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Probing the target-specific inhibition of sensitized protein tyrosine phosphatases with biarsenical probes

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Abstract

Selective control of enzyme activity is critical for elucidating the roles of specific proteins in signaling pathways. One potential means for developing truly target-specific inhibitors involves the use of protein engineering to sensitize a target enzyme to inhibition by a small molecule that does not inhibit homologous wild-type enzymes. Previously, it has been shown that protein tyrosine phosphatases (PTPs) can be sensitized to inhibition by a biarsenical probe, FlAsH-EDT_2, which inhibits PTP activity by specifically binding to cysteine residues that have been introduced into catalytically important regions. In the present study, we developed an array of biarsenical probes, some newly synthesized and some previously reported, to investigate for the first time the structure-activity relationships for PTP inhibition by biarsenicals. Our data show that biarsenical probes which contain substitutions at the 2′ and 7′ positions are more effective than FlAsH-EDT_2 at inhibiting sensitized PTPs. The increased potency of 2′,7′-substituted probes was observed when PTPs were assayed with both *para*-nitrophenylphosphate and phosphopeptide PTP substrates and at multiple probe concentrations. The data further indicate that the enhanced inhibitory properties are the result of increased binding affinity between the 2′,7′-substituted biarsenical probes and sensitized PTPs. In addition we provide previously unknown physicochemical and stability data for various biarsenical probes.

Introduction

Selective control of the activity of a single enzyme is a key criterion for chemical-biology strategies that aim to unravel a target enzyme's physiological and/or pathological functions in the cell. Chemical control of enzyme activity is most frequently achieved with small-molecule inhibitors, which can either interact with a target enzyme's active site or block its activity by allostery. However, it is often very difficult to design a cell-permeable small molecule that is specific only toward a target enzyme, particularly if the enzyme is a member of a large protein family. A complementary approach for achieving target-specific inhibition of an enzyme involves sensitizing the enzyme of interest toward a selected molecule that does not inhibit the wild-type enzyme or other homologous proteins.[1,2,3] In a successful sensitization strategy, which requires engineering of the enzyme to introduce a binding site for the inhibitor, suppression of enzyme activity is target-specific, a feature that is especially desirable in cell-signaling studies.

The classical protein tyrosine phosphatases (PTPs) are a large family of enzymes that catalyze the dephosphorylation of phosphotyrosine in metazoan signaling networks. Because the PTPs share a highly conserved active site and because many known inhibitors of wild-type PTPs are not cell-permeable,[4] this protein family potentially presents an ideal case for sensitization strategies. Previous studies on PTP sensitization have shown that introduction of spatially arranged cysteine residues into a loop of a PTP domain is sufficient to confer strong affinity for the membrane-permeable biarsenical probe, FlAsH-EDT_2, initially designed to specifically visualize proteins in the cell.[5,6,7,8] Binding of FlAsH-EDT_2 to the sensitized PTP can cause strong inhibition of PTP activity, which relies on the specific conjugation of the compound's two arsenic atoms with the cysteine-rich motif that is incorporated into the target PTP (Fig. 1A). The activities of wild-type PTPs, therefore, are generally unaffected by FlAsH-EDT_2.

An array of sensitized T-cell PTP (TCPTP) mutants, which contain tetracysteine (TC) motifs inserted at various loops within the enzyme's PTP domain, has been previously developed.[5] These TCPTP mutants exhibited varying degrees of inhibition by FlAsH-EDT_2, the most highly sensitive being a construct in which the tetracysteine insertion was placed directly adjacent to the PTP domain's WPD loop (4C@187 TCPTP).[6] The opening and closing of the conserved WPD loop is critical for the activity of all classical PTPs,[9,10] and it was hypothesized that FlAsH-EDT_2 inhibits 4C@187 TCPTP by rigidifying its WPD-loop's local structure and disrupting its normal dynamics. Subsequent studies showed that TCPTP mutants with two or three cysteines placed within the WPD loop could also be potently inhibited by FlAsH-EDT_2.[6]

Work to date on PTP sensitization has focused solely on the optimization of protein engineering, using only FlAsH-EDT_2 as the inhibitory biarsenical compound. Since multiple types of biarsenical probes with differing structures and physicochemical properties have been reported[11] we aimed to obtain a library of biarsenical probes, composed of both known and novel compounds, to test the probes' abilities to act as inhibitors of sensitized PTPs. We hypothesized that screening an array of probes with previously engineered PTP mutants would allow for the identification of optimized biarsenical/enzyme pairs, in which the degree of engineered PTP inhibition is maximized. In the course of these studies we also made unexpected observations concerning the differing stabilities of biarsenical probes in solution; these findings, also reported here, will be of utility to the entire biarsenical-research community.

Results and discussion

Synthesis of biarsenical probes

The biarsenical probes used in this study were prepared based on resorufin, fluorescein, and seven fluorescein derivatives. This library includes both new (Et2FlAsH-EDT_2, Cl4FlAsH-EDT_2, and 5-Bu-CrAsH-EDT_2) and previously described compounds (Fig. 2).[12,13,14,15] Parent fluorophores that are not commercially available (2',7'-difluorofluorescein[16], 2'7'-dichlorofluorescein[17] and 2'7'-diethylfluorescein[18]) were synthesized using the appropriate resorcinol derivative (4-chloro-, 4-fluoro-, and 4-ethyl, respectively) and phthalic anhydride. Similarly, 4,5,6,7-tetrafluorofluorescein[16] and 4,5,6,7-tetrachlorofluorescein[17] were obtained using appropriately substituted phthalic anhydrides in reaction with resorcinol (Fig. 3). 5-(N-butylamide)-fluorescein was obtained in the reaction of 5-carboxyfluorescein and *n*-buthylamide using *O*-(benzotriazol-1-yl)-*N,N,N′,N′*-tetramethyluronium hexafluorophosphate (HBTU) and N,N,N-diisopropylethylamine (DIEA) as coupling agents (Fig.4).

Using a standard synthetic route involving mercurization of a parent fluorophore and subsequent transmetalation with $AsCl_3$,[19] all compounds were successfully obtained with high purity. Details regarding each synthetic step and purification are included in Supplementary information (Fig. S1 ESI†). Initially, we also attempted to synthesize biarsenical probes based on 2′,4,5,6,7,7′-hexafluoro- and 2′,4,5,6,7,7′-hexafluorofluorescein[16,17]. Although the dimercury synthetic intermediates were obtained for both hexa-halogen fluorophores, the subsequent transmetalation was not successful, despite several attempts (*data not shown*).

Physicochemical characterization of biarsenical probes

Since a full complement of physicochemical data for our library of biarsenical probes has not been reported, we performed a systematic analysis of the probes in complex with an

optimized tetracysteine peptide (TC12),[20] the results of which are summarized in Table 1. The full set of spectra can be found in Figure S2 (ESI†). The characterization data are in good agreement with previously published results (where available), with one exception: our absorbance (510 nm) and emission (530 nm) maxima wavelengths for the F2FlAsH–TC12 complex are higher than previously reported values by 10 and 8 nm, respectively.[14]

We suspect that, since experimental conditions (pH and tetracysteine peptide) are essentially identical for the differing measurements, the discrepancy may be due to the significant degradation of the F2FlAsH-EDT_2 used in the previous study. F2FlAsH's corresponding monoarsenical species, has an absorbance maximum of 500 nm (Fig. S3, ESI†),[20] and we observed that DMSO solutions of 2′,7′-substituted probes (Cl2FlAsH-EDT_2, Et2FlAsH-EDT_2 and F2FlAsH-EDT_2) degrade significantly when stored as DMSO solution overnight at -20 °C (see below for further discussion of biarsenical stability).

