Facile exchange of arsenic between adducts and implications to drug discovery

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Abstract

Arsenic is a well-known poison that lives a double life as a therapeutic agent. Recently, arsenic trioxide has been used to treat the cancer acute promyelocytic leukemia with high efficacy. Unfortunately, arsenic drug development has been road blocked by a lack of understanding of the mechanisms of interaction. This thesis attempts to elucidate these mechanisms by looking at the reaction kinetics and thermodynamics of various arsenic species, including potent new organic arsenicals such as S-dimethylarsino-glutathione (ZIO-101) and S-dimethylarsino-cysteine (DMAC).

Data shows that rapid thiolate exchange of dimethylarsonim, Me_2As^+, occurs when the two compounds are dissolved in aqueous solution. The equilibrium constants of this interthiol transfer were characterised by the integrals of the diastereotopic methylarsonium resonances of the species in ^{1}H NMR. Dynamic NMR was used to characterise rapid intra molecular conformational dynamics which lead to the coalescence of diastereotopic methyl resonances. In addition, rapid thiolate exchange was also shown to occur in monomethylarsonium species as well.

The discovery and characterisation of these facile arsenium transfers allows us to think of the arsenicals in a different manner, instead of binding statically to vicinal thiols, arsenic could hop around various thiolates around the cell.

Abstrait

L'Arsenic est un poison bien connu qui mène une double vie en tant qu'agent thérapeutique. Récemment, le trioxide d'arsenic à été utilisé pour le traitement du cancer de la leucémie aiguë promyélocytaire avec une grande efficacité. Malheureusement, le développement des médicaments basé sur l'arsenic connait un ralentissement à cause du manque de compréhension de leurs mécanismes d'interaction. Cette thèse tente d'élucider ces mécanismes en regardant les réactions cinétiques et thermodynamiques de plusieurs espèces de molécules contenant de l'arsenic, incluant de nouveaux composés organique arsenicales comme le S-diméthylarsinoglutathione (ZIO-101) and S-diméthylarsino-cysteine (DMAC).

Les donnés démontrent que l'échange rapide du thiolate de diméthylarsomium, Me2As+, survient lorsque les deux composés sont dissous en solution aqueuse. Les constantes d'équilibres de cet échange interthiole ont étées caractérisé par les intégraux des résonances diastéréotopiques du méthylarsonium des espèces en conformation dynamique intramoléculaire qui ont mené à la coalescence des méthyles diastéréotopiques en résonance des espèces en RMN de 1H. La RMN dynamique à été utilisé pour caractériser les conformations dynamiques intramoléculaires rapides qui ont menées à la coalescence des résonances des méthyles diastéréotopiques. De plus, il à été

démontré que l'échange rapide des thiolate survient aussi dans les espèces de monométhylarsoniums.

La découverte et caractérisation de ces échanges faciles d'espèces d'arseniums nous force à voir ces arsenicales d'un autre œil, au lieu de se lier statiquement aux thiols vicinaux, l'arsenic pourrait sauter entre différent thiolates autour de la cellule.

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Contributions of Authors

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Statement of originality and contribution to knowledge

The author performed all work outlined in this thesis, including all the work presented in the paper as specified above, under the supervision of Professor D. Scott Bohle. All work presented in this thesis, with the exception of the introductory literature review, is declared by the author to be original scholarship and distinct contributions to knowledge as is mandatory for doctoral theses.

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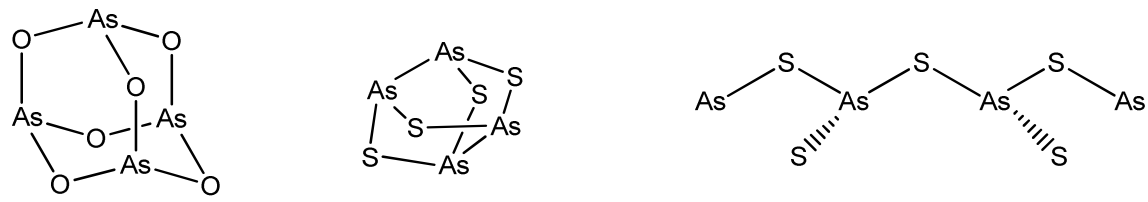
Introduction

1.1 The BioInorganic Chemistry of Arsenic

Arsenic is a group 15 metalloid that is abundant in the earth's crust (ca, 1.8 ppm)[1] in the form of various minerals. In order to fully understand the bioinorganic chemistry of arsenic, the periodicity and chemical reactivity of arsenic must be appreciated. As a metalloid, arsenic exhibits a range of class defying properties and has the characteristics of both metal and non-metals. Elemental arsenic is semiconductor, non-ductile and has the capability of forming various allotropes with various crystal structures[2]. Common inorganic forms of arsenic include arsenic trioxide (As_2O_3) and varies sulfur compounds such as realgar (As_4S_4) and Oripment (As_2S_3).



Arsenic Trioxide Realgar Orpiment

Figure 1: Inorganic forms of arsenic.

In this way arsenic is similar to phosphorus which also forms a variety of sulfur bond compounds.

The chemistry of organic arsenic compounds is just as diverse and varied as its inorganic counterparts. As a pnictogen, arsenic can readily access V, III and -3 oxidation states. Arsenic is closely related to phosphorus in terms of bioinorganic chemistry, and as a result biologically arsenic can occasionally act as a phosphorus analogue. For example enzymes that use phosphate as a substrate recognises arsenate[3]. However one key difference between arsenic and phosphorus is that Arsenic has filled inner *3d* orbitals and phosphorus does not. The *3d* orbital sits between *4s* and *4p* which makes the *4s* orbital less accessible[4]. Consequently Arsenic has a more stable oxidation (III) state whilst keeping the oxidation (V) state available, giving it access to a wider range of chemistry when compared to phosphorus. Ironically it is for this reason arsenic is not as ubiquitous in biology, as nature prefers the more inert phosphorus which is readily used in components such as the phosphate backbone of DNA. In fact many arsenic compounds are toxic to biological species, to the point where arsenic trioxide has gained the notoriety as the "*King of poisons*". Despite its toxicity, arsenic compounds may be required by some biological species. This contrasts to the two heavier elements in group 15, antimony and bismuth, which have no known natural biological function[2]. In terms of toxicity, antimony has similar toxicity to arsenic, whilst bismuth has minimal toxicity. Bismuth has been used in over the counter products such as Pepto-Bismol to treat heartburn.

Recently the interest in the bioinorganic chemistry arsenic has gained a lot of attention. The FDA has approved the arsenic based drug Trisenox in 2000 for the treatment of Acute Promeyloctyic Leukemia, leading to renewed interest in the medical uses of arsenic. The use of arsenicals in other mammals have also received a lot of attention after the FDA revealed a study in 2011[5] linking the use of arsenicals in chicken feed to increased concentrations of inorganic arsenic in chicken livers. The scope of the bioinorganic chemistry of arsenic has also been expanded by the proposal of the arsenic based lifeform GFAJ-1 – offering whole new perspective on arsenic's role in biology. Whilst interest in the arsenic has risen in the past decade, advances have been road blocked by a lack of understanding the mechanism of interaction of arsenic at a chemical level. The objective of this project is to help elucide these mechanisms with a focus on common arsenic reactions in the human body.

