Preparation of the membrane-permeant biarsenicals FlAsH-EDT$_2$ and ReAsH-EDT$_2$ for fluorescent labeling of tetracysteine-tagged proteins

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The membrane-permeant fluorogenic biarsenicals FlAsH-EDT$_2$ and ReAsH-EDT$_2$ can be prepared in good yields by a straightforward two-step procedure from the inexpensive precursor dyes fluorescein and resorufin, respectively. Handling of toxic reagents such as arsenic trichloride is minimized so the synthesis can be carried out in a typical chemistry laboratory, usually taking about 2–3 d. A wide range of other biarsenical reagents and intermediates that also bind to tetracysteine-tagged (CysCysProGlyCysCys) proteins can be prepared similarly using this general procedure.

INTRODUCTION

The introduction of genetically encoded tags such as green fluorescent protein (GFP) for visualizing proteins in living cells, tissues and organisms has been a major advance in cell biology[1,2]. Fluorescent proteins, however, are large (~30 kDa) and can perturb the activity or localization of the protein being studied. We have introduced an alternative and much smaller genetically encoded tetracysteine tag (<1 kDa) that has a high affinity for membrane-permeant fluorogenic biarsenicals such as FlAsH-EDT$_2$ and ReAsH-EDT$_2$ (**Fig. 1**)[3,4]. The minimal tetracysteine sequence is CysCysProGlyCysCys, although optimized motifs with higher affinity are preferable[5]. A unique feature of the biarsenicals is that their fluorescence is quenched until bound by the tetracysteine tag, which allows the protein to be imaged without exhaustive washing to remove unbound dye[3].

In addition to fluorescent marking of the tagged protein, biarsenicals have enabled many other techniques such as the determination of protein age by two-color pulse chase[6], dye-triggered protein aggregation[7], localized protein inactivation by chromophore-activated light inactivation[8,9], correlative fluorescent and electron microscopy[6,10] and the generation of improved Ca^{2+} and fluorescent resonance energy transfer (FRET) indicators[11,12]. The major limitations of the method are its reduced sensitivity compared with FPs because of nonspecific staining (although the optimized tetracysteine sequences have reduced this considerably) and restriction (so far) to cultured cells and tissue preparations. The requirement for washing with dithiols after staining limits the methods use in animals, although long incubation times with very low (nM) concentrations of biarsenicals without any subsequent destaining may be one solution. Surprisingly, toxicity has not been an issue in most cellular applications. Many similar genetically targeted labeling strategies have been described,[2] but the genetically encoded tag is either large (protein)[13] or small (peptide), in which case it can only be used for extracellular labeling[14,15].

FlAsH emits green-yellow fluorescence and is a good FRET acceptor from cyan fluorescence protein (spectrally it is very similar to yellow fluorescence protein) and has been used mostly in cell labeling experiments. ReAsH fluorescence peaks in the red (609 nm), is a FRET acceptor from longer wavelength FPs such as GFP and yellow fluorescence protein and is useful for pulse-chase labeling, chromophore-activated light inactivation and correlated electron microscopy. Although FlAsH and ReAsH are commercially available, their straightforward synthesis in two steps from inexpensive starting materials using standard chemical techniques allows the preparation of the substantial amounts required for many experiments. For other biarsenicals (**Fig. 2**), synthesis similar to these basic protocols is often the only option, although nonacidic mercuration conditions are required for rhodamines[4]. This

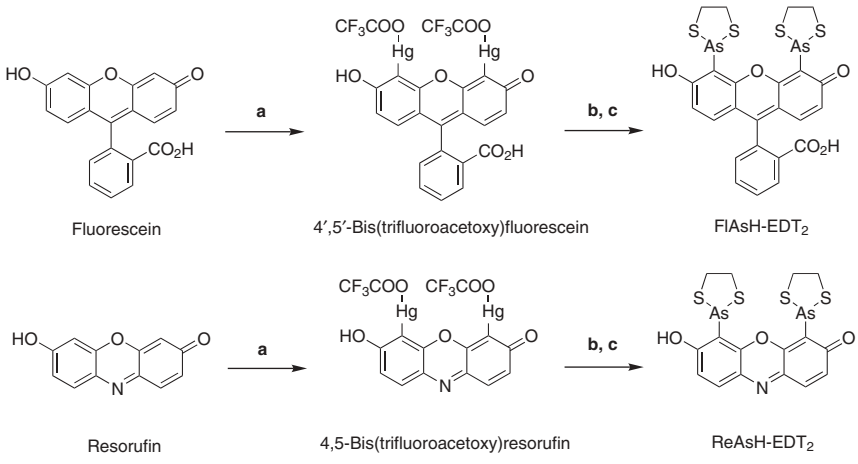

Figure 1 | Synthesis of FlAsH-EDT$_2$ and ReAsH-EDT$_2$. (**a**) HgO, TFA; (**b**) $AsCl_3$, $Pd(OAc)_2$, DIEA, NMP; (**c**) EDT, aqueous acetone.

