4-azido-2-nitrophenyl)butane-1,4-diamine. We showed that the photoprobes enter cells and specifically bind to a protein with a tetracysteine motif. Therefore, the new photoprobes are expected to be useful tools for crosslinking DNA or proteins associated with the tagged protein, allowing identification of DNA regulatory sequences and protein partners.

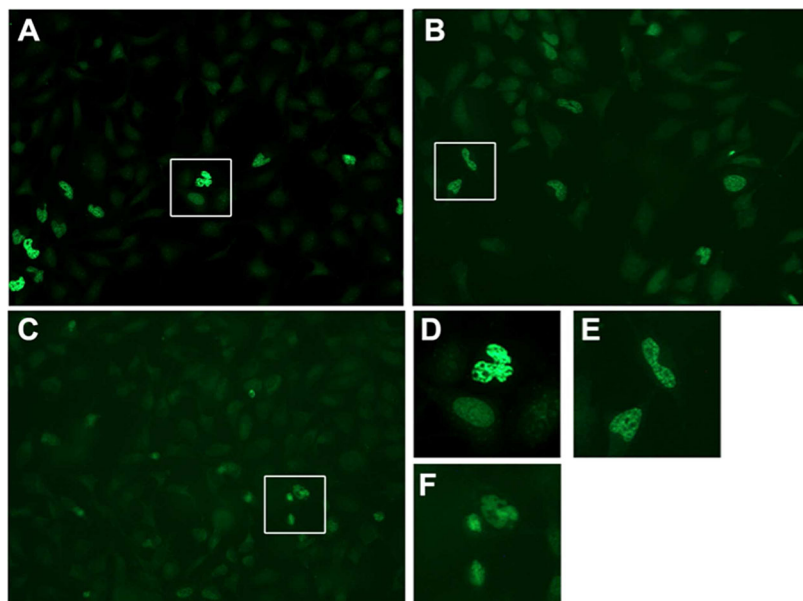
## Experimental

### Chemistry

All chemicals and reagents were purchased from commercial sources and used directly without further purification. Solvents were dried using standard procedures. All non-aqueous reactions were performed under an atmosphere of



Scheme 3. Synthesis of diazirin 13.



**Figure 2.** Live cell imaging with FIAsh-EDT<sub>2</sub>, azide-TRAP (1) and diazirine-TRAP (2). HeLa cells were transfected with an expression vector for DMRT1 containing the optimized biarsenical binding tetracysteine motif on its amino terminus (FLN-DMRT1). Cells were then treated with either FIAsh-EDT<sub>2</sub> (A and D), compound 1 (B and E) or compound 2 (C and F). The images A–F were generated from the FLN-DMRT1 transfected cells with the background subtracted. Images are 200× (A–C) or enlargements of the boxed areas (D–F).

nitrogen in oven-dried glassware. Reaction progress was monitored by thin layer chromatography using silica gel plates (silica gel 60 F<sub>254</sub>) and eluted TLC plates were visualized with UV light (254 nm) or developing the plate with Ce(SO<sub>4</sub>)<sub>2</sub> stain. The products were isolated and purified by flash chromatography. NMR experiments were performed on a 400/100 MHz instrument. NMR spectra were processed with the MestReNova program. Chemical shifts were reported as ppm relative to CDCl<sub>3</sub> (7.26 ppm for <sup>1</sup>H, 77.0 ppm for <sup>13</sup>C) and CD<sub>3</sub>OD (4.87 ppm for <sup>1</sup>H, 49.1 ppm for <sup>13</sup>C). <sup>1</sup>H NMR coupling constants (*J*) are expressed in hertz, and multiplicity is described as follows: s = singlet; d = doublet; t = triplet; q = quartet; br = broad; m = multiplet. High-resolution mass spectra and electrospray (ESI) experiments were recorded with electron-spray ionization. Melting points were determined with an Electrothermal Digital Mel-Temp 3.0 melting point apparatus and are uncorrected. Please see the Supporting Information for the InChI codes of the compounds under investigation.