This chapter will give an overview of current state of research of arsenic in terms of both biology and chemistry, starting with the metabolism of arsenic in the human body and followed by an in-depth overview the mechanism of arsenic at chemical bond level.

1.1.1 Metabolism of arsenic

In order to fully understand arsenic's metabolism by the body, it is necessary to understand that many arsenic species are in rapidly established dynamic equilibria. It is therefore insufficient to just consider one single species, instead an easier way to rationalise the interaction is to consider arsenic as a general system that involves a rapid equilibrium all of the above states. The dynamic nature of the arsenic species will be discussed in greater detail in section 1.2.3. It could be also argued that the chemistry of arsenic has mostly revolved around the stable forms of arsenic[6], whilst the actual picture is much more distorted as arsenic goes through various unstable and hard to detect species.

Arsenic is ubiquitous in the environment and hence uptake of arsenic by humans is inevitable through food and water. The main form of arsenic that is being absorbed by the body in the inorganic form As (III)[7]. As arsenic is absorbed by the body, it goes through a complicated metabolism pathway that involves a range of oxidation states in addition to methylation on the arsenic. The concern of this section will be focused on the human metabolism of arsenic, however it is important to keep in mind that the metabolism of arsenic between different organisms follows similar patterns[8]. One of the earliest and most referenced studies into arsenic metabolism was done by Challenger in 1945[9] which introduced the formulation of the Challenger pathway. Despite its age, the Challenger pathway is the framework for which the metabolism of arsenic is rationalised.

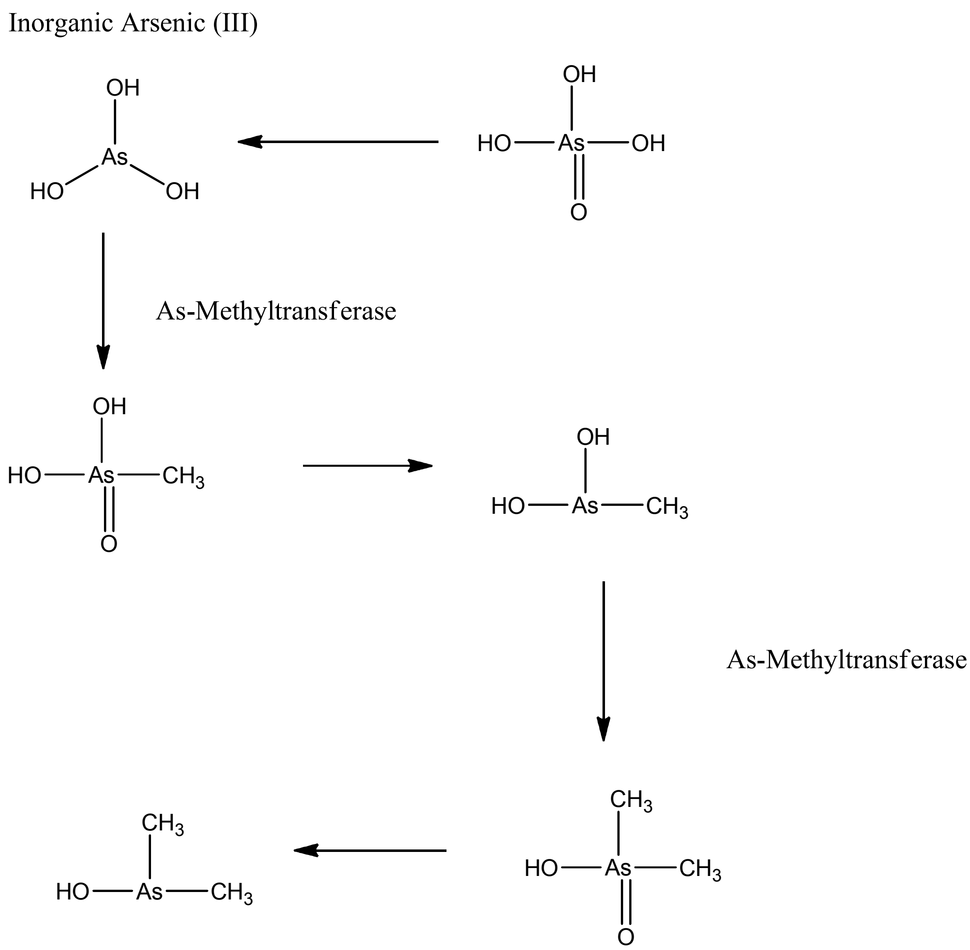


Figure 2: Challenger's proposed pathway of arsenic metabolism

Challenger proposed that arsenic is absorbed as As (V), and is reduced to inorganic As (III) by glutathione (GSH). The arsenite is then methylated and oxidised by As-methyltransferase into mono-methylarsinic acid (V). This is followed by a subsequent reduction to monomethylarsenious acid by GSH. A second methylation then proceeds with the formation of dimethylarsenic acid. Finally the dimethyl arsenious acid. Both methylated and non-methylated species are arsenite are capable of being removed from the body via the liver into urine. Recent developments[10] in the metabolism of arsenic has shown there are additional processes that allows arsenic to exchange between various states such as thiodimethylarsenopropanoic acid and thiodimethylarsenobutanic acid[11]. There have be additional revisions to the pathway proposed by Naranmandura and Hayakawa[12].

The exact reason for the why the body chooses to methylate arsenic in the body is currently not well understood. It has been suggested that reason for the methylation is to reduce the toxicity of arsenic. However, recently this has be disputed as methylated forms of arsenic are shown to be just as toxic as its inorganic counterparts[13].

1.1.2 Arsenic as a poison

Arsenic has an infamous history of being the “King of Poisons”, and in famous deaths such as Napoleon and the horse Phar Lap. The LD_{50} for arsenic trioxide is 35 mg per kg and a fatal dose of could be as low as 100 to 200 mg. The effects of arsenic poisoning appear from half an hour of ingestion. The immediate symptoms include abdominal pain, vomiting diarrhea and salvation and death. There are also long term effects to arsenic poison that include rashes in the form of "*arsenicosis*", cancer and damage to the cardiovascular system.

Figure 3: Photo of arsenic induced arsenicosis, retrieved 2013[14]

The exact mechanism of the arsenic interaction is not well established, but it is believed that different species of arsenic are responsible for a range of toxic responses. The initial symptoms have been proposed to be the result of arsenite (III) binding to viscinal thiols of active enzymes. The binding of arsenic may either cause conformational change in the enzyme or block active sites, hence inhibiting enzyme activity. Although it has been widely regarded as the mechanism of action, there has been little direct evidence to substantiate this[15]. Arsenate (V) have been proposed be toxic in another manner, they are transported into cells via phosphate transport pathways. They then mimic phosphates and is subsequently included into ATP and DNA[8]. As arsenic can do additional chemistry, this may cause mutations in the cell or cellular damage.

Arsenic's poisonous attributes is a large issue today as arsenic is found in ground water. This is a result of the leeching of arsenic from the soil into ground water. This is a major problem in many countries, but most particularly in developing economies where there is little recourse but to these wells which brings this toxic ground water up and causes health problems in the local population.