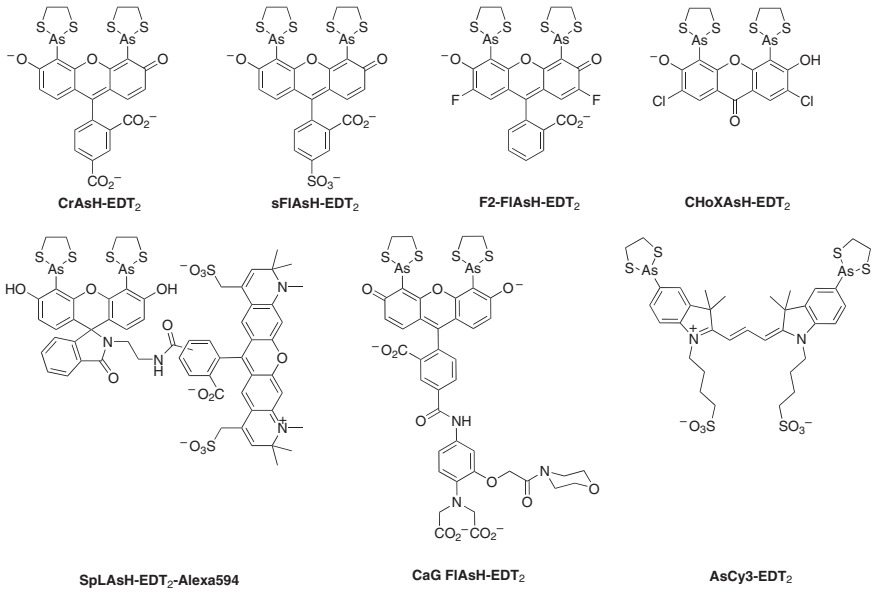

Figure 2 | Structures of some other useful biarsenicals reagents and intermediates. CrAsH-EDT_2 (refs. 4,11,17), fluorogenic biarsenicals and intermediate for CaG FlAsH and immobilized biarsenicals; sFlAsH-EDT_2 (ref. 4) membrane-impermeable fluorogenic biarsenicals; F2-FlAsH (ref. 18), photostable fluorogenic biarsenicals; CHoXAsH-EDT_2 (refs. 4,17), aggregator for BA-GFP; SpLAsH (ref. 19) colorless and nonfluorescent biarsenicals for targeting other dyes, e.g., Alexa594; CaG FlAsH (ref. 11), targetable low-affinity Ca^{2+} indicator; AsCy3 (ref. 20), fluorogenic biarsenical based on cyanine dye.

procedure for FlAsH-EDT_2 differs from previously published methods[3,16] in the first step; reaction of fluorescein using mercuric trifluoroacetate proceeds with near-quantitative yield to give 4′,5′-bis(trifluoroacetoxymercuri)fluorescein and a higher yield of FlAsH-EDT_2 in the next step. Earlier reports used commercially available 4′,5′-bis(acetoxymercuri)fluorescein (fluorescein mercuric acetate). The subsequent transmetallation step appears generally applicable to the synthesis of a wide range of biarsenicals (**Fig. 2**).

MATERIALS

REAGENTS

- Fluorescein (purity >97%; Acros, cat. no. 11924)
- Resorufin (Free acid, dye content >95%; Aldrich, cat. no. 424455; or sodium salt, dye content 75%; Aldrich, cat. no. 230154)
- Mercuric oxide, HgO (purity >99%; Aldrich, cat. no. 213357) **! CAUTION** Handle with care (extremely toxic).
- Trifluoroacetic acid (TFA; purity 99%; Aldrich, cat. no. T62200, and other suppliers) **! CAUTION** Handle with care (corrosive).
- Phosphorus pentoxide
- 1-Methyl-2-pyrrolidinone (NMP; anhydrous; Aldrich, cat. no. 328634, and other suppliers)
- Arsenic (III) chloride ($AsCl_3$; purity >99.9%; Aldrich, cat. no. 200077) **! CAUTION** Handle with care in a well-ventilated fume hood (extremely toxic, carcinogenic).
- *N*,*N*-diisopropylethylamine (DIEA; purity >99.5%; Aldrich, cat. no. 387649)
- Palladium (II) acetate ($Pd(OAc)_2$; purity 98%; Aldrich, cat. no. 205869)
- Acetone **! CAUTION** Inflammable.
- Phosphate buffer, pH 6.9, prepared from equal volumes of 1 M solutions of the monobasic and dibasic potassium salts, KH_2PO_4 and K_2HPO_4
- 1,2-Ethanedithiol (EDT; purity >98%; Fluka, cat. no. 02390) **! CAUTION** Stench. Handle in a well-ventilated fume hood.
- Chloroform **! CAUTION** Toxic.
- Brine (saturated aqueous solution of sodium chloride)
- Sodium sulfate (anhydrous; Fisher, cat. no. S429)
- Toluene
- Ethyl acetate **! CAUTION** Inflammable.
- Molecular sieve 4A beads 8–12 mesh (Aldrich, cat. no. 208604)
- Sand
- Silica gel 60 (230–400 mesh; ASTM, EMD)
- 95% (vol/vol) ethanol
- TLC plates (silica gel 60 F_{254}, EMD)
- Nanopure water
- 2-mercaptoethanesulfonate, sodium salt (Sigma, cat. no. M1511)
- Dimethylsulfoxide (DMSO; anhydrous; Aldrich, cat. no. 276855, and other suppliers)
- 4-Morpholinepropanesulfonic acid (MOPS)
- Sodium hydroxide, pellets or solution
- Tetracysteine peptide containing the motif, Cys-Cys-Pro-Gly-Cys-Cys. Optimal results are obtained with FLNCCPGCCMEP (H_2N-Phe-Leu-Asn-Cys-Cys-Pro-Gly-Met-Gln-Pro-$CONH_2$). HPLC-purified or crude peptide is suitable ▲ **CRITICAL** All solvents used for reactions should be of the highest quality possible. Solvents for chromatography are HPLC grade.