Next, we determined the pK_a values for the 3′-phenolic groups of the biarsenical probes in the library. Although proton dissociation from 3′-phenol is required for the fluorescent properties of fluorescein and resorufin derivatives, and pK_a values are highly sensitive reporters of the electron rich/poor character of a particular functional group, the pK_a values for almost all of the library's probe–peptide complexes have not been previously reported. Our systematic acid-base study shows that in every case the biarsenical probe–peptide complex has a lower phenolic pK_a value than the parent fluorophore (Table 1). The highest acidity of the phenolic group is observed for Cl2FlAsH-TC12 and F2FlAsH-TC12 complexes due to the high electronegativity of the halogen substituents. These probes demonstrate stable fluorescence properties over a broad pH range, potentially enabling their use in staining tetracysteine-tagged proteins in a variety of cellular compartments, including lysosomes. Interestingly, in the biarsenical complexes containing TC12 peptide, a second dissociation event with a pK_a value of ~7 is also observed (Fig. S4, ESI†). We did not

observe the second dissociation event, however, when a minimal tetracysteine peptide (Ac-CCPGCC-amide) was used (Fig. S5, ESI†*)*. Based on the second dissociation constant values (Table 1), we believe that the dissociation can be linked to the binding of the fourth cysteine residue in a tetracysteine motif to an arsenic atom. Our previous results on the Zn(II) coordination properties of tetracysteine peptides showed that the fourth cysteine residue has a significantly increased pK_a value, resulting in the formation of two complexes, one with three bound sulfur donors (ZnS_3) at neutral pH and one with four donors (ZnS_4) at slightly alkaline conditions.[12] A reasonable explanation of this phenomenon is the steric hindrance of four sulfurs bound to Zn(II) at weakly acidic pH. However, further experiments must be performed to confirm this hypothesis.

Stability of biarsenical probes

The main route of biarsenical probe degradation in aqueous solution is the breaking of As-C bond, forming monoarsenical species and subsequently the parent fluorophore, exemplified by mass spectra of decomposed Et2FlAsH (Fig. S6, ESI†). However, the extent or timeline of this process has not been precisely measured in previous studies. Experiments with inhibition of sensitized PTP with biarsenical probe include a 2.5 h pre-incubation of enzyme with biarsenical probe (vide infra). We therefore tested whether significant amounts of monoarsenical species would be formed under these conditions, as significant degradation of the biarsenicals would, of course, alter the outcome of the inhibition experiments. Using analytical HPLC we determined that most of the probes do not degrade in significant amounts (Fig. 5 and Table S1, ESI†). For example, a 6 h incubation gave rise to only a 0.92% increase of monoarsenical FlAsH-EDT and a 0.42% increase in monoarsenical F4FlAsH-EDT. By contrast, the extent of hydrolysis was significant for Et2FlAsH-EDT_2, which showed a 14.2% increase in monoarsenical species after 6 h. We also observed that, although all of the

biarsenical probes described here can be stored in solid form for prolonged times without degradation, DMSO stock solutions may degrade overnight, even when kept at -20°C, as we observed for Cl2FlAsH-EDT_2, Et2FlAsH-EDT_2 and F2FlAsH-EDT_2 (*data not shown*).

Inhibition of PTP activities by biarsenical probes

In order to assess the PTP-inhibitory activity for the library of biarsenical probes we expressed and purified both wild-type and engineered variants of two PTPs: T-cell PTP (TCPTP) and hematopoietic PTP (HePTP). The PTP set included four mutants of TCPTP and one of HePTP (Figure 1), all of which have been previously described.[5,6,7] We chose TCPTP because the previously published optimization of localization of biarsenical-binding cysteines was performed on this enzyme; HePTP was also used to confirm that the results can be applied in other sensitized enzymes as well. The selected mutants have cysteine-enriched biarsenical binding motifs with either two (P181C/E187C TCPTP), three (P181C+2C TCPTP), or four cysteines (4C TCPTP, 4C@211 HePTP) within or adjacent to their respective WPD loops. Also included in the study to serve as a negative control was one mutant that contains a complete tetracysteine binding motif CCPGCC, but not within or near the protein's WPD loop (4C@241 TCPTP, Fig. 1B). All proteins were expressed from *E. coli* and obtained in high yields, in most cases greater than 20 mg per liter of culture. Purification of the six-histidine tagged proteins, carried out using standard protocols, provided pure protein (Figure S7, ESI†).

An initial screen of the seven PTPs (2 wild-type, 5 engineered) and nine biarsenical probes (63 PTP-probe combinations) was carried out with the small-molecule PTP substrate *para*-nitrophenylphosphate (*p*NPP) in the presence of 20 μM TCEP.[24] We used TCEP instead of DTT, since the latter can compete with tetracysteine motifs for binding of biarsenical probes.[15] TCEP has also been previously used in PTP assays,[25] and a lack of this reducing

agent causes a decrease of enzyme activity during 2.5 h incubation at room temperature, see Fig. S8 (ESI†). We started by establishing the key kinetic parameters—catalytic rate constant (k_{cat}) and the Michaelis constant (K_m) for *p*NPP—in the absence or presence (four-fold excess) of biarsenical probe. A sample Michaelis-Menten plot is shown in Figure 6, and Figure 7 contains a graphical representation of the catalytic efficiencies of different pairs of enzymes and biarsenical probes. Detailed results can be found in Tables S2-S4 (ESI†). In the case of wild-type TCPTP no significant inhibition is induced by most of the probes and only marginal inhibition with FlAsH-EDT_2, Cl2FlAsH-EDT_2 and F2FlAsH-EDT_2. Likewise, wild-type HePTP was not significantly inhibited by any of the biarsenical probes. These data are in agreement with previous results using FlAsH-EDT_2 with wild-type TCPTP and HePTP.[5,6,7] Along similar lines, 4C@241 TCPTP, an enzyme that contains a non-sensitizing tetracysteine motif that is not near its WPD loop, is not substantially inhibited by any of the biarsenicals.

For mutants that contain biarsenical-binding motifs in their respective WPD loops, the degree of inhibition varied substantially between protein-probe combinations. Out of all combinations tested, 4C TCPTP conjugated to Et2FlAsH resulted in the highest degree of inhibition (72% reduction in catalytic efficiency, Fig. 6), although other biarsenical compounds also inhibited the same sensitized PTP very effectively (Fig. 7, Table S4 ESI†). High-potency inhibitors were also identified for P181C/E187C TCPTP: both Cl2FlAsH-EDT_2 and Et2FlAsH-EDT_2 inhibited this enzyme more effectively than FlAsH-EDT_2. The 2',7'-substituted probes reduced the activity by 43% and 52%, respectively, whereas FlAsH-EDT_2 reduced the activity by only 28%. Likewise, for P181C+2C TCPTP Et2FlAsH-EDT_2 and F2FlAsH-EDT_2 offer additional 35% and 23% reduction of activity, respectively compared to FlAsH-EDT_2. Similarly, in the case of 4C@211 HePTP the F2FlAsH- and Et2FlAsH-bound enzymes were more strongly inhibited than the FlAsH-bound protein. Taken together, these data demonstrate that substitutions at positions 2′ and 7′ of the

fluorescein scaffold with fluorine, chlorine atoms or ethyl chain serve to improve the inhibition of sensitized WPD-loop PTP mutants.

