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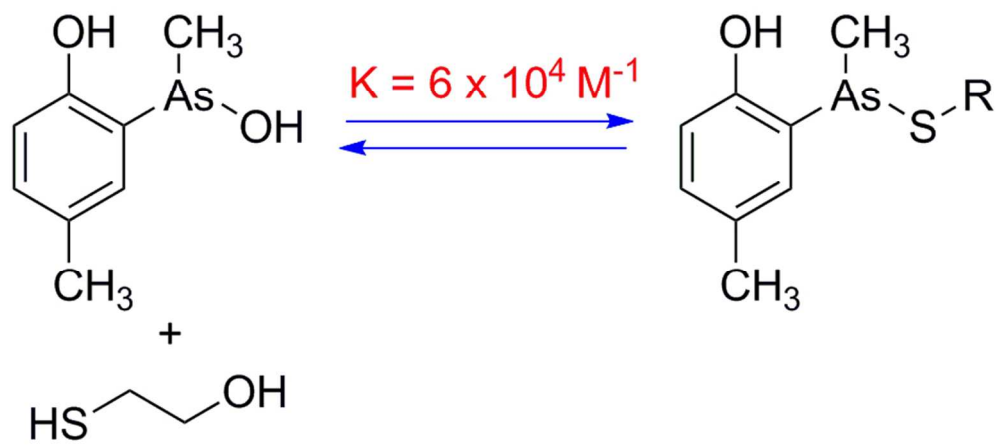


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Arsinous Acid as a Thiol Binding Group: Potential Cysteine Peptide Tagging Functionality that Binds a Single Thiol[†]

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A simple aryl arsinous acid (ArAs(CH₃)OH) was prepared by ortho mercuration of p-cresol followed by Pd-catalyzed reaction with methylarsenic dibromide, purification as the mercaptoethanol adduct, and deprotection using a silver salt. Isothermal titration calorimetry experiments showed reaction with mercaptoethanol with an association constant of $6 \times 10^4 \text{ M}^{-1}$. Rapid exchange of thiols at the arsenic atom was demonstrated by ¹H-NMR.

The fluorescent labeling of proteins, especially within living cells, has been a valuable tool for studying protein function.¹⁻⁴ Of particular value has been fusion of the green fluorescent protein and its variants for visualization and tracking of the protein of interest.⁵ The substantial size of the fluorescent protein domain and the time delay from translation to formation of the fluorophore limit many applications. A very useful development has been the fluorescent bisarsenical probe FIAsh, commonly used as the bis-ethanedithiol adduct **1** and related analogues **2** pioneered by Tsien's group.⁶ FIAsh usually labels a simple tetracysteine motif CCXXCC (commonly CCPGCC), though binding to other sequences and "bipartite" dicysteine pairs has also been demonstrated.⁷⁻⁸ The need for orthogonal probes that bind alternate cysteine arrangements has been recognized, and demonstrated with some success in the probe AsCy₃ **3**, which targets a sequence of two cysteine pairs separated by five amino acids.⁹

A limitation in design of arsinous acid-based tags is that the binding of two cysteine thiols to a single arsenic atom requires that the peptide or protein adopt a structure which allows formation

Fig. 1. FIAsh and Other Fluorescent Bisarsenical Probes as their Ethanedithiol Adducts.

of a cyclic complex. While providing high affinity per arsenic functionality, this may require major conformation change of the dithiol structure, as illustrated in Fig. 2, and may not permit binding to a regular repeating peptide structure. Indeed, FIAsh and other arsenic (III) compounds do not generally bind cysteine pairs within

α -helical peptides but show highest affinity for a structure designed for hairpin formation.^{6, 10-11} Cline et al. have shown that $\text{CH}_3\text{As}(\text{OH})_2$ binds to $i,i+1$; $i,i+2$; $i,i+3$; and $i,i+4$ dicysteine pairs with almost equal affinity, while CD measurements indicate the α -helical population is increased in binding to the $i,i+4$ peptide but decreased in other dicysteine arrangements.¹⁰ An alternate and perhaps more flexible approach would be to utilize arsenic functionality in which each arsenic atom binds to only a single thiol group, displayed on a backbone structure such that they are positioned for binding to a specifically spaced cysteine pair within an α -helical peptide or other regular structure. Such an approach might be achieved using arsinous acids ($\text{RR}'\text{AsOH}$), having only one arsenic-oxygen bond for displacement by a thiol. Such compounds might also be less toxic than the arsine oxides ($\text{RAs}=\text{O}$) or the hydrated arsonous acid form ($\text{RAs}(\text{OH})_2$), which apparently exhibit their toxicity by forming cyclic adducts with essential dithiols.¹²⁻¹⁴

Fig. 2. Major Conformational Change Required for Formation of a Cyclic Adduct 6 between a Dithiol 4 and an Arsonous Acid or Arsine Oxide 5.