1.1.3 Treatment of arsenic poisoning

Dimercaprol, also known as British anti-Lewisite (BAL), is used medically as a treatment against arsenic poisoning. BAL has a capability of binding very strongly to arsenic via a bi-dentate bond.

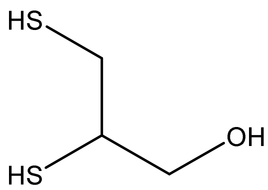


Figure 4: Structure of British Anti-Lewisite (Dimercaprol)

It has been proposed that **BAL** competes with metabolic enzymes for arsenic binding and hence reduces arsenic's toxicity. The chelation would result in subsequent removal of the bound compound from the body via urine.

1.1.4 Medicinal applications of arsenic

It has been proposed by Ehrlich[REF] that arsenic based drugs would get converted to "RAsO" and would interact with metallothionein. The exact mechanism of interaction is not known as little direct evidence was found to support this theory. The underlying pattern is that many enzymes are inhibited by arsenic, especially those containing thiol (SH) groups.

More recently arsenic trioxide (As_2O_3) was discovered to be effective against the cancer Acute Promeylocytic Leumkemia (APL). APL is a rare form of myeloid leukemia that affects an estimated 1500 new patients each year. The discovery of the drug initially came from traditional Chinese therapy followed by major research developments in the US, leading to FDA approval in 2000 under tradename of Trisenox. In the clinical trial for Trisenox, it was reported that 77% of the 582 patients treated were alive after 3 years, compared with just 50% for non-arsenic treatment. More recently, Ziopharm oncology has developed Darinaparsin, an oxidation state III organoarsenical bound to glutathione for the treatment of APL.

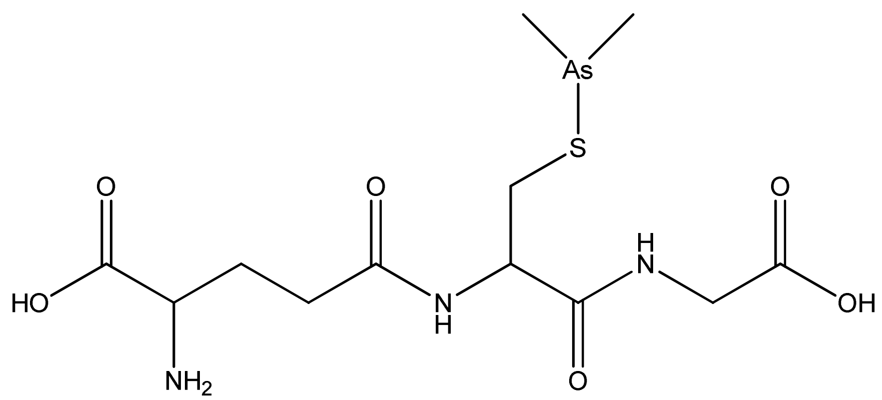


Figure 5: Structure of Darinparsin

Currently the drug is in Phase III trials and has been shown to be more effective that arsenic trioxide with reduced side effects. This is an interesting drug because it is a possible metabolite of arsenic trioxide (see section 1.1.1, the metabolism of arsenic) as Me_2AsOH forms an equilibrium with GSH to form Darinparsin in solution. In addition there are two methyls bound to the arsenic already, preventing it from forming more than 1 bond to thiols in the As(III) ground state.

The mechanism for arsenic's activity in the treatment of APL is not fully understood. Luckily APL is a well-studied cancer and work has been done on the oncoprotein PML-RARα. PML-RARα a fusion protein that of Promyelocytic Leukemia protein PML, and retinoic acid receptor RARα. Zhang et al[16] proposed in 2010 that arsenic trioxide interacts directly with the two zinc fingers in PML by replacing the zinc in the zinc finger as shown in **Figure 6**. They showed using Extended X-ray absortpion fine structure (EXAFS) that arsenic (III) can interact directly with the sulfurs on the cysteine in the zinc finger.

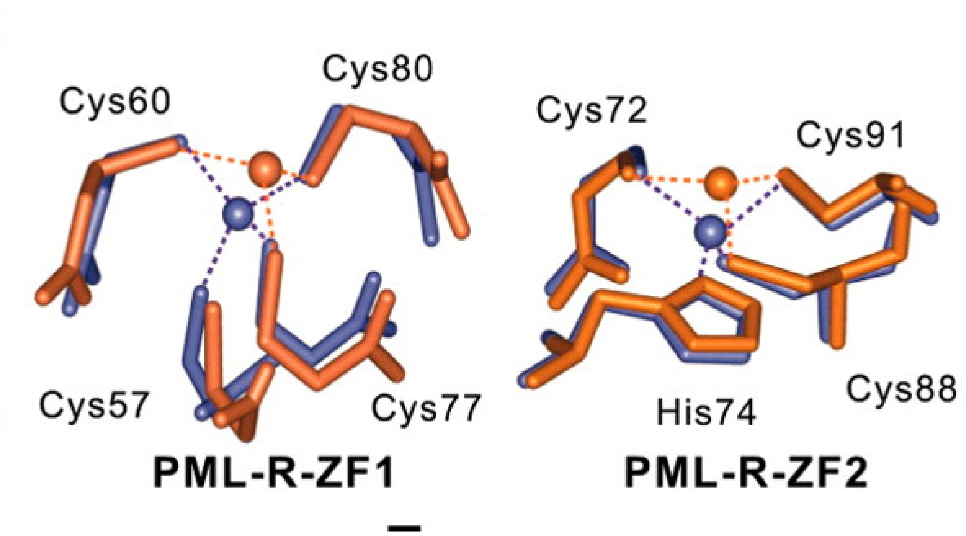


Figure 6: Binding of zinc and arsenic to the PML-R-ZF1 and PML-R-ZF2 Purple: interaction of zinc Orange: Predicted interaction of arsenic.

(Figure from Zhang, et al, 2010, Science16)

They proposed that the coordination of the arsenic causes conformation changes in the protein. These changes would lead to aggregation of this protein and trigger a chain of events that lead to the eventual cell death. The exact mechanism in which the arsenic displaces the zinc is relatively unknown and not much additional work has been done on the arsenic interaction in the zinc finger domain. In addition this proposal does not apply for dimethylated arsenic species such as Darinaparsin as the methyl groups take space in arsenic's co-ordination sphere.

Another proposal for the mechanism of arsenic[17] has been proposed by Lemmand-Breitenbach involves the ability of arsenic to bind to thiols on PML proteins. Once bound, arsenic would form intermolecular disulfide bridges that result in the multimerization of PML into a mesh. It is unclear if arsenic has specificity for the thiols in PML.

1.2 Chemistry of Arsenic compounds

Despite the frequent use of this concept to explain arsenic's poisonous attributes, the chemistry of this interaction is not well understood. This section will outline the approaches taken in chemistry to resolve the kinetics and thermodynamics of the arsenic thiol interaction.

1.2.1 Redox of arsenic by GSH

Cullen et al.[18] first discovered the ability of thiols to reduce Me_2AsOOH(V) to form Me_2AsGSH (III).