#### 5-Carboxyfluorescein-4',5'-bis(mercuric trifluoroacetate) (4) [23, 28]

Mercuric oxide (1.72 g, 7.97 mmol) was added to TFA (12 mL) and stirred for 10 min to obtain a clear solution. Commercially available 5-carboxyfluorescein (3) (1.50 g, 3.98 mmol) was dissolved in NaOH (1 N, 8 mL) and added to the above mixture and stirred at room temperature. A heavy yellow-colored precipitate formed over the next few hours. After 5 h, the precipitate was filtered off and washed with excess water and dried *in vacuo* to obtain 3.07 g (72%) of 5-carboxyfluorescein 4',5'-bis(mercuric trifluoroacetate) (4) as a yellow-orange solid, which was used for the next step without further purification.

#### 4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5-carboxylic acid (5-carboxy-FIAsh-EDT<sub>2</sub> or 5-CrAsH-EDT<sub>2</sub>) (5)

Crude 5-carboxyfluorescein 4',5'-bis(mercuric trifluoroacetate) (4, 0.50 g, 0.49 mmol) was suspended in THF (10 mL) and DIPEA (0.69 mL, 3.99 mmol) was added at ambient temperature. The reaction mixture was stirred for 5 min, and then AsCl<sub>3</sub> (0.84 mL, 9.99 mmol) was added. The reaction mixture turned into a red solution. Pd(OAc)<sub>2</sub> (10 mg) was added to the reaction mixture and heated to 50°C for 2 h. The dark-colored reaction mixture was allowed to cool to room temperature and stirred overnight. The reaction mixture was poured into phosphate buffer (pH 7) and stirred for 5 min, and then EDT (2.6 mL, 60 mmol) was added to the above solution and stirred for 30 min [20, 23, 29]. Next, CHCl<sub>3</sub> (50 mL) and AcOH (9 mL) were added and the reaction mixture was stirred for another 1.5 h. The reaction mixture was then filtered and extracted with CHCl<sub>3</sub> (3 × 25 mL) and dried over Na<sub>2</sub>SO<sub>4</sub>. The solvent was removed *in vacuo*. The resulting orange residue, which was purified by flash column chromatography (silica gel, toluene/EtOAc, 4:1) to obtain 0.21 g (61%) of 5-CrAsH-EDT<sub>2</sub> (5) as a yellow solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub> + CD<sub>3</sub>OD) δ 8.60 (s, 1H), 8.30 (d, *J* = 8.0 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 1H), 6.53 (d, *J* = 8.8 Hz, 2H), 6.45 (d, *J* = 8.7 Hz, 2H), 3.63–3.42 (m, 8H); HRMS (ESI) calcd. for C<sub>25</sub>H<sub>19</sub>O<sub>7</sub>S<sub>4</sub>As<sub>2</sub> (M+H<sup>+</sup>) 708.8446, found 708.8440.

#### 4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5-carboxylic acid succinimidyl ester (6)

5-Carboxy-FIAsh-EDT<sub>2</sub> (5) (0.04 g, 0.06 mmol) was dissolved in anhydrous THF (2 mL) under N<sub>2</sub>. Then diisopropylcarbodiimide (0.01 g, 0.07 mmol) and *N*-hydroxysuccinimide (0.027 g, 0.23 mmol) were added. After 3 h, the mixture was evaporated under reduced pressure and the resultant crude product

was purified by flash column chromatography (silica gel, toluene/EtOAc, 4:1) to furnish 0.04 g (86%) of **6** as yellow solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.98 (s, 2H), 8.81 (d, *J* = 0.8 Hz, 1H), 8.44 (dd, *J* = 8.1, 1.5 Hz, 1H), 7.40 (d, *J* = 8.1 Hz, 1H), 6.63 (d, *J* = 8.8 Hz, 2H), 6.57 (d, *J* = 8.8 Hz, 2H), 3.84–3.38 (m, 8H), 2.97 (s, 4H); HRMS (ESI) calcd. for C<sub>29</sub>H<sub>22</sub>O<sub>9</sub>S<sub>4</sub>As<sub>2</sub> (M+H<sup>+</sup>) 805.8609, found 805.8616.