EQUIPMENT

- 2-necked round-bottomed flask
- Round-bottomed flask
- Fume hood
- Rotary evaporator
- Dessicator
- Constant volume pipettes (Microman, Gilson Inc.) or glass syringes
- Filter funnels, filter paper
- Graduated cylinders
- Heating mantle
- Heat gun or Bunsen burner
- Glass chromatographic columns (air-pressurizable)
- Separatory funnels
- Spatulas
- Teflon-coated magnetic stir bars
- Thermometers
- TLC-developing chambers
- UV handheld lamp
- Pipettes

REAGENT SETUP

Solvents Dry NMP and DIEA over 4A molecular sieve for at least several days before use in reactions or use from bottles of anhydrous material kept and withdrawn under inert gas. All reactions, workup and chromatography should be carried out in a well-ventilated fume hood.

Arsenic trichloride handling Constant volume pipettes (Microman) are recommended for this toxic and volatile liquid as the plastic pistons and tips are both disposable and can be decontaminated with dilute bleach solution. (Alternatively, dry glass syringes with needles may be used.) Used reaction glassware can similarly be treated with dilute bleach. Solid and liquid waste generated should be disposed of according to appropriate regulations.

Solutions Approximately 0.1 M potassium phosphate buffer in water–acetone. Make a buffer containing an equimolar (0.25 M)

concentration of NaH_2PO_4 and Na_2HPO_4, then add acetone until cloudiness develops.

0.1 M MOPS adjusted to pH 7.2 by adding sodium hydroxide solution.

EQUIPMENT SETUP

Glassware for the transmetallation step should be dried before use in an oven or by flame-drying.

A convenient but optional way to produce a N_2 atmosphere in a reaction flask is to use a Firestone valve (Aldrich, cat. no. Z103616) attached by plastic or rubber tubing to N_2 (g) tank, house vacuum and one of the necks of the round-bottomed flak via a septum-inlet adapter (e.g., Aldrich, cat. no. Z102288).

PROCEDURE

1| Prepare FlAsH-EDT_2 or ReAsH-EDT_2 by following the steps in Options A or B, respectively.

(A) Preparation of FlAsH-EDT_2 (synthesis of fluorescein 4′,5′-bis(mercuric trifluoroacetate) (Steps (i–iv)

● TIMING ~24 h); synthesis of FlAsH-EDT_2 (4′,5′-bis(1,3,2-dithiarsolan-2-yl)-fluorescein) (Steps (v–xx) ● TIMING ~8 h))

(i) Weigh 1.3 g of mercuric oxide (HgO) (6 mmol) and transfer to a 50-ml round-bottomed flask equipped with a Teflon-coated magnetic stir bar.

! CAUTION HgO is poisonous; avoid contact and breathing dust.

(ii) Transfer 15 ml of TFA to the flask and gently heat the stirred suspension with a heat gun until the solid has dissolved to form a colorless solution. Allow the solution to cool to room temperature (18–25 °C).

(iii) Weigh out 1.0 g of fluorescein (3 mmol) and add to the solution of mercuric trifluoroacetate to give a yellow solution. Protection from moisture is not necessary. A heavy yellow precipitate forms over the next few hours.

(iv) After 5 h, evaporate off the TFA (bath temperature <40 °C) and suspend the resulting solid in 25 ml of water. Filter off the product, wash several portions of water (10 ml), allow to air-dry, then place overnight in a vacuum desiccator containing a glass beaker of phosphorus pentoxide (~20 g) evacuated using a high vacuum pump. If the P_2O_5 becomes liquid, replace with fresh solid and continue desiccation. The product is a yellow-orange solid. Typical yields are ~2.8 g (over 96%).

■ **PAUSE POINT** Stable at room temperature indefinitely, but protect from light.

(v) Weigh 0.96 g of fluorescein 4′,5′-bis(mercuric trifluoroacetate) (1.0 mmol) and transfer to a dried 50-ml two-necked round-bottomed flask, equipped with a Teflon-coated magnetic stir bar, and under N_2 atmosphere (preferably with a slight positive-pressure of N_2 gas) through one neck of the flask. Seal neck with rubber septum.

(vi) Transfer 10 ml of anhydrous NMP through a disposable plastic or a glass syringe with attached dry needle into the flask by injecting through the septum.

(vii) Transfer 1.69 ml of arsenic trichloride (20 mmol) into the flask through a syringe or positive-displacement pipette by injecting through the septum or by briefly removing the septum if the reaction vessel is under positive N_2 pressure.