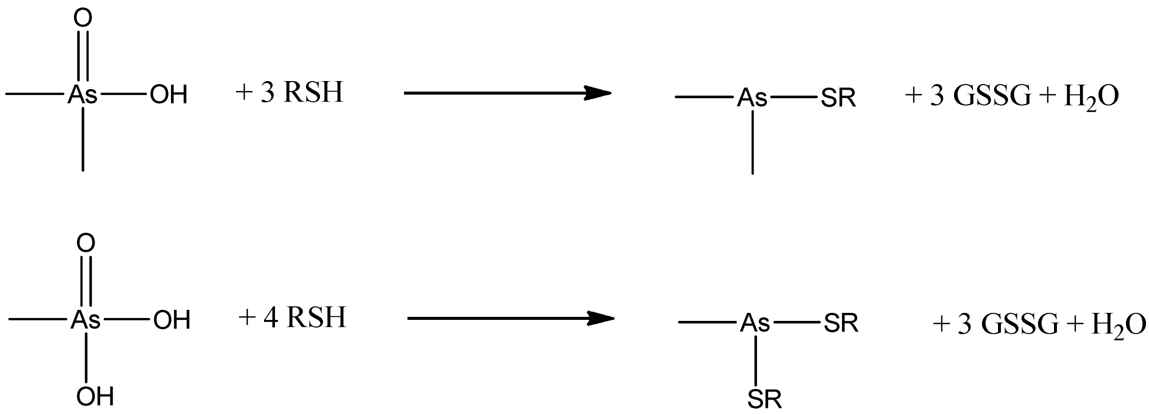


Figure 7: Reduction of dimethylarsinic acid and monomethylarsinic acid by thiols.

They used a range of thiols such as GSH, Cys and mercaptoethanol which had a range of pKas. They concluded that the thiols did not have to be deprotonated before this reduction took place as they could perform this reaction in a range of pH below that of the pKa of the thiol.

Delnomdedieu et al[19] built upon this work using modern NMR spectroscopy. They also noticed at as the pH increased, there is the possibility of the formation of free Me2As$^+$. This is because even though there was no direct peak for that of free Me2As$^+$, the noticed the chemical shift of the methyl peaks would shift slightly as the pH was increased. They proposed change in the chemical shift was caused by the involvement of an increased amount of Me2As$^+$ as solution was basified. Based on this they proposed that the reduction occurred before binding to the thiol. They also noted changes in the spectrum at high pH; the two diastereoteopic methyl peaks would become a single peak. However no kinetic measurements were done on the system.

Carter et al[20] showed the reduction of arsenate to form $As(GS)_3$

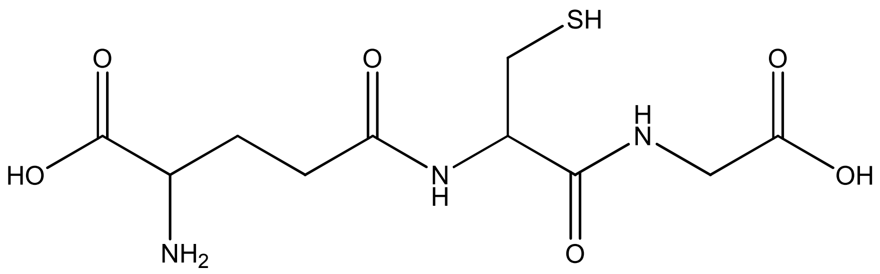
$$3H^+ + AsO_4^{3-} + 5GSH \rightleftharpoons As(SG)_3 + GS\text{-}SG + 4H_2O$$

This reaction was found to happen readily at pH 7 and has been fully characterised by ^1H and ^{13}C NMR. They proposed that like for the methylated systems, the arsenate reduction occurred before the binding to GS as a when used a ratio of 1:2 or less, the formation of $As(SG)_3$ was not observed.

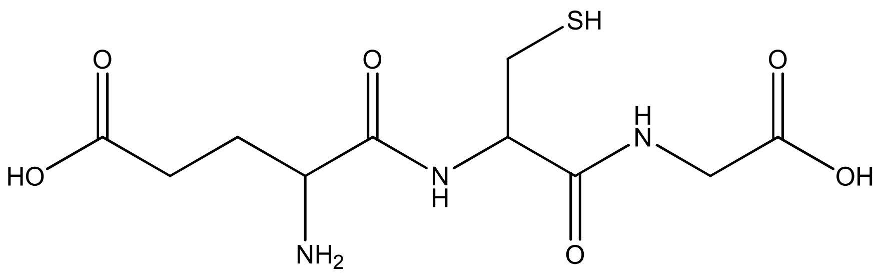
Arsenic (III) species are formed in the presence of a reducing environments. However, in non-reducing aqueous environments, arsenic (III) species are unstable. This has been noted by Cullen et al as they found the Me_2AsCys and Me2AsGS were unstable in solution and rapidly decomposed to their parent oxidation state V acid along with the production of the disulfide.

$$2Me_2AsGS + 2H_2O \xrightarrow{\text{Air}} 2Me_2AsOOH + GSSG$$

As a result, they degassed all their solvents prior to use and noted this improved their yields when compared to that of Zingaro et al[21] who performed similar synthesis under aerobic conditions. However, the exact mechanism of this degradation was not fully understood. More recently Zhao et al[22] examined the degradation of the similar system $As(GS)_3$ using HPLC-ESI-MS. They found that an isomer of α-GSH that was the result of the cleavage of GSSG.



glutathione



alpha - glutathione

Figure 8: Structures of glutathione and alpha-glutathione.

The α -GSH was identified comparing the fragmentation patters of degradation products of $As(GS)_3$ compared to that of standard GSH. They noted that α-GSH is not normally formed from the degradation of GSSG in the absence of arsenic. The mechanism and reason for the formation of this isomer is not known.

1.2.2 Interaction of arsenic with thiols

One of the well-studied systems in terms of thermodynamics is that of $As(OH)_3$ and its interaction with Glutathione to form $As(GSH)_3$. Rey et al[23] used potentiometric and spectroscopic data to show that the formation constant, log K, of $As(GS)_3$ to be 32. They noted that pH played important role in this interaction:

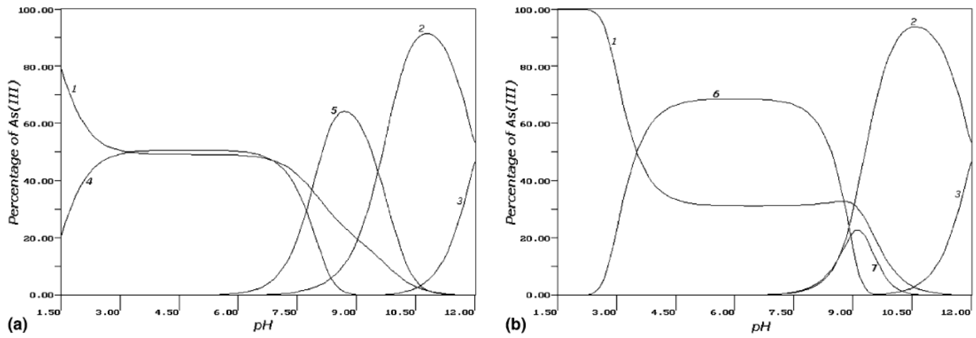


Fig. 2. Species distribution curves for the systems: (a) As–Cys (1) $As(OH)_3$; (2) $As(OH)_2O^-$; (3) $As(OH)O_2^{2-}$; (4) $[As(HCys)_3]$; (5) $As(Cys)(OH)_2]^-$; and (b) As–GSH (1) $As(OH)_3$; (2) $As(OH)_2O^-$; (3) $As(OH)O_2^{2-}$; (6) $[As(HGS)_3]^{3-}$; (7) $[As(GS)(OH)_2]^{2-}$. In both cases the total ligand concentration

Figure 9: Effect of pH on the speciation of arsenic

(Figure from Rey et al, 2004, *J. Inorg. Biochem.*[24])

Further research has been done by Wilcox[15] et al. who used colormetric and calorimetric studies to quantify the formation constants of arsenothiolates.