#### 4-Azido-1-fluoro-2-nitrobenzene (**8**) [30]

4-Fluoro-3-nitroaniline (**7**) (2.00 g, 12.80 mmol) was dissolved in a mixture of concentrated hydrochloric acid (12 mL) and water (2 mL) by heating (50°C). The resulting brown mixture was cooled to –70°C and then NaNO<sub>2</sub> (0.96 g, 13.84 mmol) dissolved in water (2 mL) was slowly added and then stirred at the same temperature for 10 min. The reaction mixture was rapidly filtered at –70°C. At this temperature NaN<sub>3</sub> (0.88 g, 13.58 mmol), dissolved in water (3.2 mL) was added. An orange to tan-colored precipitate was observed. The reaction was stirred for a few minutes and then ice-cold water was added to the reaction mixture. The solid was filtered and washed with water and dried under vacuum to provide 2.29 g (98%) of **8** as a tan-colored solid: mp 53.7–54.8°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 7.73 (dd, *J* = 6.1, 2.6 Hz, 1H), 7.36–7.25 (m, 2H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 153.7, 151.1, 137.1 (d, *J* = 3.6 Hz), 125.7 (d, *J* = 8.3 Hz), 119.9 (d, *J* = 22.7 Hz), 116.1 (d, *J* = 2.4 Hz); HRMS (ESI) calcd. for C<sub>12</sub>H<sub>4</sub>N<sub>4</sub>O<sub>2</sub> (M+H<sup>+</sup>) 183.0318, found 183.0307.

#### N<sup>1</sup>-(4-Azido-2-nitrophenyl)hexane-1,6-diamine (**9**)

To a stirred solution of 1-fluoro-2-nitro-4-azidobenzene (**8**) (0.18 g, 0.99 mmol) in MeOH (2 mL) was added hexamethylenediamine (0.58 g, 5.00 mmol) in MeOH (20 mL) at room temperature and the reaction mixture was stirred for 12 h. The solvent was removed *in vacuo* and EtOAc was added to the residue, unreacted diamine precipitated, and was removed by filtration and the filtrate was extracted with EtOAc and washed with H<sub>2</sub>O (4 × 25 mL), dried over Na<sub>2</sub>SO<sub>4</sub> and concentrated *in vacuo* to furnish 0.22 g (80%) of **9** as brown-colored solid: mp 59.1–60.8°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.03 (s, 1H), 7.86 (d, *J* = 2.7 Hz, 1H), 7.13 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.87 (d, *J* = 9.2 Hz, 1H), 3.36–3.22 (m, 2H), 2.75 (t, *J* = 7.0 Hz, 2H), 1.83–1.64 (m, 2H), 1.62–1.31 (m, 6H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 143.3, 131.4, 128.2, 127.7, 115.9, 115.5, 43.1, 40.5, 30.4, 28.8, 26.6, 26.3; HRMS (ESI) calcd. for C<sub>12</sub>H<sub>19</sub>N<sub>6</sub>O<sub>2</sub> (M+H<sup>+</sup>) 279.1569, found 279.1566.

#### N-(6-(5-Azido-2-nitrophenylamino)hexyl)-4',5'-di(1,3,2-dithiarsolan-2-yl)-3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-carboxamide (azide-TRAP) (**1**)

To a stirred solution of NHS ester **6** (0.02 g, 0.03 mmol) in THF (5 mL) was added **9** (0.01 g, 0.05 mmol) at room temperature. After stirring for 3 h, THF was removed *in vacuo* and the residue was purified by column chromatography (silica gel, EtOAc/toluene 5:1) to yield 0.02 g (77%) of probe **1** as an orange solid: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.86 (s, 2H), 8.23 (s, 1H), 8.14 (dd,

*J* = 8.0, 1.4 Hz, 1H), 7.96 (s, 1H), 7.79 (d, *J* = 2.7 Hz, 1H), 7.22 (d, *J* = 8.0 Hz, 1H), 7.07 (dd, *J* = 9.2, 2.7 Hz, 1H), 6.80 (d, *J* = 9.2 Hz, 1H), 6.51 (d, *J* = 8.8 Hz, 2H), 6.44 (d, *J* = 8.8 Hz, 2H), 6.34 (t, *J* = 5.7 Hz, 1H), 3.65–3.36 (m, 10H), 3.24 (dd, *J* = 12.2, 6.9 Hz, 2H), 1.78–0.94 (m, 8H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 168.3, 165.5, 162.9, 154.6, 152.3, 143.1, 136.7, 134.7, 131.4, 130.4, 128.1, 127.5, 127.0, 124.5, 122.6, 115.8, 115.3, 114.8, 112.4, 109.6, 82.7, 43.3, 43.0, 40.0, 29.5, 29.3, 28.6, 26.5, 26.4; HRMS (ESI) calcd. for C<sub>37</sub>H<sub>35</sub>N<sub>6</sub>O<sub>8</sub>S<sub>4</sub>As<sub>2</sub> (M+H<sup>+</sup>) 968.9831, found 968.9822.