Based on the results described above we selected the most promising pairs of PTP mutants and biarsenical probes to test them using a phosphopeptide substrate. Peptide substrates mimic the natural PTP substrates (phosphoproteins) more closely than the small-molecule substrate *p*NPP.[26] Moreover, an assay for inhibition of phosphopeptide dephosphorylation provides a test for the substrate-independence of biarsenical-induced PTP inhibition. To measure PTP activity we used a continuous PTP-chymotrypsin coupled assay in which increased fluorescence is caused by a peptide-cleavage event that can occur on non-phosphorylated peptide (PTP product), but not on phosphopeptide (PTP substrate). Although this assay has previously been used in a stopped format, in which the PTP inhibitor sodium orthovanadate and chymotrypsin are added at the end of the PTP reaction, we found that the assay worked well in continuous format. Under our continuous-assay conditions, PTP activity is rate-limiting; therefore the observed rate of fluorescence increase is proportional to PTP concentration. By measuring the activities of enzymes incubated in various molar ratios of biarsenical probe to enzyme (Fig. 8), we were able to determine the values of dissociation constant for the 2',7'-substituted biarsenical probes (Table 2). Similarly to results obtained with *p*NPP as a substrate, wild-type TCPTP was not inhibited in this assay by any of the biarsenical probes. In the case of the double cysteine mutant (P181C/E187C TCPTP) there is no significant difference between FlAsH-EDT_2 and 2',7'-substituted probes in dissociation constant. However, when more cysteines are present (P181C+2C and 4C TCPTP), F2FlAsH-EDT_2 demonstrates substantially improved inhibitory capabilities in comparison with FlAsH-EDT_2. F2FlAsH-EDT_2 not only inhibits the enzyme to higher extent at a 4:1 ratio of biarsenical probe to protein but also has 14- (P181C+2C) and 9-fold (4C) higher affinities toward the target PTPs (Table 2). These values corroborate results obtained using *p*NPP as a

substrate, showing that improved inhibition is substrate-independent, as would be expected if the biarsenical dye blocks the conformational changes of the WPD loop that are required for catalysis.[9,10] Since Et2FlAsH-EDT$_2$ also provided improved reduction of catalytic efficiency, the molecular basis can be explained with two factors. First, the 2',7'-substituted biarsenical probes can potentially induce steric hindrance during closing of the WPD loop, due to the sheer size of the compounds. Second, the presence of halogen atoms may result in the increased Lewis acidity of the compounds' arsenic atoms, thereby increasing the stability of binding with the engineered cysteine residues of the target proteins.

Collectively, our results based on both *p*NPP and phosphopeptide assays confirm that cysteine-enriched WPD loops can be effectively targeted and that substantial improvements in inhibition can be realized by using optimized biarsenical probes.

Conclusions

In conclusion, we have developed a library of known and new biarsenical probes and systematically characterized their physicochemical properties, many of which were previously unknown. In the course of these studies we found that some biarsenical probes are unstable in aqueous buffered or DMSO solution, which can affect their long-term application and storage. Our biarsenical library was screened against a panel of wild-type and sensitized protein tyrosine phosphatases (PTPs) in order to identify optimal pairs of engineered PTPs and biarsenicals for selective and potent inhibition of inhibitor-sensitized PTPs. Based on inhibition assays with both small-molecule and phosphopeptide substrates, we found that the 2′,7′-substituted biarsenical probes Cl2FlAsH-EDT$_2$, F2FlAsH-EDT$_2$ and Et2FlAsH-EDT$_2$ are more effective inhibitors of sensitized PTPs than the previously investigated compound FlAsH-EDT$_2$, with F2FlAsH-EDT$_2$ being the most effective inhibitor identified to date. Our results show that substantial improvements in inhibitor effectiveness can be realized through

the systematic investigation of biarsenical/enzyme pairs and suggest that future strategies for controlling protein activity with biarsenicals should give considerable attention to optimization of both components of the conjugate, protein and inhibitor.

Experimental procedures

Materials

Unless otherwise noted, all chemicals and reagents were obtained from commercial suppliers (Sigma-Aldrich, Merck, Roth, Avantor Performance Materials, or Iris Biotech) at the highest quality available and were used without further purification. Buffers were prepared using Milli-Q water (Merck Millipore system). Fluorescein, 5-carboxyfluorescein, resorcinol, 4-chlororesorcinol, 4-ethylresorcinol, phthalic anhydride, 4,5,6,7-tetrachlorophthalic anhydride, resorufin were from Sigma-Aldrich, 4-fluororesorcinol, 4,5,6,7-tetrafluorophthalic anhydride were from Fluorochem and were used directly as received. NMP, DMSO and DIEA from Iris Biotech were dried and stored over 3Å molecular sieve.

Synthesis of biarsenical probes

The biarsenical probes used in this study were prepared based on a general synthesis protocol published previously.[12,19] The first step of fluorescein-based probe synthesis involved the reaction of the appropriate derivative of phthalic anhydride and resorcinol in methanesulfonic acid (for details see ESI†). After stirring at 70 ^{o}C for 12 h, reactions were terminated by addition of cold water, and the precipitates were collected by centrifugation at 4 ^{o}C. All synthesized or commercially purchased fluorescein or resorufin platforms were subsequently converted to 4′,5′-dimercury derivatives. Probe synthesis was completed by palladium-catalyzed transmetalation of the mercuric derivatives with $AsCl_3$ and capping of the arsenic

atoms with 1,2-ethanedithiol (EDT). Biarsenical probes were purified by FLASH chromatography using a gradient of ethyl acetate or methanol in toluene (Gilson PLC 2020 using Interchim PuriFlash SIHP 30 µm, 20 g column, see ESI† for details). Fractions with at least 95% purity, as estimated by HPLC described below, were collected. The pooled fractions were concentrated on a rotary evaporator, aliquoted, and dried by Speed Vac. Once solvent was evaporated, tubes were immediately removed to avoid degradation by over-drying.[27] Because of that solid contained small amounts of solvents used during purification, which prohibited the estimation of the synthesis yield of biarsenical derivatives. Identification and purity assessment of the fractions and final product were carried out on by ESI-MS (Applied Biosystems API 2000) and HPLC (Dionex Ultimate 3000 using a Phenomenex Aeris Peptide C18 column, see ESI† for details). All biarsenical probes were stored as solid in -20ºC. Prior to use probes were dissolved in anhydrous DMSO.

4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (FlAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 14.9 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 99 %. ^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.60 (m, 8H, S-CH_2), 6.52 (d, 2H, *J* = 10 Hz, H-2', 7'), 6.63 (d, 2H, *J* = 10 Hz, H-1', 8'), 7.20 (d, 1H, *J* = 5 Hz, H-7), 7.63 (t, *J* = 5 Hz, H-6), 7.69 (t, *J* = 5 Hz, H-5), 8.02 (d, 1H, *J* = 5 Hz, H-4). ^{13}C NMR ($CDCl_3$): 43.45, 110.68, 112.39, 114.87, 125.29, 128.22, 129.03, 130.78, 135.06, 137.87, 152.50, 162.82, 169.07. MS (*m/z*) calculated for $C_{24}H_{18}As_2O_5S_4$ $[M-H]^-$ 662.8, found 663.3.