Table 3. Thermodynamic Properties for the Formation of As(III)−Thiolate Complexes

	$As(OH)_3$	MMA
GSH	1:3 $\beta_3 = 1.8 \times 10^6$ $\Delta G = -8.8$ kcal/mol $\Delta H = -38.7$ kcal/mol $\Delta S = -96$ cal/(mol·K)	1:2 $\beta_2 = 1.3 \times 10^7$ $\Delta G = -10.1$ kcal/mol $\Delta H = -17.8$ kcal/mol $\Delta S = -25$ cal/(mol·K)
DMSA (5)^a	1:2 $\beta_2 = 8.3 \times 10^8$ $\Delta G = -12.7$ kcal/mol $\Delta H = -27.3$ kcal/mol $\Delta S = -47$ cal/(mol·K)	1:1 $K = 1.0 \times 10^7$ $\Delta G = -9.9$ kcal/mol $\Delta H = -13.2$ kcal/mol $\Delta S = -9$ cal/(mol·K)
DHLA (6)^a	2:3 $\beta_{2:3} = 4 \times 10^{18\,b}$ $\Delta G = -25$ kcal/mol $\Delta H = -43$ kcal/mol^c $\Delta S = -59$ cal/(mol·K)	1:1 $K = 1.1 \times 10^7$ $\Delta G = -10.0$ kcal/mol $\Delta H = -17.0$ kcal/mol $\Delta S = -20$ cal/(mol·K)
DTT (7)^a	1:1 $K = 9.5 \times 10^5$ $\Delta G = -8.5$ kcal/mol $\Delta H = -13.7$ kcal/mol $\Delta S = -17$ cal/(mol·K)	1:1 $K = 8.2 \times 10^5$ $\Delta G = -8.4$ kcal/mol $\Delta H = -15.9$ kcal/mol $\Delta S = -24$ cal/(mol·K)

Figure 10: Thermodynamic for the formation of As(III)-Thiolate complexes

(Figure from Wilcox et al. 2008[25])

They found that for $As(OH)_3$ the binding of glutathione has a stability constant β_3 =2 x10^6. In addition, they found a co-operative effect in the binding of thiols and the formation constant after each binding in increased. For example ΔH_3 was found to be -33.1 kcal /mol compared to ΔH_1 of -2.5 kcal/mol. They also looked at monomethylarsenite and its equilvalent binding to GSH and found that the binding of GSH to a monomethylated species gave comparable numbers to that of the non-methylated species. Gailer[26] offered an alternative view and proposed after passing both methylated and non-methylated compounds through size exclusion chromatography, the methylated versions were found to be more stable.

Finally they worked out the enthalpy of thiolate displacement to be -2.8 kcal/mol. They also found that there is a large unfavourable ΔS term involved in the binding as a result of the loss of conformational degrees of freedom. As a result they proposed that viscinal thiols that are conformationally constrained will have a higher affinity for arsenic III. This argument extends to conformationally unconstrained Cys residues found in zinc fingers would by this theory have a lower affinity for As (III).

Zingaro et al used the ability of thiols to displace the hydroxyl group in numerous synthesis of new dimethyl arsenious derivatives[27].

1.2.3 Lability of the Arsenic Thiol bond

It was found experimentally that as $As(SG)_3$ was passed through a size exclusion chromatography (SEC)[26], an increase of temperature of the column would cause retention shifts of the arsenic peaks towards small-molecular-mass regions. This is indicative of labile arsenic sulfur bonds which could allow the GS to break away from the molecule.

Arsenic III compounds have the ability are known have labile bonds. The is shown by the ability of meso-2,3-dimercaptosuccinic acid to displace GSH from the complex from $As(GS)_3$[28]

Arsenic systems have been studied by theoretical chemistry by Orthaber et al[29] using DFT calculations with B3lYP/6-31G basis set. They were interested in the interaction of H_2S with Arsenic (V) species

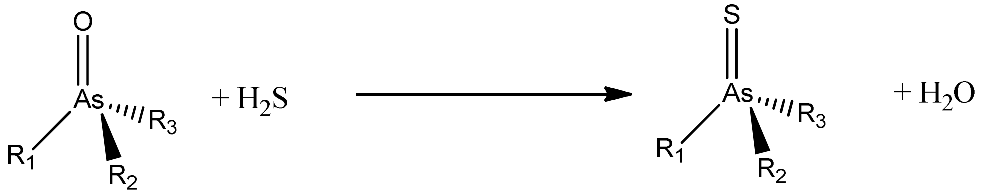


Figure 11: Interaction of H_2S with arsenic V species

They found that the formation constants is very favorable with ΔG of up to 51 kJ/mol for dimethylarsinoylethanol. Interesting they found that the pentavalent state, the –OH and –SH could interconvert between axial and equatorial positions similar to that of the Berry pseudorotation. They found that the relative intermediate of the interconversion is 6.7 kJ/mol.

One analogous system for the lability of arsenic is that of Mercury. The mercury thiolate bond has been shown to be labile and capable of rapidly breaking and reforming[30].

1.2.4 Co-ordination and geometry of arsenic compounds

The co-ordination chemistry of arsenic is both rich and diverse. As previously discussed in chapter 1.1, arsenic is a metalloid and can both accept and donate lone pairs. As it result it can accept co-ordination and act as a metal, in addition to donating its lone pair and acting as a ligand.