We sought to develop synthetic methodology and explore thiol binding properties of an arsinous acid. The limited literature on arsinous acid synthesis rely on either reaction of a Grignard reagent with an alkyl arsenic dihalide or similar reagent (Fig. 3a) or nucleophilic substitution by an arsonous acid dianion on an alkyl halide followed by reduction of the initial product (Fig. 3b).¹⁵ Synthesis of FIAsH and related arsonous acids is accomplished by mercuration ortho to a phenolic hydroxyl group followed by a Pd-

Fig. 3 Synthetic Approaches to Arsinic Acid and Aryl Arsonic Acid Derivatives.

catalyzed substitution of the resulting aryl-mercurial with arsenic trichloride (Fig. 3c).⁶ The resulting aryl arsenic dichloride product is isolated as a dithiol adduct and subsequently hydrolyzed to the arsonic acid.

We chose *p*-cresol **7** as the precursor for a simple model compound, with the methyl group serving to block substitution at the *para*-position. The *ortho*-mercuration of **7** followed standard procedures for the mercuration of phenols also used in the synthesis of FIAsH and derivatives (scheme 1). Formation of the methyl-substituted arsinous acid derivative required reaction with a methylarsenic dihalide rather than the trihalide. While methylarsenic dibromide and the corresponding dichloride are both known compounds, they are not available from common sources and a convenient synthesis is not described.¹⁶⁻¹⁷ We chose to make and use the dibromide in part because of its lower volatility and resulting greater ease of handling relative to the dichloride. Reaction of the commercially available cacodylic acid with concentrated HBr formed the product in a retro-Arbusov type reaction (scheme 1),¹⁸ and the product was isolated by extraction into dichloromethane. The Pd-catalyzed mercury to arsenic substitution followed procedures for the reaction with arsenic trichloride but to our knowledge had not been demonstrated with an arsenic bromide or with any alkyl dihaloarsenic derivative.¹⁹ The reaction proceeded and the product was isolated as the mercaptoethanol adduct **8**, which was purified by chromatography on silica gel. Mercaptoethanol was chosen to improve the aqueous solubility of the thiol adduct, though that feature was not utilized in the work reported here. Efforts to cleave the arsenic-sulfur bond with a mercury salt by published procedures gave a mixture of products.⁶ We developed an alternate deprotection procedure using silver nitrate in methanol. The silver-thiolate complex was removed by filtration after addition of a small amount of lithium chloride to precipitate excess silver ion.

Scheme 1. Completed Synthesis of an Arsinous Acid Model Compound 9.

The methanol adduct isolated upon evaporation of methanol was converted to the arsenious acid form **9** when dissolved in water and was used directly without further purification. The products **8** and **9** appear quite stable and show no decomposition upon standing in pure form or in DMSO or aq. solution.

Formation of the complex **8** between **9** and mercaptoethanol was studied by isothermal titration calorimetry (Fig. 4). An association constant of $6 \times 10^4 \text{ M}^{-1}$ was determined. This represents approximately 4-fold and 30-fold greater affinity than the values of $1.6 \times 10^4 \text{ M}^{-1}$ and $1.9 \times 10^3 \text{ M}^{-1}$ for the first and second association constants for binding of methylarsonous acid [$\text{CH}_3\text{As}(\text{OH})_2$] to two equivalents of thiol.¹² The high affinity of **9** for thiols further supports this approach to the development of selective dicysteine peptide receptors. Selectivity of arsenic functionality for thiol groups over other functional groups present in proteins is illustrated in the binding of arsenous and arsenious acids to glutathione, mercaptoalcohols, and related compounds.^{12,20} Formation of the arsenic-thiol complex is rapid on the time-scale of the ITC experiment.