2',7'-difluoro-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (F2FlAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 10.7 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min.

showed purity of 97 %. ^{1}H NMR (500Hz, $CDCl_3$, ppm): 3.60 (m, 8H, S-CH_2), 6.41 (d, 2H, *J* = 10 Hz, H-1', 8'), 7.21 (d, 1H, *J* = 5 Hz, H-4), 7.66 (t, *J* = 5 Hz, H-6), 7.72 (t, *J* = 5 Hz, H-5), 8.03 (d, 1H, *J* = 5 Hz, H-7). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 43.39, 108.76, 115.28, 115.44, 123.66, 125.27, 126.32, 127.97, 128.78, 130.19, 132.34, 134.19, 135.17, 145.29 (d, J_{CF} = 240 Hz), 154.61 (d, J_{CF} = 246 Hz), 151.15, 168.20.. MS (*m/z*) calculated for $C_{24}H_{18}As_2F_2O_5S_4$ $[M-H]^-$ 699.8, found 699.3.

2',7'-dichloro-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (Cl2FlAsH-EDT$_2$).

A red solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 9.7 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 90 %. ^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.62 (m, 8H, S-CH_2), 7.21 (d, 1H, *J* = 5Hz, H-4), 7.70 (t, *J* = 5 Hz, H-6), 7.75 (t, *J* = 5 Hz, H-5), 8.06 (d, 1H, *J* = 5 Hz, H-7). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 43.42, 110.91, 113.80, 118.42, 123.65, 125.04, 127.97128.78, 130.07, 135.31, 157.54. MS (*m/z*) calculated for $C_{24}H_{18}As_2Cl_2O_5S_4$ (monoisotopic peak) $[M-H]^-$ 730.8, found 731.1.

2',7'-diethyl-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (Et2FlAsH-EDT$_2$).

A red solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 16.1 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 96 %. ^{1}H NMR (500 Hz, $CDCl_3$, ppm): 1.0 (t, 3H, *J* = 5 Hz), 2.40 (m, 2H), 3.58 (m, 8H, S-CH_2), 7.21 (d, 1H, *J* = 5 Hz, H-4), 7.64 (t, *J* = 5 Hz, H-6), 7.69 (t, *J* = 5 Hz, H-5), 8.04 (d, 1H, *J* = 5 Hz, H-7). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 14.06, 23.56, 43.66, 110.13, 112.14, 128.47, 129.28, 129.74, 135.22, 160.6. MS (*m/z*) calculated for $C_{28}H_{26}As_2O_5S_4$ $[M-H]^-$ 718.9, found 719.3.

4,5,6,7-tetrachloro-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (F4FlAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 11.6 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 97 %.^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.57 (m, 8H, S-CH_2), 6.60 (d, 2H, *J* = 10 Hz, H-2', 7'), 6.78 (d, 2H, *J* = 10 Hz, H-1', 8'). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 43.29, 107.59, 112.81, 115.00, 125.04, 127.97, 128.78, 134.30, 135.27 (m), 141.04 (m), 143.35 (dd, J_{CF} =250, 4 Hz), 146.92 (dd, J_{CF} = 255 Hz, 4 Hz), 151.79, 163.17. MS (*m/z*) calculated for $C_{24}H_{14}As_2F_4O_5S_4$ $[M-H]^-$ 734.8, found 735.1.

4,5,6,7-tetrachloro-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (Cl4FlAsH-EDT$_2$).

A red solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 8.7 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 97 %. ^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.56 (m, 8H, S-CH_2), 6.58 (d, 2H, *J* = 10 Hz, H-2', 7'), 6.75 (d, 2H, *J* = 10 Hz, H-1', 8'). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 43.30, 107.09, 112.63, 115.08, 122.46, 125.29, 128.21, 129.02, 129.38, 149.35, 152.31, 163.11. MS (*m/z*) calculated for $C_{24}H_{14}As_2Cl_4O_5S_4$ (monoisotopic peak) $[M-H]^-$ 798.7, found 799.1.

5-Carboxy-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (5-CrAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 9.8 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 97 %.^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.60 (m, 8H, S-CH_2), 6.53 (d, 2H, *J* = 10 Hz, H-2', 7'), 6.62 (d, 2H, *J* = 10 Hz, H-1', 8'), 8.00 (d, 1H, *J* = 10Hz, H-7), 8.40 (d, 1H, *J* = 10 Hz, H-6), 8.72 (s, 1H, H-4). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 15.50, 17.91, 29.90, 30.91,

43.73, 49.76, 66.10, 110.21, 112.83, 115.29, 124.51, 127.54, 130.89, 136.74, 152.63, 168.34. MS (*m/z*) calculated for $C_{25}H_{18}As_2O_7S_4$ $[M-H]^-$ 706.8, found 707.3.

5-(N-butylamide)-4',5'-bis(1,3,2-dithiarsolan-2-yl)-fluorescein (5-BuCrAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 9.7 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 95 %. ^{1}H NMR (500 Hz, DMSO, ppm): 0.92 (t, 3H, *J* = 5 Hz), 1.35 (m, 2H), 1.54 (m, 2H), 2.18 (t, 2H, *J* = 5 Hz), 3.30 (m, 8H, S-CH_2), 6.58 (d, 2H, *J* = 10 Hz, H-2', 7'), 6.72 (d, 2H, *J* = 10 Hz, H- 1', 8'), 7.2 (m, 2H, H-5, 6), 8.25 (d, 1H, *J* = 10 Hz, H-7), 8.39 (d, 1H, *J* = 10 Hz, H-6), 8.45 (s, 1H, H-4). ^{13}C NMR (500 Hz, DMSO-d_6, ppm): 13.68, 17.20, 30.08, 39.50, 42.13, 113.29 118.42, 121.42, 128.19, 128.88, 129.61, 132.06, 142.62, 147.32, 156.58, 164.13, 172.02. MS (*m/z*) calculated for $C_{29}H_{27}As_2NO_6S_4$ $[M-H]^-$ 763.9, found 763.9.

4,5-Bis(1,3,2-dithiarosolan-2-yl)-resorufin (ReAsH-EDT$_2$).

An orange solid was obtained. Analysis on HPLC (Phenomenex Aeris Peptide 3.6 µm C_{18}): t_R = 13.3 min. with gradient from 100 : 0 to 10 : 90 (5 mM NH_4CO_3 : ACN) in 20 min. showed purity of 97 %.^{1}H NMR (500 Hz, $CDCl_3$, ppm): 3.54 (m, 8H, S-CH_2), 6.22 (d, 2H, *J* = 10 Hz, H-2, 7), 6.30 (d, 2H, *J* = 10 Hz, H-1, 8). ^{13}C NMR (500 Hz, $CDCl_3$, ppm): 14.11, 29.69, 31.92, 33.46, 125.29. MS (*m/z*) calculated for $C_{29}H_{27}As_2NO_6S_4$ $[M-H]^-$ 543.8, found 544.2.

Peptide synthesis

Tetracysteine peptides Ac-FLNCCPGCCMEP-amide (TC12) and Ac-CCPGCC-amide (TC6) were synthesized by solid phase synthesis using the Fmoc strategy. Tenta Gel R Ram resin was utilized for amide peptides and 2-chlorotrityl for carboxyl peptides. Peptides were

synthesized in Liberty 1 microwave-assisted synthesizer (CEM). Couplings of amino acids were performed with 3 eq. of N-α-Fmoc-protected amino acid, HBTU (3 eq.) and DIEA (5 eq.) in DMF. Peptides were terminated by acetylation with Ac_2O. For that purpose resin was mixed with 4 eq. of Ac_2O, 4 eq. of DIEA in DMF for 4 h. Peptide cleavage was achieved with mixture of 90% of TFA, 5% thioanisole, 3% anisole and 2% 1,2-ethanedithiol over 1.5 h, followed by precipitation in cold (-80°C) diethyl ether. Crude peptide pellets were collected by centrifugation. Peptides were purified on HPLC (Dionex Ultimate 3000) using semi-preparative Phenomenex Gemini-NX C18 column and gradient of 0.1% TFA in acetonitrile with 0.1% TFA. The purified peptide was identified by ESI mass spectrometry using API 2000 (Applied Biosystems) instrument. MS (*m/z*) calculated for TC12 and TC6 $[M+H]^+$ were 1358.7, 626.8 and found 1358.4, 626.6, respectively.