Edmonds et al[31] found that chiral arsenite species are capable rapidly racemizing. The studied this using the chiral arsenite, methylphenylarsinic acid and reacted it with (L)-glutathione to form two diastereomers. The then tried to separate the diastereomers using HPLC

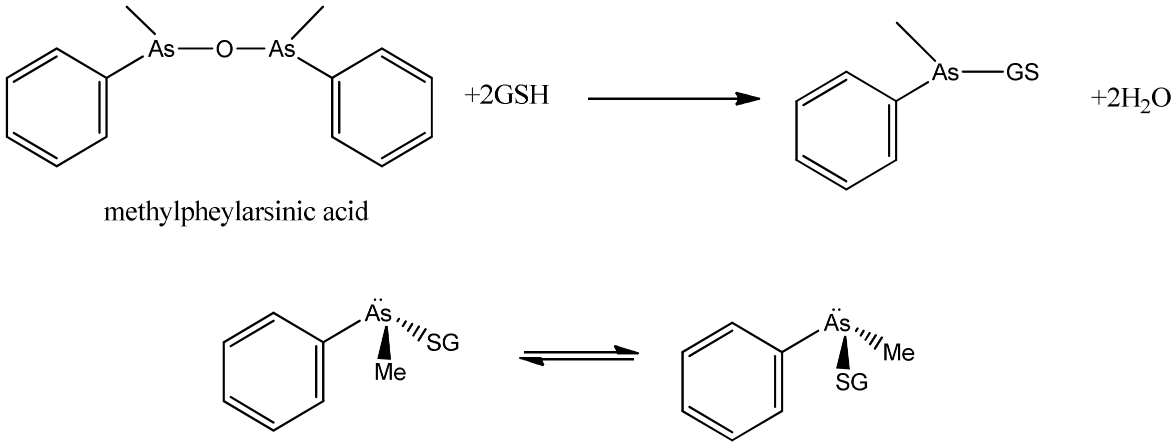


Figure 12: Reaction of methylpheylarsinic acid with glutathione.

They tried to resolve the diastereomers using NMR spectroscopy with the aid of lanthanide shift reagents and COSY 2D techniques. They were surprised to find that the species have racemized during the purification process (with heating was less than 40°C). The concluded that no pyramidal inversion of the arsenic was seen but could not give a definitive mechanism for this inversion.

1.2.5 Mechanism of arsenic bond lablity

The mechanism under which arsenic can perform its bond lability is not fully understood. Zampella et al[32] tried to calculate the binding of structures of arsenite systems using DFT calculations in order to understand how arsenite interacts with thiols of proteins. They modelled the arsenic binding systems with $As(CH_3S)_3$ and determined that there are two major forms of binding: the endo and exo isomers.

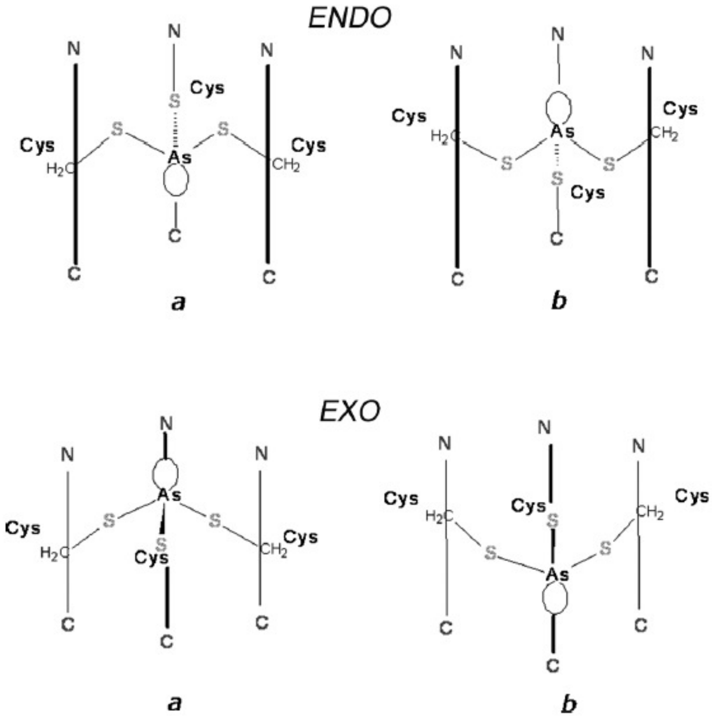


Figure 13: endo and exo binding forms of $As(CH_3S)_3$

(Figure from Zampella et al, 2012, Chemistry[32])

They concluded that the calculated lowest energy forms were similar to that found in protein systems. This meant that the mode of binding was strongly influenced by the steric bulk of the substituents. They also determined that the direction of the lone pair was a crucial factor influencing the selectivity binding for particular metal ions.

1.3 Summary

The mechanism of arsenic based drugs is currently not well understood and poses a road-block to arsenic based drug development. It is hypothesised that whilst arsenic is often thought react in an analogous fashion phosphorus, the kinetics and thermodynamics of pnicogen bonds formed by these two species far from analogous. The Challenger pathway shows that arsenic can be prone to both oxidation and methylation, a sharp contrast to phosphorus bonds which are stable enough to be used in genetic material. In addition to having different reactivity than phosphorus, arsenic seems to have bond lability, where arsenic appears to move between different thiol containing compounds. The mechanism of this interaction has not been thoroughly characterised, the thermodynamics of arsenic thiol bonds remains unknown. This project aims at using modern kinetic techniques to study the kinetics and thermodynamics of arsenic compounds. This is a pivotal step in the greater picture of understanding of arsenic drug interaction and the transportation of arsenic within the body.

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Chapter 2

Facile dimethylarsenic exchange in dimethylarsenous adducts of cysteine and glutathione.

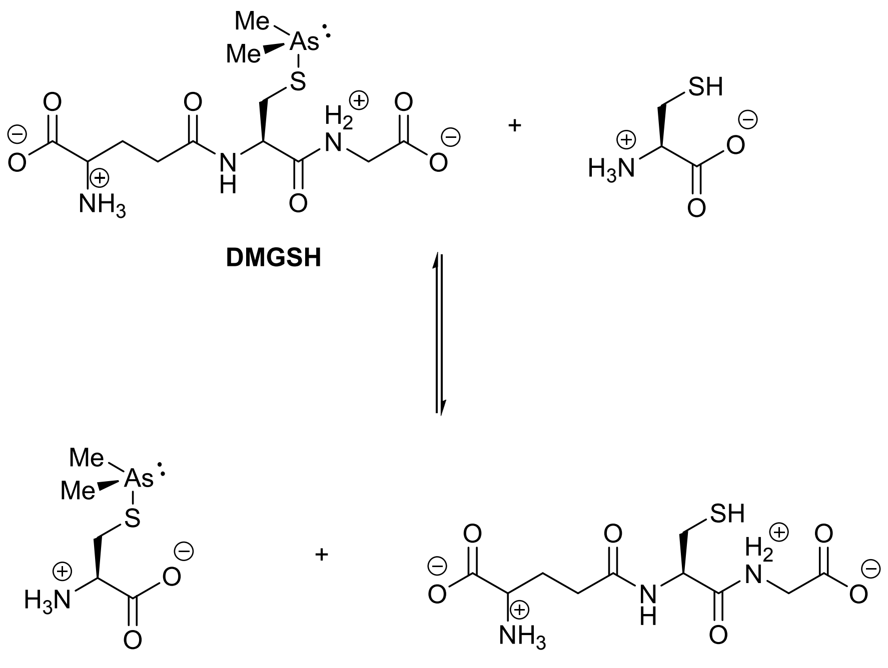
Introduction

The previous chapter established the need to identify the mechanics of arsenic interactions in the body. One of the primary interactions of arsenics within the body is with sulfurs on the amino acids cysteine and glutathione. The previous chapter has established that two types of chemical reactions occur between arsenic and thiols: the labile arsenic sulfur bond results in arsenic exchange between adducts and arsenic can undergo oxidation reduction reactions. The facile exchange of arsenic is between adducts of particular interest as it would improve understanding of the biochemistry of arsenic and offer new insights into its bio availability. The following section has been adapted from the paper *"Facile dimethylarsenic exchange and pyramidal inversion in its cysteine and glutathione adducts*", regarding the mechanics of dimethyl arsenic exchange between its glutathione and cysteine adducts.