! CAUTION Arsenic trichloride is volatile, corrosive, acutely toxic and a carcinogen. Handle only in a well-ventilated fume hood wearing suitable protective clothing (gloves, labcoat and safety glasses). Keep bottle tightly closed. Dispose of residues in syringes, needles, pipette tips and gloves by rinsing with diluted bleach (sodium hypochlorite solution).

(viii) Transfer 1.4 ml of anhydrous DIEA (8 mmol) into the flask by syringe or pipette by injecting through the septum or by briefly removing the septum.

(ix) Transfer a few milligrams of palladium (II) acetate into the flask by a spatula and by briefly removing the septum.

(x) The dark-colored reaction mix is heated at 60 °C for about 3 h.

■ **PAUSE POINT** The reaction can be left with stirring at room temperature overnight under N_2 at this stage.

(xi) Transfer the reaction mixture with a disposable plastic or glass pipette into a 250-ml glass Erlenmeyer containing a well-stirred (with a Teflon-coated magnetic stir bar) mixture of 100 ml of 1:1 vol/vol 0.25 M potassium phosphate buffer pH 6.9 and acetone to give an orange solution. Note that this amount of buffer is insufficient to maintain the pH at ~7 during quenching of the excess $AsCl_3$ in the reaction mix but will prevent the solution from becoming too acidic and causing decomposition of the product.

(xii) Transfer ~4 ml of EDT to the stirred solution with a disposable plastic or glass pipette. The solution decolorizes, turns milky and a solid deposits.

! CAUTION Stench from EDT; use only in a well-ventilated fume hood. Quench the transfer pipette in a diluted bleach solution.

(xiii) Transfer ~50 ml of chloroform to the rapidly stirred solution. Rinse out the reaction vessel by transferring the mixture to and from the flask. Stir for 15–30 min at room temperature.

(xiv) Separate the aqueous and chloroform layers in a separatory funnel. Extract the aqueous layer twice with fresh ~50-ml portions of chloroform. Dry the combined organic layers with anhydrous Na_2SO_4. After ~30 min, remove the drying agent by filtration through filter paper. Remove the chloroform by rotary evaporation at 40 °C to give an orange liquid.

Note: A large amount of Na_2SO_4 will be required, so add until free solid remains suspended rather than clumped after swirling the mixture. Alternatively, residual NMP can be removed by evaporation on a high-vacuum pump with gentle warming. This avoids the following step, but traces of NMP may affect the chromatography step.

(xv) Dissolve the oil in 100 ml of toluene and wash (three times) with 50 ml of water in a separatory funnel. One or two back extractions of the pooled aqueous washes with ~20 ml of toluene are recommended. After ~30 min, remove the drying

agent by filtration through filter paper. Remove the toluene by rotary evaporation at 40 °C to give an orange oil. Dissolve in ~5 ml of toluene.

■ **PAUSE POINT** The crude product can be stoppered and left overnight in the fume hood. $As_2(EDT)_3$ may precipitate as a white solid; redissolve by gentle heating or by adding a few milliliters of chloroform before proceeding to the next step.

? TROUBLESHOOTING

(xvi) Prepare a chromatography column (1 inch i.d. × 12 inches with 250 ml of reservoir) of silica gel (~50 g) packed in toluene. Cover the top with a layer of sand (0.5 cm thick).

(xvii) Load the column with the crude product obtained from Step A(xv) and elute the column with toluene (~500 ml) under air pressure (or gravity) until the eluent shows no UV absorbance when spotted on to TLC silica gel plates.

(xviii) Elute the product with 10–20% (vol/vol) ethyl acetate–toluene (~500 ml). An orange band will move down the column and elute but the fractions will be colorless. Identify fractions of the product by running TLC silica gel plates eluting with 20% (vol/vol) ethyl acetate–toluene (retention factor = 0.48). Gently warming the developed plate with a hot air gun reveals the product as a yellow spot.

(xix) Remove the solvents from the pooled collected fractions by rotary evaporation (<40 °C) to yield the product as an off-white solid. Remove immediately as soon as it becomes dry.

▲ **CRITICAL STEP** Continuing to evaporate the solid on the rotary evaporator or by further evaporation on a vacuum pump leads to loss of EDT from the arsenic groups and some polymerization. The solid will no longer dissolve completely in chloroform and toluene and will give a baseline spot under the TLC conditions in Step A(xviii).

? TROUBLESHOOTING

(xx) Remove residual traces of toluene by adding absolute ethanol (~10 ml) and re-evaporating. Suspend the remaining solid in ~5 ml of 96% ethanol, chill on ice for 30 min and filter off the product, washing with some cold ethanol. Dry overnight on filter without vacuum to give the product as a pale yellow or pinkish solid. The yield is ~300 mg. Store in the dark at −20 °C.

■ **PAUSE POINT** Store at −20 °C. No deterioration detected in 10 years.

? TROUBLESHOOTING

(B) Preparation of ReAsH-EDT_2 (synthesis of resorufin 4,5-bis(mercuric trifluoroacetate) (Steps (i–iv) ● TIMING ~1 d); synthesis of ReAsH-EDT_2 (4,5-bis(1,3,2-dithiarsolan-2-yl)-resorufin) (Steps (v–xx) ● TIMING ~8 h))

(i) Weigh 120 mg of mercuric oxide (HgO) (0.5 mmol) and transfer to a 10-ml round-bottomed flask equipped with a Teflon-coated magnetic stir bar.