Physicochemical properties of biarsenical probes

Electronic spectra were obtained on a Jasco V-650 spectrophotometer. Fluorescence was recorded on a Horiba Scientific FluoroMax-4 spectrofluorimeter. All measurements were recorded in 50 mM Na^+-HEPES buffer with 100 mM NaCl and 200 μM TCEP at pH 7.4, 25°C. Excitation wavelengths were chosen based on the biarsenical-probe complex's maximal absorption. All probe–peptide conjugates were prepared with the optimized tetracysteine peptide, TC12.[15] Probe–peptide conjugates were obtained by initial incubation of 10 μM biarsenical probe with 15 μM TC peptide. All spectra were recorded after 2 h.

Determination of pK_a values.

Solutions containing 1 μM biarsenical probe with 6 μM TC12 peptide in 50 mM Na^+-borate buffer with 50 μM TCEP at pH 10.0 were incubated at room temperature until no increase of fluorescence was observed. Samples were then titrated with HCl, and the resulting and pH

and fluorescence intensities were measured and fitted using Hill's equation (Eq. S1, ESI†) in order to obtain pK_a values and Hill coefficients for the phenolic group at position 3′. When TC6 peptide was used the same methodology was applied and data was fitted to Hill's equation (Eq. S2, ESI†)

Stability of biarsenical probes.

Solutions of 25 μM biarsenical probes in 50 mM Na^+-HEPES buffer and 100 mM NaCl at pH 7.4 were incubated in darkness over periods of 1, 2, 3 or 6 h and analyzed by analytical HPLC (Dionex Ultimate 3000 using column C18 Phenomenex Aeris Peptide) using a 5 mM ammonium carbonate/acetonitrile gradient. Since monoarsenical and biarsenical species have absorption maxima that differ by ~10 nm, the percent composition of the two species was estimated by comparing the peak areas from chromatograms collected at 280 nm.

Protein expression and purification

Plasmids encoding the wild-type and mutant forms of six-histidine tagged TCPTP and HePTP were described previously (see Table 2 for mutant amino-acid sequences).[5-7] The appropriate plasmids were used to transform BL21(DE3)-codonPLUS-RIL *E. coli*. Single colonies were picked and used to inoculate 1000 ml LB cultures, which were grown to mid-log phase at 37°C, and induced with either 0.2 mM IPTG (TCPTP) or 0.04% arabinose (HePTP) for 16 h at 26°C. Bacterial cells were centrifuged and frozen at -20 ^oC. Cell lysis was achieved by pellet sonication on ice at 80 W for 5 seconds with 10 second intervals. Enzyme purification was carried out using Ni-NTA Agarose (Qiagen) according to manufacturer's instructions. Purified proteins were concentrated on 7 kDa cut-off CentriPrep Centrifugal Filter Devices (Milipore) and exchanged into pH 7.0 buffer containing 50 mM 3,3-dimethylglutarate, 1 mM EDTA, 150 mM NaCl, and 1 mM dithiothreitol (DTT), flash-

frozen in liquid nitrogen, and stored at -80°C. Protein purity was assessed by SDS-PAGE. Protein concentrations were determined spectrophotometrically utilizing Bradford method using BSA as a standard.[28]

PTP activity assay with *p*NPP substrate

Solutions of TCPTP or HePTP (0.24 μM) in PTP activity buffer (50 mM 3,3-dimethylglutaric acid pH 7.0, 50 mM NaCl, 1 mM EDTA with 20 μM TCEP) were incubated in darkness with or without biarsenical probe (0.96 μM) in protein low-binding tubes (Eppendorf). The final concentration of DTT from storage buffer was less than 1 μM. After 2.5 h incubation at room temperature, the enzymatic activity was assayed using *para*-nitrophenylphosphate (*p*NPP). Reactions were carried out in a total volume of 200 μL at 22°C. To a solution of 0.25-10 mM *p*NPP in PTP activity buffer, 100 nM of appropriate PTP enzyme was added. After 3-8 min, depending on the enzyme activity, reactions were quenched by the addition of 40 μL of 5 M NaOH. The absorbance of the hydrolyzed product was measured at 405 nm in a microplate reader (Asys UVM 340, Biochrom). All experiments were performed in triplicates. Enzymatic kinetic parameters were obtained by nonlinear regression fitted to the Michaelis-Menten equation using Origin 8.6 software. The calculation of catalytic efficiency error is described in Supplementary information (ESI†).

PTP activity assays with phosphorylated peptide substrate

PTP activities were assayed using the fluorogenic (FRET) phosphorylated peptide MCA-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-amide (Sigma-Aldrich, SCP0229) in a modified PTP-chymotrypsin coupled assay.[26] MCA and DNP correspond to 7-methoxycumarin-4-acetyl (donor) and 2,4-dinitrophenyl (quencher) moiety, respectively. The assay, which monitors the fluorescence increase induced by peptide cleavage once the

peptide is dephosphorylated, was performed using enzymes with and without biarsenical probes. To a cuvette containing PTP activity buffer with 50 μM TCEP, 500 nM phosphorylated peptide (dissolved in DMSO) was added. Next, chymotrypsin in PTP activity buffer (final concentration 0.05% (w/v) – 20 μM) was added and the reaction was monitored on a Fluoromax-4 spectrofluorimeter (excitation: 328 nm, emission: 395 nm). Once chymotrypsin had cleaved all of the dephosphorylated peptide present in the phosphopeptide solution, TCPTP was added (wild-type or mutant, with or without a biarsenical probe) to a final concentration of 1 nM. Prior to the measurement the enzyme was incubated for 2.5 h with in molar ratios of TCPTP to biarsenical probes of 1 to 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4 or 8, prepared as described above. All experiments were performed in triplicates.

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Table 1. Spectral and chemical properties of biarsenical probes conjugated to the peptide Ac-FLNCCPGCCMEP-amide (TC12). Superscripted numbers indicate references for previously published values.

Probe-peptide complex	λ_{ex} / λ_{em} (nm)	pK_{a1}	pK_{a2}	pK_a of parent fluorophore
FlAsH-TC12	509/530 (511/527)[20]	5.07 ± 0.01 (5.4)[8]	6.71 ± 0.06	6.43[21]
F2FlAsH-TC12	510/530 (500/522)[14]	3.66 ± 0.02	6.24 ± 0.04	4.8[16]
Cl2FlAsH-TC12	519/539 (520/539)[12]	3.32 ± 0.03	6.36 ± 0.08	4.72[17]
Et2FlAsH-TC12	521/542	4.93 ± 0.01	7.09 ± 0.04	6.61 ± 0.03[18]
F4FlAsH-TC12	528/545 (528/544)[14]	4.95 ± 0.02	7.2 ± 0.1	6.1[16]
Cl4FlAsH-TC12	530/546	5.02 ± 0.01	6.96 ± 0.08	5.97[17]
5-CrAsH-TC12	511/536 (-/534)[a,13]	5.36 ± 0.01	7.6 ± 0.1	6.4[22]
5-BuCrAsH-TC12	512/538	5.19 ± 0.01	6.74 ± 0.05	-
ReAsH-TC12	597/606 (597/608)[15]	4.180 ± 0.007	6.21 ± 0.02	5.8[23]

[a] Theses values were determined using peptides with unoptimized TC sequence - CCXXCC.