2.1 Facile dimethylarsenic exchange

Regardless of its oxidation state or its substitution arsenic and its compounds are to varying degrees universally toxic[1]. Surprisingly though As_4O_6, arsenous oxide, is the FDA approved therapy for acute promyelocytic leukemia (APL)[2]. As with many metals and metalloids our current understanding of arsenic detoxification centers on its methyl derivatives, their transport, and localization [3]. In addition to its methylation arsenic binding and transport frequently involves thiols and thiol proteins, which reflects arsenic's strong thiophilicity[4,5,6]. For example, arsenic(III) compounds form very strong bonds to glutathione, GSH

$$3RSH + Me_2AsO_2H \longrightarrow RSAsMe_2 + RS^{-}SR + 2H_2O$$



DMGSH

DMCYS

Figure 14, and the tris glutathione adduct, $As(SG)_3$, has a high formation constant of log Kf = 32.0[7]. Nevertheless, thiolate exchange even from this tris-thiol adduct by either meso-2,3-dimercaptosuccinic acid or British Anti-Lewisite is well established[8,9,10]. The mechanisms for these facile exchange reactions have not been examined in any detail, but recent studies for As(III) species suggested that their Lewis basicity is a factor in their lability[11]. However the kinetics of arsenic-thiol exchange remains poorly characterised. In the course of characterising darinaparsin, dimethyl-arsino-gluatathione, **DMGSH**, and dimethyl-arsino-cysteine[12], **DMCYS**, as part of a program to understand the formers anticancer role in APL[13], we have discovered examples of rapid thiolate exchange. Herein we report: 1. Equilibrium constants for the rapid exchange of dimethylarsenium groups between cysteine and glutathione; 2. Dynamic NMR studies for the self-exchange of the methyl sites in these dimethylarsenic thiolate adducts; and 3. The dependence of these self-exchange reactions on pH, concentration, and thiol. Taken together these studies lay a foundation for understanding biochemical arsenic lability and transport.

$$3RSH + Me_2AsO_2H \longrightarrow RSAsMe_2 + RS^-SR + 2H_2O$$

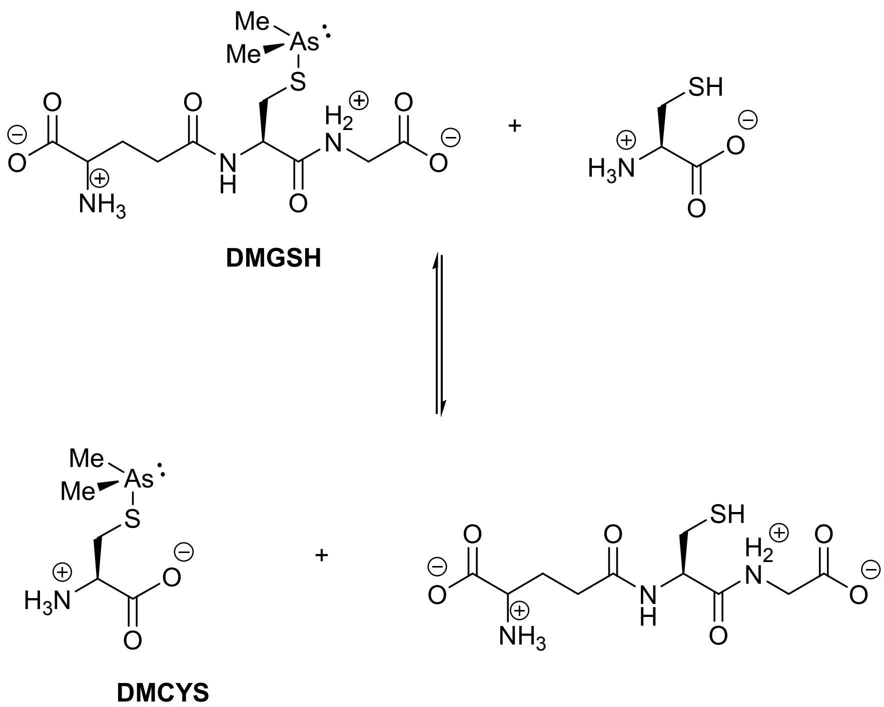
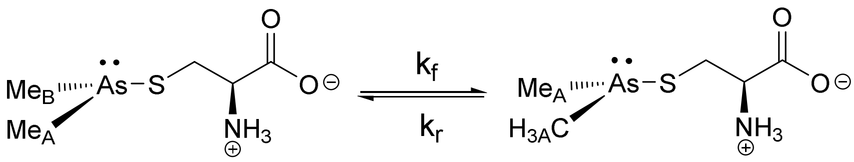


Figure 14: Synthesis and equilibrium of DMGSH and DMCYS. In H_2O at 25 °C

In aqueous solution GSH rapidly exchanges Me2As+ with **DMCYS** to give $GSAsMe_2$, **DMGSH**, ESI mass spectroscopy reveals the presence of the two dimethylarsenio derivatives in solution, with the peaks at 225.889 and 411.991 m/z corresponding to **DMGSH** and **DMCYS**. In the ^1H NMR spectra of these solutions at room temperature and high field, 500 MHz, **Figure 16**, there are total of 4 peaks between 1-2 ppm which correspond to the diastereotopic, non-equivalent methyl resonances of compounds **DMCYS** and **DMGSH**.



Me_2AsCys

Figure 15: Methyl site exchange in DMCYS.

Warming this mixture leads to reversible coalescence of first the methyls resonances of the cysteine derivative, and then at higher temperature the glutathione, and finally all four methyls. Up until 50 ˚C the ratio of 1:2 remains constant. Subsequent titration and integration of related samples gives the equilibrium constant (K_{eq}) for