#### tert-Butyl (6-(3-(3-methyl-3H-diazirin-3-yl)propanamido)hexyl)carbamate (**12**)

To a stirred solution of commercially available **11** (0.02 g, 0.10 mmol) in THF was added commercially available amine **10** (0.03 g, 0.15 mmol) at ambient temperature and the reaction mixture was stirred for 3 h. After completion of the reaction (monitored by TLC), THF was removed *in vacuo* and the residue was purified by column chromatography (silica gel, hexanes/EtOAc, 4:1) to provide (0.03 g) 97% of **12** as a white solid: mp 97.4–100.6°C; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 5.64 (bs, 1H), 4.53 (bs, 1H), 3.23 (dd, *J* = 13.0, 6.7 Hz, 2H), 3.11 (d, *J* = 5.9 Hz, 2H), 1.98 (t, *J* = 7.7 Hz, 2H), 1.76 (t, *J* = 7.7 Hz, 2H), 1.58–1.24 (m, 17H), 1.03 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 171.22, 156.11, 79.13, 40.09, 39.17, 30.69, 30.06, 29.99, 29.34, 28.41, 26.06, 25.91, 25.46, 19.94.

#### N-(6-Aminohexyl)-3-(3-methyl-3H-diazirin-3-yl)propanamide (**13**)

To a solution of **12** (0.03 g, 0.10 mmol) in dichloromethane (2 mL) was added trifluoroacetic acid (1 mL) at 0°C. After stirring for 1 h at 0°C, the reaction mixture was concentrated *in vacuo* and the residue was dissolved in EtOAc and washed with saturated aqueous sodium bicarbonate solution and extracted with EtOAc. The combined organic layers were dried over Na<sub>2</sub>SO<sub>4</sub> and dried to furnish **13** (0.02 g) in 87% yield, which was used for the preparation of probe **2** without further purification.

#### 4',5'-Di(1,3,2-dithiarsolan-2-yl)-3',6'-dihydroxy-N-(6-(3-(3-methyl-3H-diazirin-3-yl)propanamido)hexyl)-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-carboxamide (diazirine-TRAP) (**2**)

To a stirred solution of NHS ester **6** (0.05 g, 0.06 mmol) in THF (5 mL) was added **13** (0.01 g, 0.07 mmol) at room temperature. After being stirred for 3 h, THF was removed *in vacuo* and the residue was purified by column chromatography (silica gel, EtOAc/toluene 5:1) to provide probe **2** (0.03 g) as an orange solid in 53% yield: <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) δ 9.90 (s, 2H), 8.37 (d, *J* = 1.6 Hz, 1H), 8.22 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.26 (d, *J* = 8.1 Hz, 1H), 6.83 (s, 1H), 6.64–6.39 (m, 4H), 5.63 (t, *J* = 6.0 Hz, 1H), 3.74–3.37 (m, 10H), 3.24 (q, *J* = 6.6 Hz, 2H), 1.97 (dd, *J* = 8.5, 6.6 Hz, 2H), 1.75 (dd, *J* = 8.6, 6.7 Hz, 2H), 1.63 (m, 8H), 0.99 (s, 3H); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) δ 170.56, 167.41, 164.69, 162.01, 153.61, 151.46, 135.98, 133.83, 129.60, 126.16, 123.56, 122.07, 114.00, 111.60, 108.94, 82.23, 38.60, 37.86, 30.91, 29.71, 28.98, 28.69, 28.46, 28.19, 26.29, 21.68;

HRMS (ESI) calcd. for  $C_{36}H_{39}N_4O_7S_4As_2$  ( $M+H^+$ ) 917.0134, found 917.0139.