Table 2. Dissociation constants of biarsenical-PTP enzyme complexes. The values were determined by dose-dependent inhibition of the indicated probe-responsive TCPTP mutants. The enzyme (final concentration 1 nM) was incubated for 2.5h with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4 or 8 equivalents of biarsenical probe. Experimental data, partially shown in Fig. 8 were fitted to Equation S6 (ESI†)

Biarsenical probe	P181C/E187C (nM)	P181C+2C (nM)	4C (nM)
FlAsH-EDT_2	0.60 ± 0.09	2.1 ± 0.3	0.8 ± 0.1
F2FlAsH-EDT_2	1.1 ± 0.3	0.15 ± 0.04	0.09 ± 0.03
Cl2FlAsH-EDT_2	1.2 ± 0.3	0.9 ± 0.2	1.2 ± 0.4
Et2FlAsH-EDT_2	0.8 ± 0.2	0.6 ± 0.2	1.1 ± 0.3

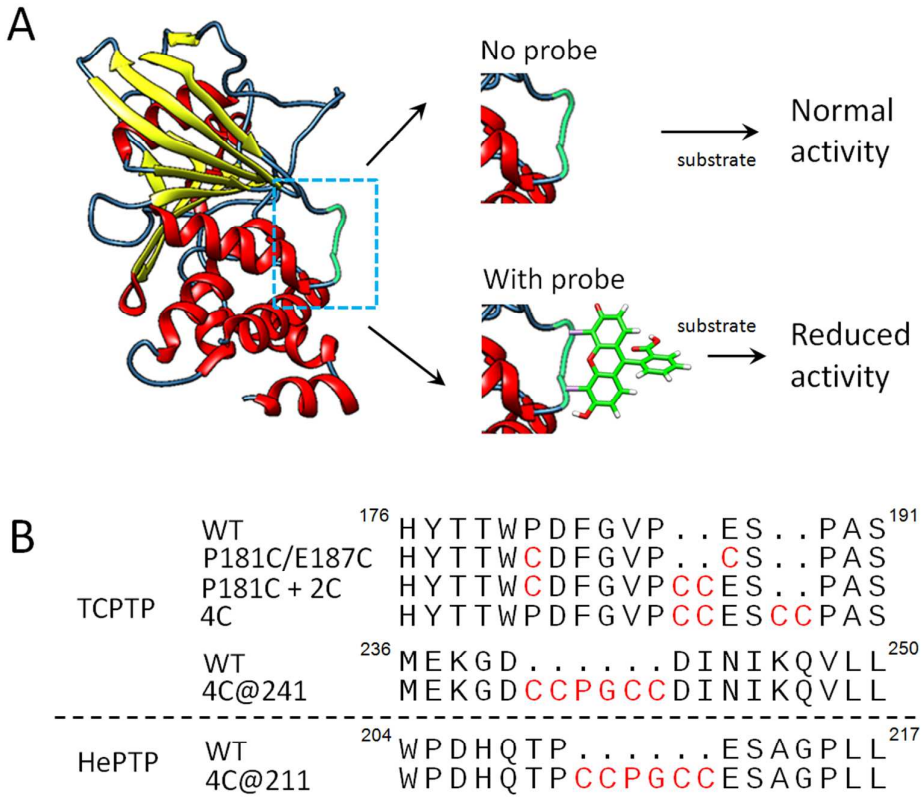
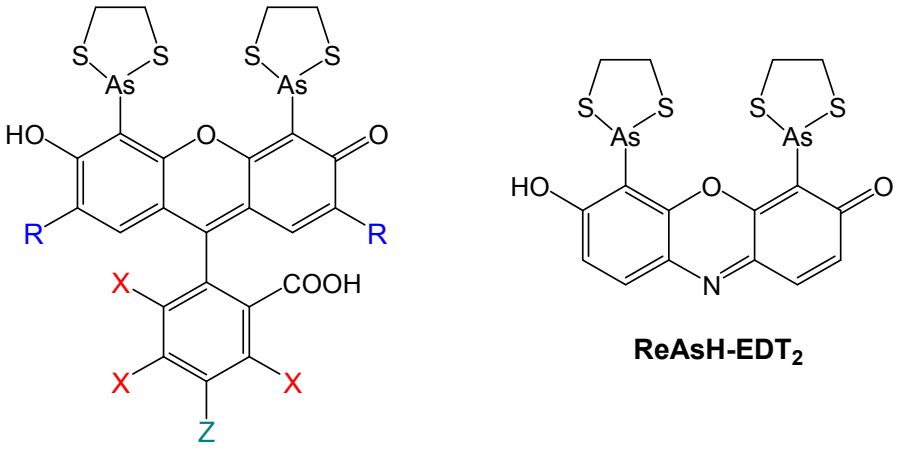


Figure 1. Biarsenical probe acting as an allosteric inhibitor. (A) Spatially arranged cysteines are introduced (light green) in the WPD loop of a protein tyrosine phosphatase (PTP). The mutation causes only limited changes in the catalytic efficiency of the enzyme. However, when the biarsenical probe FlAsH is conjugated, the enzyme is strongly inhibited. (B) Amino acid sequences of wild type and cysteine mutants of TCPTP and HePTP used in this study. Red letters highlight amino acid residues substituted or added to the wild type protein sequence. Protein model was based on structure of TCPTP (PBD: 1L8K).



R,X,Z = H ---------- **FlAsH-EDT$_2$**

R = F X,Z = H -------- **F2FlAsH-EDT$_2$**

R = Cl X,Z = H ------- **Cl2FlAsH-EDT$_2$**

R = C_2H_5 X,Z = H---- **Et2FlAsH-EDT$_2$**

R,X = H Z = COOH ---------- **5-CrAsH-EDT$_2$**

R = H X,Z = F ---------- **F4FlAsH-EDT$_2$**

R = H X,Z = Cl ---------- **Cl4FlAsH-EDT$_2$**

R,X = H Z = CO-NH-C_4H_9 -- **5-BuCrAsH-EDT$_2$**

Figure 2. Structures of biarsenical EDT-capped probes based on different parent fluorophores used in this work.