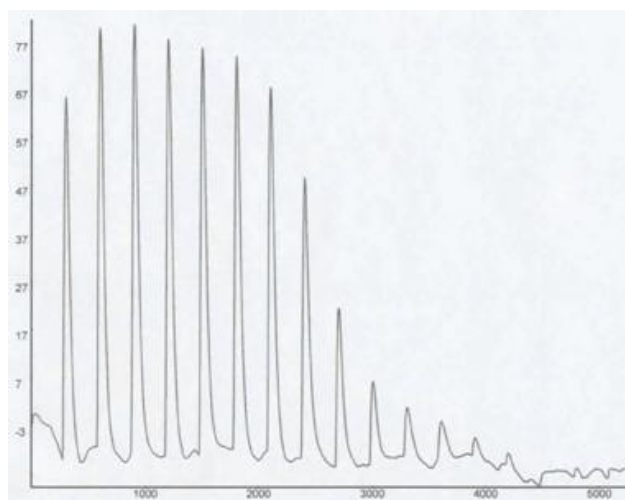


Figure 4. Isothermal titration calorimetry profile for titration of **0.5 mM 9** in pH 7 phosphate buffer (1.3 mL) with **10 μL** injections of **6 mM 2-mercaptoethanol**.

To address the rate of thiol exchange, 3 eq. of cysteine were added to a 9 mM solution of **8** in D_2O in an NMR tube. The $^1\text{H-NMR}$ spectrum was taken immediately (about 2 min to mid-point of acquisition) and again after 30 minutes and the ratio of the mercaptoethanol and cysteine adducts was determined by integration. The spectra indicated that complete equilibration was achieved prior to the first spectrum. While this does not provide an accurate rate measurement or controlled conditions of pH, etc., it does indicate the kind of rapid exchange necessary for a peptide binding agent.

This work has demonstrated a synthetic approach to an arsenious acid and the high affinity and reversible binding to a thiol. These results provide a foundation for a new design approach to arsenic-based receptors for cysteine peptides in which each arsenic functionality binds a single cysteine thiol group.

Experimental

Methylarsenic dibromide

Cacodylic acid (10.0 g, 0.0725 mol) was dissolved in 48% aq. HBr (57 mL) and the mixture was heated at 130°C for 5 hr. The mixture was allowed to warm to room temp. and was extracted with dichloromethane (3 x 30 mL). The combined organic layers were dried over MgSO_4 and evaporated to give methylarsenic dibromide (15.3 g, .0612 mol, 84% yield) which was used without further purification. $^1\text{H-NMR}$ (CDCl_3) δ 2.61 (s) (lit.,¹⁷ δ 2.61 (CCl_4)).

Mercuric acetate derivative of p-cresol

To a solution of p-cresol **7** (4.32 g, 40 mmol) in methanol (180 mL) at room temp. was added acetic acid (2 mL) and mercuric acetate (12.8 g, 40 mmol). The mixture was heated in a 70°C bath for 24 h, then allowed to cool to room temp. The suspension was filtered to remove the insoluble solid which by $^1\text{H-NMR}$ appears to be the 2,6-bis-mercurated product (aromatic singlet at δ 6.94 in DMSO-d_6). The filtrate was evaporated to about 10 mL, 40 mL water was added, and the mixture was cooled to 4°C for 40 minutes. The resulting suspension was vacuum filtered to recover the solid product. $^1\text{H-NMR}$ indicated about 20% unreacted **7** along with the desired monomercurated product. $^1\text{H-NMR}$ δ_{H} (300 MHz: DMSO-d_6) 2.02 (3 H, s, OAc), 2.20 (3 H, s, Ar- CH_3), 6.73 (1 H, d, $J = 8 \text{ Hz}$), 6.93 (1 H, dd, $J = 2, 8 \text{ Hz}$), 7.01 (1 H, d, $J = 2 \text{ Hz}$). This crude product was stable indefinitely at room temp., and was used in the next step without purification.