! **CAUTION** HgO is poisonous, avoid contact and breathing dust.

(ii) Transfer 2 ml of TFA to the flask and gently heat the stirred suspension with a heat gun until the solid has dissolved to form a colorless solution. Allow the solution to cool to room temperature.

(iii) Weigh out 59 mg of resorufin sodium salt (0.25 mmol) and add to the solution of mercuric trifluoroacetate to give a dark red solution. Equip with a reflux condenser with water cooling and gently reflux the solution overnight. Protection from moisture is not necessary. A dark red precipitate is formed. Note that an equivalent amount of resorufin-free acid can be substituted for the sodium salt.

(iv) After cooling to room temperature, evaporate off the TFA (bath temperature <40 °C) and suspend the resulting solid in 5 ml of water. Filter off the product, wash with several portions of water (5 ml), allow to air-dry, then dessicate over phosphorus pentoxide (~5 g) under reduced pressure overnight. If the P_2O_5 becomes liquid, replace with fresh solid and continue dessication. The product is a yellow-orange solid. Typical yields are ~132 mg (over 60%).

■ **PAUSE POINT** Stable at room temperature indefinitely but protect from light.

(v) Weigh 73 mg of resorufin 4,5-bis(mercuric trifluoroacetate) (0.087 mmol) and transfer to a dried 5-ml two-necked round-bottomed flask, equipped with a Teflon-coated magnetic stir bar, and under N_2 atmosphere (preferably with a slight positive pressure of N_2 gas) through one neck of the flask. Seal neck with rubber septum.

(vi) Transfer 1.5 ml of anhydrous NMP through a disposable plastic or a glass syringe with attached dry needle into the flask by injecting through the septum.

(vii) Transfer 0.17 ml of arsenic trichloride (4 mmol) into the flask through a syringe or positive-displacement pipette by injecting through the septum or by briefly removing the septum if the reaction vessel is under positive N_2 pressure.

! **CAUTION** Arsenic trichloride is volatile, corrosive, acutely toxic and a carcinogen. Handle only in a well-ventilated fume hood wearing suitable protective clothing (gloves, labcoat and safety glasses). Keep bottle tightly closed. Dispose of residues in syringes, needles, pipette tips and gloves by rinsing with diluted bleach (sodium hypochlorite solution).

(viii) Transfer 0.14 ml of anhydrous DIEA (1.6 mmol) into the flask by syringe or pipette by injecting through the septum or by briefly removing the septum.

(ix) Transfer a few milligrams of palladium (II) acetate into the flask by spatula and by briefly removing the septum.

(x) The dark-colored reaction mix is heated to 60 °C for about 3 h.

■ **PAUSE POINT** The reaction can be left stirring overnight at room temperature under N_2 at this stage.

(xi) Transfer the cooled reaction mixture with a disposable plastic or glass pipette into a 100-ml glass Erlenmeyer containing a well-stirred (with a Teflon-coated magnetic stir bar) mixture of 25 ml of 1:1 vol/vol 0.25 M potassium phosphate buffer pH 6.9 and acetone to give an orange solution.

(xii) Transfer ~0.5 ml of EDT to the stirred solution with a disposable plastic or glass pipette. The solution decolorizes, turns milky and a solid deposits.

! CAUTION Stench from EDT; use only in a well-ventilated fume hood. Quench the transfer pipette in a diluted bleach solution.

(xiii) Transfer ~25 ml of chloroform to the rapidly stirred solution. Rinse out the reaction vessel by transferring the mixture to and from the flask. Stir for 15–30 min.

(xiv) Separate the aqueous and chloroform layers in a separatory funnel. Extract the aqueous layer twice with fresh ~20 ml portions of chloroform. Dry the combined organic layers with anhydrous Na_2SO_4. After ~30 min, remove the drying agent by filtration through filter paper. Remove the chloroform by rotary evaporation at 40 °C to give a red liquid.

Note: A large amount of Na_2SO_4 will be required; add until free solid remains suspended after swirling the mixture. Alternatively, residual NMP can be removed by evaporation on a high-vacuum pump with gentle warming. This avoids the following step but traces of NMP may affect the chromatography step.

(xv) Dissolve the oil in 30 ml of toluene and wash with 10 ml of water in a separatory funnel. One or two back extractions of the pooled aqueous washes with ~5 ml of toluene are recommended. Dry the combined toluene layers with anhydrous Na_2SO_4. After ~30 min, remove the drying agent by filtration through filter paper. Remove the toluene by rotary evaporation at 40 °C to give a dark red oil. Dissolve in ~5 ml of toluene.

■ PAUSE POINT The crude product can be stoppered and left overnight in the fume hood. $As_2(EDT)_3$ may precipitate as a white solid; redissolve by gentle heating or by adding a few milliliters of chloroform before proceeding to the next step.

? TROUBLESHOOTING

(xvi) Prepare a chromatography column (0.5 inch i.d. × 12 inches with 100 ml reservoir) of silica gel (~20 g) packed in toluene. Cover the top with a layer of sand (0.5 cm thick).

(xvii) Load the column with the crude product from Step B(xv) and elute the column with toluene (~200 ml) under air pressure (or gravity) until the eluent shows no UV absorbance when spotted on to TLC silica gel plates.