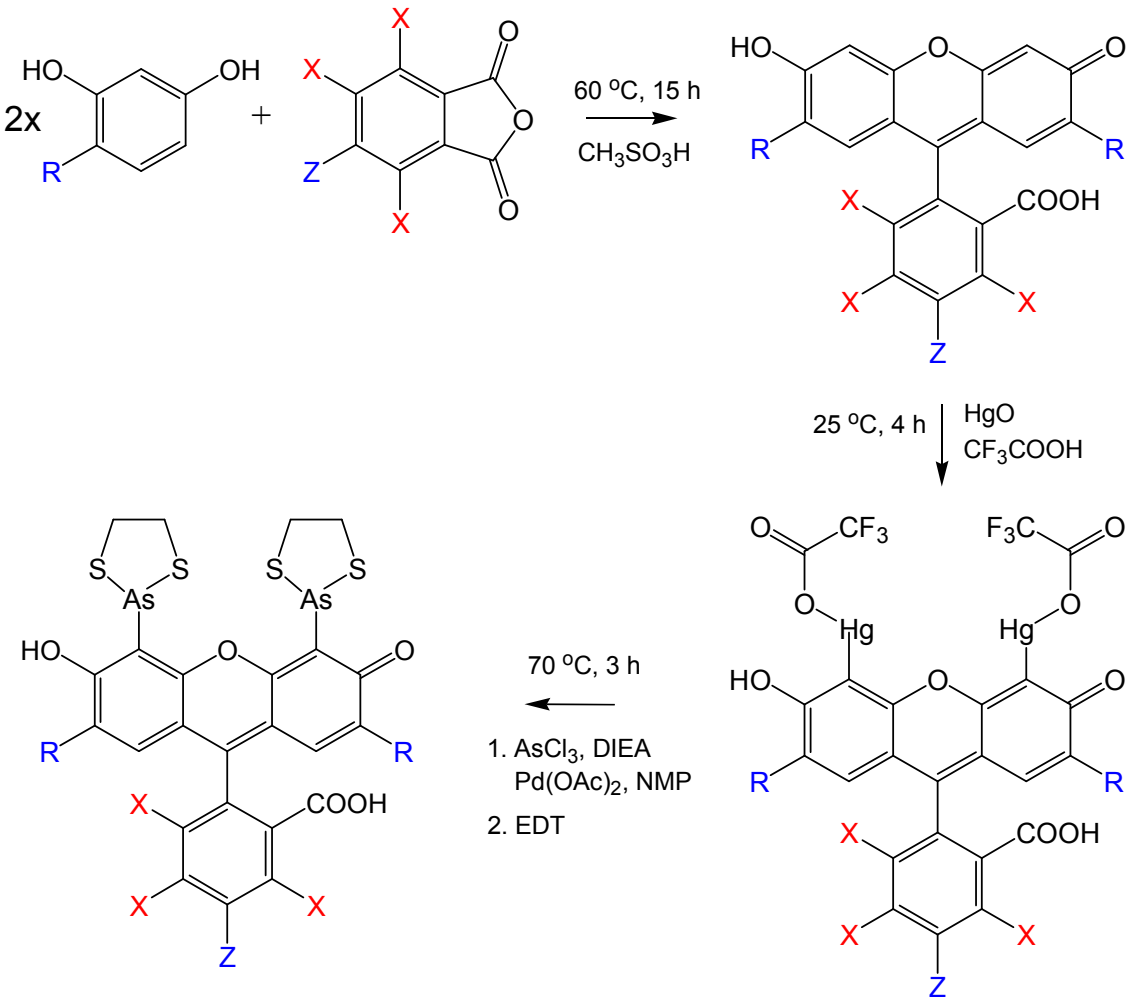


Figure 3. Schematic pathway for the synthesis of biarsenical probes built on the fluorescein platform. Stages include the synthesis of parent the fluorophore, its subsequent mercurization, transmetalation with $AsCl_3$, and final capping with EDT. It should be noted that first reaction results in forming of two isomers, where Z is located at position 5 or 6 in the product. Since the probes used in this study are single isomers the Z was placed at position 5.

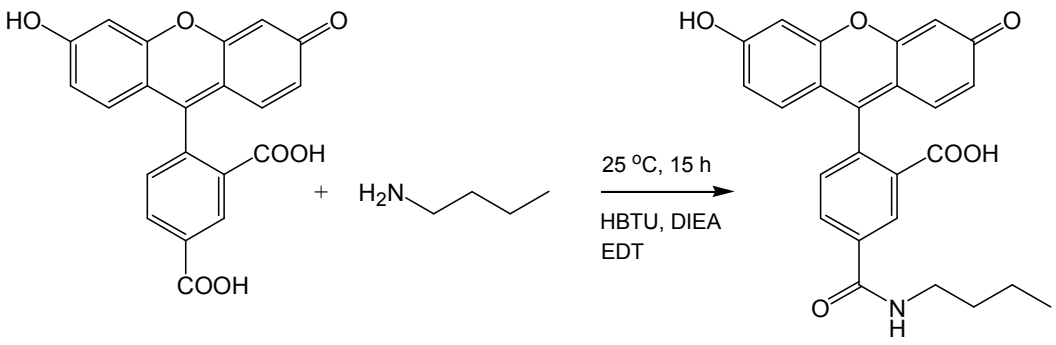


Figure 4. Synthesis of 5-(N-butylamide)-fluorescein, a parent platform used for the synthesis of 5-BuCrAsH-EDT_2.

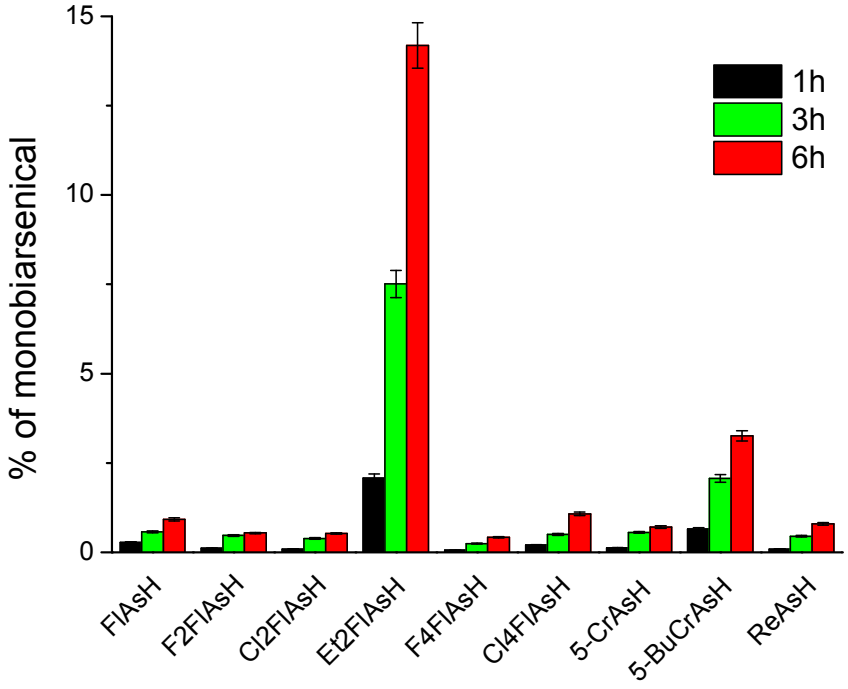


Figure 5. Decomposition of biarsenical probes in aqueous pH buffered solution. 25 μM biarsenical probes were incubated for 1, 3 and 6 h in 50 mM Na+-HEPES buffer, 100 mM NaCl, pH 7.4. The percentage of monoarsenical species was determined using analytical HPLC. Details can be found in Table S1 (ESI†).