2-(((2-hydroxyethyl)thio)(methyl)arsino)-4-methylphenol **8**

To the crude mercuric acetate derivative of p-cresol (0.54 g, 1.47 mmol) and palladium(II) acetate trimer (8 mg, .036 mmol Pd) under N_2 was added a solution of methylarsenic dibromide (1.30 g, 5.0 mmol) in dry THF (17 mL). Diisopropylethylamine (1.20 mL, 7.5 mmol) was added and the solution was stirred at room temp. for 18 h. The reaction mixture was poured into a solution of 2-mercaptoethanol (0.80 mL, 0.89 g, 11.4 mmol) in aq. sodium phosphate (70 mL, 0.25 M, pH 7.0) and acetone (70 mL). The mixture was stirred at room temp. for 1 hr and extracted with ether (3 x 50 mL). The combined organic layers were dried over MgSO_4 , evaporated, and purified by chromatography on silica gel (25 to 65% acetone in heptane)²¹ to give **8** as a white powder (0.23 g, 0.84 mmol, 57% yield). $^1\text{H-NMR}$ δ_{H} (300 MHz: CDCl_3) 1.62 (3 H, s, As- CH_3), 2.28 (3 H, s, Ar- CH_3), 2.92 (2 H, t, $J = \text{Hz}$, S- CH_2), 3.79 (2 H, m, $\text{CH}_2\text{-OH}$), 6.75 (1 H, d, $J = 8.1 \text{ Hz}$), 7.05 (1 H, dd, $J = 2, 8.1 \text{ Hz}$), 7.20 (1 H, d, $J = 2 \text{ Hz}$). $^{13}\text{C NMR}$ (75 MHz, CDCl_3): $\delta = 11.80$ (CH_3As), 20.50 (CH_2S), 34.96 (Ar- CH_3), 62.87 (CH_2O), 116.29 (CH), 124.88 (C), 130.30(CH), 131.78 (C), 132.24 (CH), 156.46 (C); HRMS (ESI): m/z calcd for $\text{C}_{10}\text{H}_{15}\text{AsO}_2\text{S}$ [M-H]⁻: 272.9936; found: 272.9939

2-(hydroxy(methyl)arsino)-4-methylphenol **9**

8 (0.0079 g, 28.5 μmol) was dissolved in 8 drops methanol in a test tube and a solution of silver nitrate in methanol (1.25 mL, 0.025 M, 31.25 μmol) was added, with immediate formation of a light yellow

precipitate. After 30 minutes, a solution of lithium chloride in methanol (50 μ L, 0.2 M, 10 μ mol) was added. After 10 minutes the suspension was filtered through a cotton plug, the filtrate was evaporated, and the residue was dissolved in DMSO- d_6 (0.7 mL). $^1\text{H-NMR}$ δ_{H} (300 MHz; DMSO- d_6) 1.46 (3 H, s, As- CH_3), 2.20 (3 H, s, Ar- CH_3), 6.68 (1 H, d, $J = 8$ Hz), 6.99 (1 H, dd, $J = 2, 8$ Hz), 7.26 (1 H, d, $J = 2$ Hz). 0.1 mL of the solution was mixed with 0.5 mL D_2O and 0.1 mL of a 0.021 M solution of pinacol in D_2O and the concentration of **9** in the NMR sample was determined to be 0.026 M by relative integration of the $^1\text{H-NMR}$ signals of **9** and the methyl singlet of pinacol at δ 1.05. This corresponds to a total yield of **9** of 18.5 μ mol (59% yield).

ITC experiments

ITC experiments were performed on a Model CSC 4200 microcalorimeter (Calorimetry Sciences Corp.) at 25° C with stirring at 297 rpm. The cell (1.3 mL) contained a 0.5 mM solution of **9** (0.65 μ mol) in 5 mM potassium phosphate buffer pH 7.0 containing 2% DMSO. A solution of 2-mercaptoethanol (6.0 mM) in the same buffer with 2% DMSO was added in 23 injections of 10 μ L each, with 300 s between injections. Data was processed using BindWorks 3.0 software.

NMR thiol-exchange experiment

The $^1\text{H-NMR}$ spectrum was taken of a solution of **8** (9 μ mol) in D_2O (1 mL). A solution of L-cysteine hydrochloride in D_2O (27 μ L, 1 M, 27 μ mol) was added and the spectrum was taken immediately and again after 30 minutes. Both spectra showed formation of equal amounts of the free mercaptoethanol S- CH_2 triplet at δ 2.55.

Notes and references

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Electronic Supplementary Information (ESI) available: [$^1\text{H-NMR}$ spectra of **8** and **9**]. See DOI: 10.1039/c000000x/

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