(xviii) Elute the product with 2.5–5% (vol/vol) ethyl acetate–toluene (~200 ml). A pale orange band will move down the column following a blue band. Identify fractions of the product by spotting TLC silica gel plates eluting with 20% (vol/vol) ethyl acetate–toluene (retention factor = 0.53). Gently warming the developed plate with a hot air gun reveals the product as a purple spot.

▲ CRITICAL STEP Carefully separate the product from the blue-colored, more nonpolar side-product (retention factor ~0.6), ReAsH-EDT_3, as this gives background staining in mammalian cells that is not removable with the usual post-staining washing concentrations of the dithiol arsenic andidotes EDT or BAL (British anti-Lewisite; 2,3-dimercaptopropanol), and therefore reduces the sensitivity of the method in live cells. Traces of ReAsH-EDT_3 are best identified by TLC or by HPLC.

? TROUBLESHOOTING

(xix) Remove the solvents from the pooled collected fractions by rotary evaporation (<40 °C) to yield the product as an off-white pinkish solid. Remove immediately.

▲ CRITICAL STEP Continuing to evaporate the solid on the rotary evaporator or by further evaporation on a vacuum pump leads to loss of EDT from the arsenic groups and some polymerization. The solid will no longer dissolve completely in chloroform and toluene and will give a baseline spot under the TLC conditions mentioned in Step B(xviii).

(xx) Remove residual traces of toluene by adding absolute ethanol (~5 ml) and re-evaporating. Suspend the remaining solid in ~1 ml of 96% ethanol, chill on ice for 30 min and filter off the product, washing with a little cold ethanol. Dry overnight on filter without vacuum to give the product as a dark red solid. The yield is 5–16 mg.

■ PAUSE POINT Store in the dark at −20 °C. No deterioration detected in 8 years.

? TROUBLESHOOTING

Quality control of the products by measuring fluorescence before and after addition of tetracysteine-tagged peptide

2| Prepare 1 mM solutions of FlAsH-EDT_2 or ReAsH-EDT_2 in dry DMSO and keep protected from light. Aliquot and keep at −20 °C.

? TROUBLESHOOTING

3| Prepare stock solutions of 10 mM EDT in dry DMSO (0.84 µl EDT per ml) and 1 M 2-mercaptoethanesulfonate in water.

▲ CRITICAL STEP Prepare these solutions immediately before use.

4| Prepare a 10 mM stock solution of a tetracysteine peptide in 50% (vol/vol) aqueous acetonitrile containing 0.1% (vol/vol) TFA. The optimized sequence, FLNCCPGCCMEP (H_2N-Phe-Leu-Asn-Cys-Cys-Pro-Gly-Met-Glu-Pro-$CONH_2$) is preferable.

5| Prepare a 100 mM aqueous solution of MOPS neutralized with NaOH to pH 7.2.

6| To 2.5 ml of the MOPS buffer in a fluorescence cuvette, add 25 µl of 1 M mercaptoethanesulfonate (MES) followed by 2.5 µl of 10 mM EDT to give final concentrations of 10 mM and 10 µM, respectively.

7| With a fluorimeter, measure the emission intensity of the solution at 530 nm with excitation at 508 nm (for FlAsH) or emission at 608 nm with excitation at 597 nm (for ReAsH). This is best done continuously, for example, every 1 or 5 s.

8| Add 2.5 µl of 1 mM FlAsH-EDT_2 or ReAsH-EDT_2 to give a final concentration of 1 µM. Mix well and continue to measure the fluorescence at the appropriate wavelength for 5–10 min. If there is a noticeable decrease in fluorescence during this period, this indicates that there has been some loss of EDT from the biarsenical.

9| Add 10 µl of 10 mM tetracysteine peptide to the cuvette and mix well. The fluorescence will increase rapidly but will take up to 1 h to plateau as the complex forms.

10| Measure the percent fluorescence of the FlAsH-EDT_2 or ReAsH-EDT_2 compared with that of the complex with peptide after subtracting the baseline fluorescence (before addition of the biarsenical).

? TROUBLESHOOTING

? TROUBLESHOOTING

Heavy emulsions when washing toluene extract with water (Steps 1A(xv), 1B(xv)). Heavy emulsions are usually formed but these can be broken by adding ~10 ml of saturated NaCl solution to the funnel. Do not reshake the funnel. Separate the two layers as much as possible and repeat the washing with water twice, each time breaking the emulsion as described. Note that this step produces heavy precipitation of salts that are best carried through the extraction rather than removed before by filtration. Dry the combined toluene layers with anhydrous Na_2SO_4.

FlAsH-EDT_2 or ReAsH-EDT_2 that have lost EDT (Steps 1A(xix), 1B(xix)). Excess fresh EDT can be added to DMSO solutions of FlAsH and ReAsH that have lost EDT but reaction is incomplete and repurification is necessary. (Addition of EDT occurs quickest at pH ~7, so mixing the DMSO solution with an aqueous buffer might be advantageous.) However, repeating the synthesis is probably the best option.

