Synthesis of Arylazide- and Diazirine-Containing CrAsH-EDT$_2$ Photoaffinity Probes

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Two photo-crosslinking biarsenical (CrAsH-EDT$_2$)-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$_2$ and N$^1$-(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
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 FlAsH-EDT$_2$ (fluorescein arsenical helix binder), which is a fluorescein containing 4',5'-di(1,3,2-dithiarsolan-2-yl) groups (Fig. 1). When FlAsH 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

![Figure 1. Structures of non-fluorescent FlAsH-EDT$_2$ and CrAsH-EDT$_2$ and green fluorescent FlAsH-EDT$_2$ and CrAsH-EDT$_2$ complexed with Cys-Cys-Xaa-Xaa-Cys-Cys and structures of TRAP, azide-TRAP (1) and diazirine-TRAP (2) photo-crosslinking biarsenical probes.](image-url)
partners in situ. Use of a CrAsH-EDT$_2$ (carboxy-FlAsH-EDT$_2$) 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 tetracystein-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 photoprobe 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$_2$, 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 dehydroazoepine 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 CH-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$_2$ 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$_2$ 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$_2$ 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 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 palladadium(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$_2$ (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-hydroxysuccinimide and disopropylcarbodiimide 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
followed by treatment with NaN₃ 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 N₁,N₄-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-hydroxysuccinimide ester 11 to furnish amide 12 in 97% yield. Exposure of 12 to TFA in CH₂Cl₂ 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-EDT₂ 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 1. Preparation of affinity probes azide-TRAP (1) and diazirine-TRAP (2).
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 (pc5FLN-DMRT1) and transfected into HeLa cells, which were subsequently treated with FlAsH-EDT2, 1, or 2 and visualized for fluorescence. Treatment of pc5FLN-DMRT1-transfected HeLa cells with FlAsH-EDT2 showed many cells with strong fluorescence localized to the nucleus (Fig. 2A and D). Similarly, fluorescent nuclei were also observed in 5FLN-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 FlAsH 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-EDT2) 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-EDT2 (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 N1,N4-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
nitrogen in oven-dried glassware. Reaction progress was monitored by thin layer chromatography using silica gel plates (silica gel 60 F_{254}) and eluted TLC plates were visualized with UV light (254 nm) or developing the plate with Ce(SO₄)₂ 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 CDCl₃ (7.26 ppm for ¹H, 77.0 ppm for ¹³C) and CD₃OD (4.87 ppm for ¹H, 49.1 ppm for ¹³C). ¹H 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 under reduced pressure and the resultant crude product was sublimed and extracted with CHCl₃ (3 x 25 mL) and dried over Na₂SO₄. 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-EDT₂ (5) as a yellow solid: ¹H NMR (400 MHz, CDCl₃ + CD₃OD) 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 C₄₃H₂₂O₃S₆As₂ (M+H⁺) 708.8446, found 708.8440.

4',5'-Bis(1,2,3-dithioarsolan-2-yl)-fluorescein-5-carboxylic acid (5-carboxy-FlAsH-EDT₂ or 5-CrAsH-EDT₂) (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 AsCl₃ (0.84 mL, 9.99 mmol) was added. The reaction mixture turned into a red solution. Pd(OAc)₂ (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, CHCl₃ (50 mL) and AcOH (9 mL) were added and the reaction mixture was 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 stirred for another 1.5 h. The reaction mixture was then filtered and extracted with CHCl₃ (3 x 25 mL) and dried over Na₂SO₄. 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-EDT₂ (5) as a yellow solid: ¹H NMR (400 MHz, CDCl₃ + CD₃OD) 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 C₄₃H₂₂O₃S₆As₂ (M+H⁺) 708.8446, found 708.8440.

Figure 2. Live cell imaging with FlAsH-EDT₂ 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 FlAsH-EDT₂ (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).

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was purified by flash column chromatography (silica gel, toluene/EtOAc, 4:1) to furnish 0.04 g (86%) of 6 as yellow solid: \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 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), 5.67 (d, \(J = 8.8\) Hz, 2H), 3.84–3.38 (m, 8H), 2.97 (s, 4H); HRMS (ESI) calcd. for C\(_{29}\)H\(_{22}\)O\(_9\)S\(_4\)As\(_2\) (M\(^+\)) 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 \(-70^\circ\)C and then Na\(_2\)O\(_4\) (0.96g, 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 \(-70^\circ\)C. At this temperature Na\(_2\)O\(_4\) (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; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 7.73 (dd, \(J = 6.1, 2.6\) Hz, 1H), 7.36–7.25 (m, 2H); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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\(_{12}\)H\(_4\)N\(_4\)O\(_2\) (M\(^+\)) 183.0318, found 183.0307.

N\(^1\)-(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\(_2\)O (4 × 25 mL), dried over Na\(_2\)SO\(_4\) and concentrated in vacuo to furnish 0.22 g (80%) of 9 as brown-colored solid: mp 59.1–60.8°C; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 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); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 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\(_{12}\)H\(_9\)N\(_2\)O\(_2\) (M\(^+\)) 279.1569, found 279.1566.

N-(6-Aminoethyl)-3-(3-methyl-3H-diazirin-3-yl)propanamide (hexylcarbamate) (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; \(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 5.64 (bs, \(J = 1.6\) Hz), 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); \(^13\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 71.22, 156.11, 156.07, 131.19, 135.98, 133.83, 130.4, 129.60, 126.16, 123.56, 122.07, 114.00, 111.60, 108.94, 82.23, 38.60, 37.86, 30.91, 29.71, 29.88, 28.69, 28.46, 28.19, 26.29, 21.68;
HRMS (ESI) calcd. for C$_{36}$H$_{39}$N$_4$O$_7$S$_4$As$_2$ (M$^+$H$^+$) 917.0134, found 917.0139.

**Biography**

**Materials**

FlAsH-EDT$_2$ 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'-CCGGAGACCTTGCTGCACTGCTGCGCTG CTGACTGGAGCCTCTCAACGACGAGCTTGCCTGG-3') and a reverse primer to the carboxyl-terminus of DMRT1 (DMRT1.40; 5'-CGGC GAAATTCTACTGCTCCTCATCCTTT-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 μL 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 μL HBSS containing calcium and magnesium (Cellgro) supplemented with 1 mM sodium pyruvate (Sigma). Cells were then incubated with either 250 μL 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 μM 2-mercaptoethanol, BME (EMD chemicals) for 30 min at 37°C in a cell culture incubator. After the incubation, the cells were washed once with 500 μL HBSS containing calcium and magnesium supplemented with 1 mM sodium pyruvate. After aspirating the wash solution, cells were incubated in 250 μL 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 μL 1× 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 μL 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 20x 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 20x magnification; exposure time = 87 s; gain = 5; offset = −88. Images of cells transfected with FLN-DMRT1 or DMRT1-pcDNA3 and treated with FlAsH-EDT$_2$ were captured at 20x 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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