## Biology

### Materials

FlAsH-EDT<sub>2</sub> was purchased from Invitrogen (control compound).

### Generation of DMRT1 expression vectors

DMRT1 containing a tetracysteine motif at its amino terminus was PCR-amplified using Phusion polymerase (New England Biolabs), 1 ng of DMRT1-pcDNA3 plasmid as template, a forward primer encoding the tetracysteine motif (5'FLN-TC-DMRT1; 5'-CCGGAAGCTTATGTTCTGAACTGCTGCCCTGGCTGCTGCATGGAGCTCCTAACGACGACGATTCGG-3') and a reverse primer to the carboxyl-terminus of DMRT1 (DMRT1.40; 5'-CGGC GAATTCTCACTCGTCTCATCTCTT-3'). The included tetracysteine motif, FLNCCPGCCMEP, was one previously shown to enhance biarsenical binding activity [26]. The amplified cDNA was digested with HindIII and EcoRI, column purified using UltraClean PCR Clean-Up kit (MoBio, Carlsbad, CA) and cloned into the HindIII/EcoRI site of pcDNA3 (Invitrogen, Carlsbad, CA). The pcDNA3 vector containing modified DMRT1 (pc5'FLN-DMRT1) was purified from overnight cultures using affinity column purification, according to the manufacturer protocol (Qiagen Valencia, CA), and used for transient transfection analysis.

### Transient transfections

HeLa cells were seeded onto poly-L-lysine-coated 12 mm coverslips in 24-well plates at a density of 52000 cells per well. Twenty-four hours post-seeding, cells were transfected with 350 ng plasmid DNA and 0.5  $\mu$ L of Turbofect *in vitro* Transfection Reagent (Fermentas) according to the manufacturer's instructions.

### Compound treatment and imaging

At 24 h post-transfection, the medium was removed from the wells and the cells were washed twice with 500  $\mu$ L HBSS containing calcium and magnesium (Cellgro) supplemented with 1 mM sodium pyruvate (Sigma). Cells were then incubated with either 250 mL of 0.45 mM FlAsH reagent (Invitrogen), 0.45 mM probe 1, or 0.45 mM probe 2 in HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate and 3  $\mu$ M 2-mercaptoethanol, BME (EMD chemicals) for 30 min at 37°C in a cell culture incubator. After the incubation, cells were washed once with 500 mL HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate. After aspirating the wash solution, cells were incubated in 250 mL of HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate and 100 mM BME in a 37°C cell culture incubator for 30 min. Cells were then washed twice in 250 mL 1 $\times$  BAL buffer (0.25 mM 2,3-dimercapto-1-propanol, Sigma) for 5 min in a 37°C cell culture incubator. After the BAL washes, 500 mL of HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate was added to each well and slides were

prepared for imaging. Coverslips were inverted onto slides containing a drop of HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate that was used as mounting media. Coverslips were sealed with nail polish and incubated at 37°C until dried at which time they were imaged using a Nikon Eclipse 80i equipped with a FITC filter (excitation 494 nm; emission 518 nm).

### Image analysis

Images of cells transfected with FLN-DMRT1 or DMRT1-pcDNA3 and treated with compound 1 were captured at 20 $\times$  magnification; exposure time = 1 s; gain = 5; offset = -88. Images of cells transfected with FLN-DMRT1 or DMRT1-pcDNA3 and treated with compound 2 were captured at 20 $\times$  magnification; exposure time = 87 s; gain = 5; offset = -88. Images of cells transfected with FLN-DMRT1 or DMRT1-pcDNA3 and treated with FlAsH-EDT<sub>2</sub> were captured at 20 $\times$  magnification; exposure time = 405 min; gain = 5; offset = -88. For cells treated with the same compound, background adjustment was performed using Adobe Photoshop. For this, image pairs (i.e., like compound treatment and exposure parameters) of FLN-DMRT1 and DMRT1-pcDNA-transfected cells were combined on one layer within Photoshop and the input levels adjust in the RGB channel with the default preset until the background (signal in the DMRT1-pcDNA-transfected cells) was negligible.

The authors have declared no conflicts of interest.

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