No solid on washing evaporated FlAsH-EDT_2 or ReAsH-EDT_2 with ethanol (Steps 1A(xx), 1B(xx)). This may result from incomplete removal of toluene from the pooled fractions, so reevaporate (ethanol and toluene form an azeotrope aiding the removal of residual toluene) and re-add ethanol. If still no solid is formed either before or after adding a little fresh cold ethanol, chill in ice and scratch glass with spatula. If no solid is formed after a few hours, reevaporate and dissolve residue in DMSO. Measure concentration of biarsenical by absorbance (see ANTICIPATED RESULTS).

TLC analysis of chromatography fractions and purified products gives baseline material in addition to that with expected mobility (Steps 1A(xviii), 1B(xviii)). This reflects breakdown of the products under the TLC conditions rather than impure fractions. For example, simply spotting pure FlAsH-EDT_2 or ReAsH-EDT_2 on silica TLC plates leads to formation of the corresponding arsenoxides probably by hydrolysis of EDT and subsequent oxidation by air. Excess EDT prevents this, so the problem only occurs during the chromatography step that removes free EDT. To assess the presence of any arsenoxides in a sample of FlAsH-EDT_2 or ReAsH-EDT_2, dissolve in DMSO and analyze by reverse-phase HPLC (see ANTICIPATED RESULTS for conditions; any arsenoxides will be eluted almost immediately).

Inability to get large fluorescence increases when FlAsH-EDT_2 and ReAsH-EDT_2 are mixed with tetracysteine peptides (Step 10). See the section on 'Quality control of the products by measuring fluorescence before and after addition of tetracysteine tagged peptide' for optimal conditions for this reaction. It is important to have 10 µM EDT present in the assay as millimolar concentrations of monothiols such as 2-MES or 2-mercaptoethanol slowly react with the biarsenicals to form fluorescent products and thereby decrease the final enhancement on adding peptide. MES is preferred as millimolar 2-mercaptoethanol can compete with the peptide for the biarsenicals, probably because the hydroxyl group can weakly bind to the arsenic in addition to the thiol. A slow decrease in fluorescence on adding the sample of biarsenical to this solution is indicative of a loss of EDT in the sample. Simply waiting until the EDT complex is reformed (fluorescence stops decreasing) before the addition of tetracysteine peptide should give the usual 1,000- to 2,000-fold increase in fluorescence upon peptide addition. If the fluorescence enhancement is smaller than this, fluorescent impurities or breakdown products (e.g., monoarsenicals or fluorescein or resorufin) are probably present and the sample should be repurified. Analytical HPLC (see Analytical data) will also confirm their presence.

Instability and color changes on storage of FlAsH-EDT_2 and ReasH-EDT_2 solutions in DMSO (Step 2). Although stock solutions of the biarsenicals in EDT are stable at −20 °C for many years (measured by HPLC), color changes of these (FlAsH and ReAsH solutions becoming redder and bluer, respectively) are usually seen over time and even vary from aliquot to aliquot of the same solution. This probably reflects uptake of traces of moisture into the solutions resulting in more of the quinonoid form of FlAsH (rather than the lactone) and deprotonation of ReAsH. These changes have no effect on protein or cell-labeling experiments.

ANTICIPATED RESULTS

Analytical data

Fluorescein 4′,5′-bis(mercuric trifluoroacetate): ^{1}H-NMR: (D_6-DMSO) δ 6.48 (d, 2H, H-2′), 6.63 (d, 2H, H-1′), 7.23 (d, 1H, H-7, $J = 7.5$ Hz), 7.72 (m, 1H, H-6), 7.80 (m, 1H, H-5), 7.98 (d, 1H, H-4, $J = 7.5$ Hz), 9.98 (s, 2H, OH). ^{13}C-NMR (d_6-DMSO): 110.81, 112.91, 116.50, 118.14, 120.35, 124.78, 125.44, 127.15, 130.18, 130.79, 136.24, 154.84, 159.12, 159.61, 160.09, 160.58, 162.41, 169.31. Storage: stable at room temperature indefinitely but protect from light.

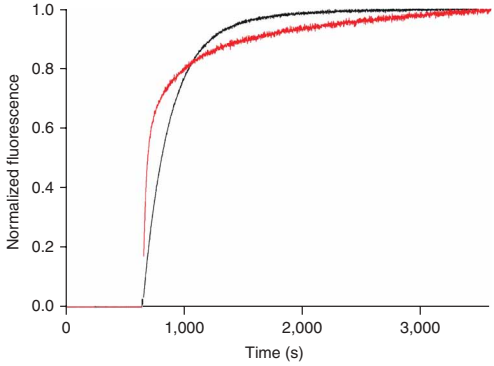

Figure 3 | Typical time courses for the reaction of FlAsH-EDT_2 (black trace) and ReAsH-EDT_2 (red trace) with the tetracysteine peptide, FLNCCPGCCMEP (added at 700 s).