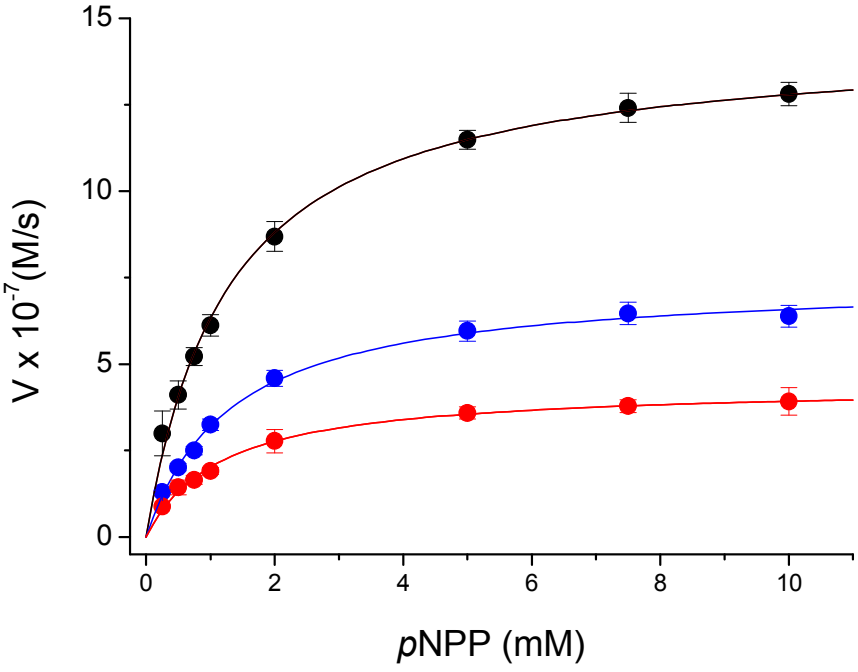


Figure 6. Michaelis-Menten plot for TCPTP 4C incubated without biarsenical probe (black), with FlAsH-EDT_2 (blue) or Et2FlAsH-EDT_2 (red). Assay was performed at various concentrations of *para*-nitrophenylphosphate (*p*NPP) using 100 nM enzyme or enzyme-biarsenical complex as described in Materials and Methods. Data was fitted to the Michaelis-Menten equation.

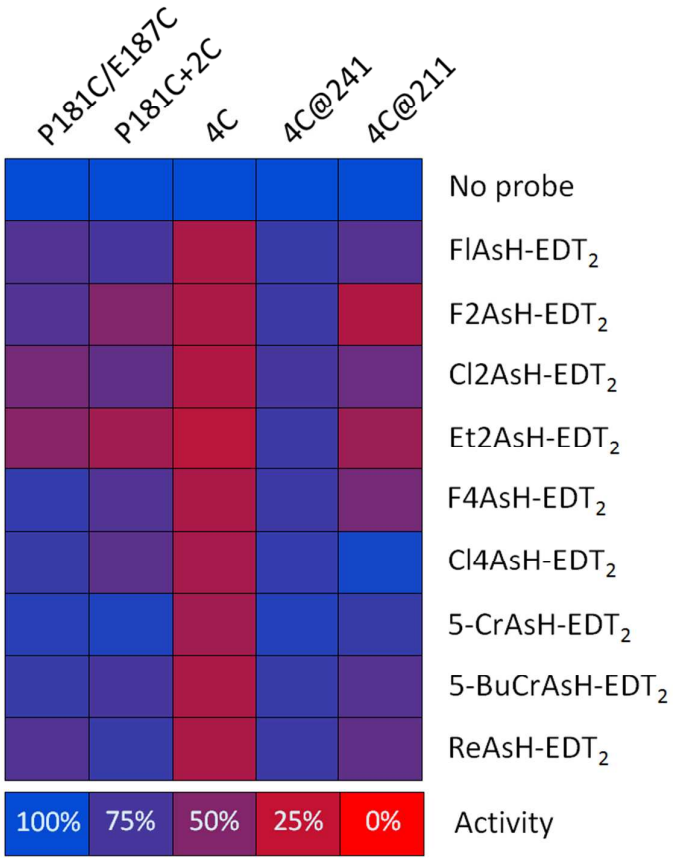


Figure 7. Graphical representation of catalytic efficiency (k_{cat}/K_m, s^{-1} mM^{-1}) values for PTP mutants in the presence of 4 equivalents of biarsenical probes and assayed with *p*NPP. Blue color demonstrate the typical enzyme activity (not inhibited) and red complete inhibition of activity. Gradient color is directly proportional to the degree of inhibition of the enzyme. k_{cat}, K_m and k_{cat}/K_m values are listed with their errors in Table S2-S4 (ESI†).

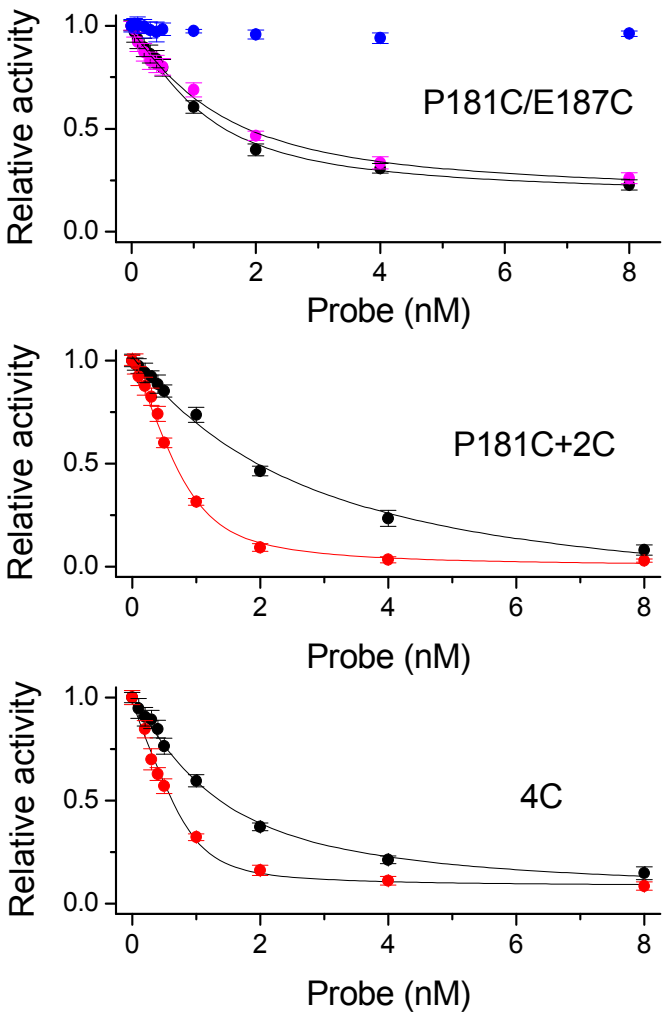


Figure 8. Dose-dependent inhibition of the biarsenical-responsive TCPTP mutants. The indicated enzyme (final concentration 1 nM) was incubated for 2.5 h with 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 1, 2, 4 or 8 molar equivalents of biarsenical probe – FlAsH-EDT_2 (black), F2FlAsH-EDT_2 (red), Cl2FlAsH EDT_2 (magenta). Blue demonstrates the activity of wild-type TCPTP incubated with F2FlAsH-EDT_2 for the comparison. The activity was measured using fluorogenic phosphorylated peptide substrate MCA-Gly-Asp-Ala-Glu-Tyr(PO_3H_2)-Ala-Ala-Lys(DNP)-Arg-amide.

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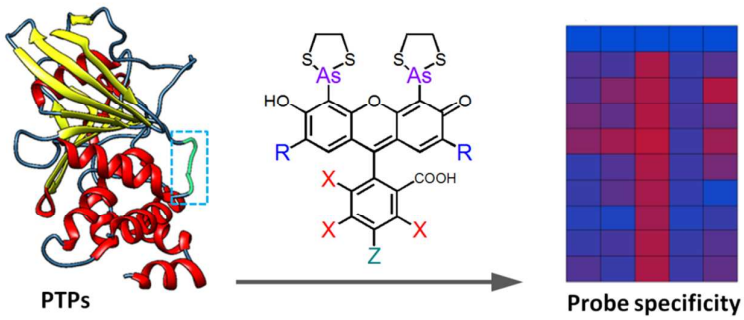
Graphical abstract



A library of biarsenical probes was developed, characterized and used to probe structure-activity relationships for inhibition of sensitized protein tyrosine phosphatases (PTPs), revealing the superior inhibitory properties of 2'7'-substituted biarsenicals.