FlAsH-EDT_2: ^{1}H- NMR (CDCl3) δ 2.3 (s, OH), 3.57 (m, -SCH2CH2S-), 6.60 (d, H-2′), 6.69 (d, H-1′), 7.19 (d, H-7), 7.66 (m, H-5, 6), 8.03 (d, H-4). ^{13}C-NMR (d_6-DMSO): δ 42.50, 42.43, 83.95, 110.36, 113.30, 116.75, 124.82, 125.44, 126.91, 130.58, 130.90, 136.36, 152.54, 153.19, 161.77, 169.18. HPLC ES-MS (4.6 mm × 250 mm Phenomenex Luna 2 C_{18}, 1 ml min^{-1} of 10–90% ACN-H_2O with 0.05% TFA linear gradient in 20 min then 90% for an additional 10 min); retention time, 23.9 min +ve mode: (M + 1) 664.8. Calculated for $C_{24}H_{18}As_2O_5S_4$, 664.85. HR-ES 664.8536 (100%), 665.8565 (30%), 668.8508 (24%). Calculated $[M+H]^+$ 664.8542 (100%), 665.8571 (30%), 666.8522 (24%). UV-visible (10 mM K.MOPS pH 7.2): λ_{max} 508 nm, ε_{max} 63,500 M^{-1} cm^{-1}; (0.1 M NaOH): λ_{max} 496 nm, $\varepsilon = 69{,}500$ M^{-1} cm^{-1}. Storage: store at −20 °C. No deterioration detected in 10 years.

Resorufin 4,5-bis(mercuric trifluoroacetate): ^{1}H-NMR: (D_6-DMSO) δ 6.97 (d, 2H, $J = 8.8$ Hz), 7.04, (s, 1H, OH), 7.26 (d, 2H, $J = 8.7$ Hz). Storage: stable at room temperature indefinitely but protect from light.

ReAsH-EDT_2: ^{1}H-NMR (CDCl3): δ 3.49 (m, 8H, S-CH2), 6.22 (d, 2H, $J = 8.3$ Hz), 6.30 (d, 2H, $J = 8$ Hz). ^{13}C-NMR (d_6-DMSO): δ 42.32, 110.94, 115.34, 117.71, 125.92, 144.02, 153.33. HPLC ES-MS (4.6 mm × 250 mm Phenomenex Luna 2 C_{18}, 1 ml min^{-1} of 10–90% ACN-H_2O with 0.05% TFA linear gradient in 20 min then 90% for an additional 10 min); retention time, 21.4 min +ve mode: (M + 1) 545.8. Calculated for $C_{16}H_{13}NO_3As_2S_4$ 545.8. HR-ES 545.8283 (100%), 546.8313 (18%), 547.8253 (17%). Calculated $[M+H]^+$ 545.8283 (100%), 546.8310 (20%), 547.8253 (19%). UV-visible (0.1 M NaOH): λ_{max} 579 nm, $\varepsilon = 63{,}000$ M^{-1} cm^{-1}. Storage: keep at −20 °C. No deterioration detected in 8 years.

Quality control of the products by measuring fluorescence before and after addition of tetracysteine-tagged peptide Absorbance. ReAsH-EDT_2 has low solubility in aqueous solutions at pH 7 and FlAsH-EDT_2 can partially form the colorless lactone form. Therefore, FlAsH-EDT_2 and ReAsH-EDT_2 solutions are best quantified by reading the absorbance of a suitable dilution in 0.1 N NaOH in which they are more soluble and any lactone forms are minimal. The measurement should be made immediately after addition to the base as the absorbance decreases with time. Note that under these conditions, EDT is lost from the biarsenicals and the product absorbs at slightly shorter wavelengths than the EDT complex.

Fluorescence enhancement of FlAsH and ReAsH upon binding to a tetracysteine peptide. Typical preparations of FlAsH-EDT_2 and ReAsH-EDT_2 from this method have 0.05–0.1% of the fluorescence of the tetracysteine complex. However, samples with higher values (up to 0.5%) were still suitable for cell staining. Typical time courses for the reaction of the biarsenicals with excess tetracysteine peptide are shown in **Figure 3**. Absorbance and emission spectra of the FlAsH and ReAsH complexes with a tetracysteine peptide are shown in **Figures 4** and **5**, respectively.

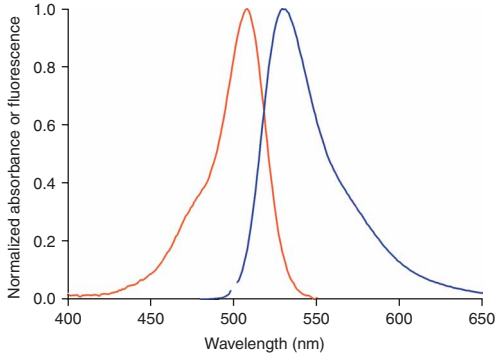

Figure 4 | Absorbance (red trace) and fluorescence emission (blue trace) of FlAsH complex with FLNCCPGCCMEP.

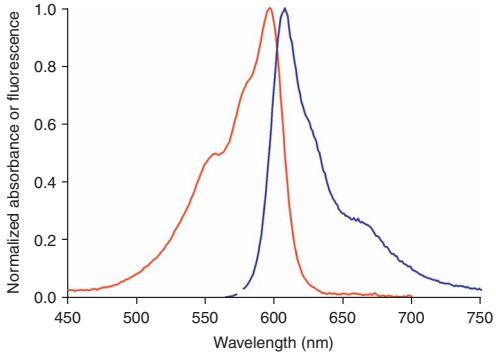

Figure 5 | Absorbance (red trace) and fluorescence emission (blue trace) of ReAsH complex with FLNCCPGCCMEP.

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