$$3RSH + Me_2AsO_2H \longrightarrow RSAsMe_2 + RS^{-}SR + 2H_2O$$

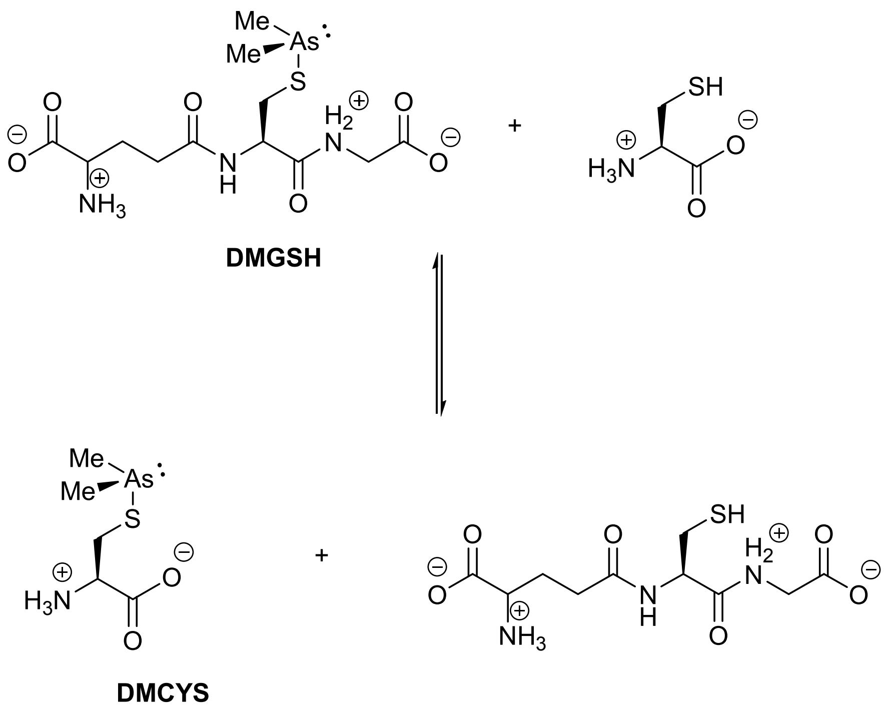


Figure 14 to be 0.6 and favoring the glutathione derivative.

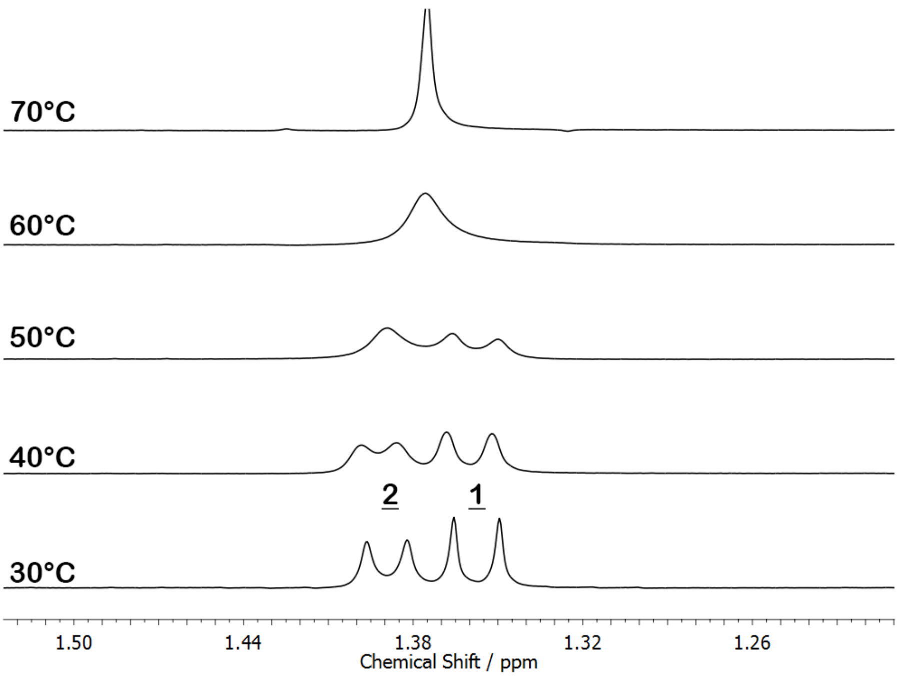
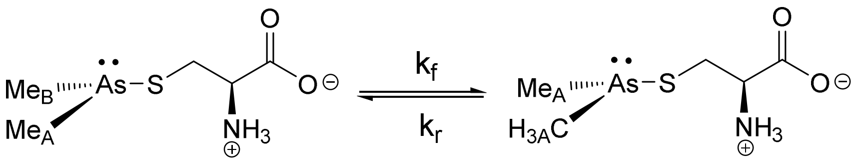


Figure 16: Variable temperature ^1H NMR spectra for the diastereotopic methyl resonances on DMGSH and DMCYS caused by dynamic exchange at equilibrium.

This corresponds to a relatively small free energy difference (ΔG) of 1.4 $kJmol^{-1}$ between the two species and suggests similar arsenic-sulfur bond energies. Raising the pH to between 5.5 and 7.0 also results in similar spectroscopic changes as shown in **Figure 16**. More basic conditions promote methyl site exchange. Attempts to measure the kinetics of these reactions have been hampered by the lack of useful UV-vis chromophores, in **DMGSH** and **DMCYS**, and that the reaction occurs in the mixing time (Supplementary material), of a typical NMR experiment. We concluded that this coalescence is due facile methyl exchange, and to test this facile exchange we opted to perform the study of DMCYS in isolation.

Individually the ^1H and ^{13}C NMR spectra of **DMGSH** and **DMCYS** are markedly temperature, pH, and concentration sensitive. For example in Figure 17 the solution spectra for a 5mM solution of **DMCYS** in 0.1M phosphate buffer at pD = 4.5 exhibited a reversible coalescence of the two methyl resonances.

Formally this corresponds to the two site exchange shown in



Me2AsCys

Figure 15, which corresponds to an effective inversion of the arsenic stereochemistry. Although this mechanism may not the same as interthiol exchange of Me_2As^+, both reactions suggest a markedly unexpected labililty for the Me_2As-S bond.

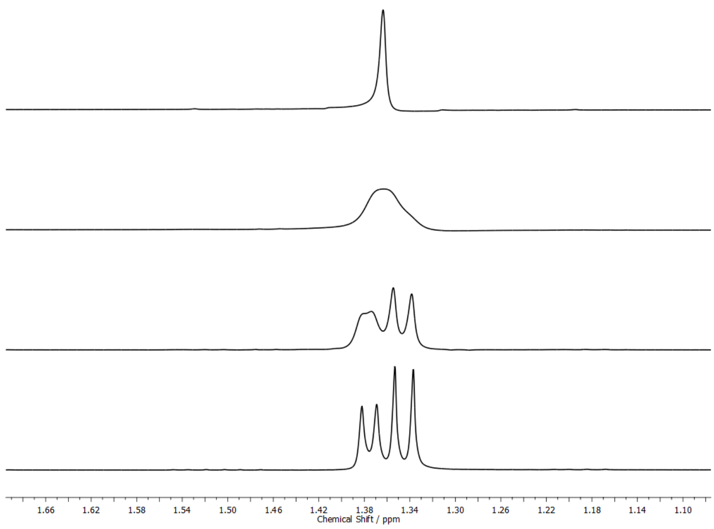


Figure 17: Variable temperature 1H NMR for the diastereotopic methyl signals in DMCYS in 0.1 M phosphate buffer at pH = 4.5. Bottom trace is at 10 ˚C, followed by 20, 30, 40, 45, 50, 55, and 70 ˚C at top.

The ^1H spectrum by the NMR experiments of **DMCYS** were performed on a 5mM phosphate buffered solution at pH = 4.6 between the temperatures of 270 K to 335 K. The rate constant $\frac{d[DMCYS]}{dt} = -kf[DMCYS]$ was calculated using the chemical shift difference between the methyl peaks using the Sandstrom's equation[14] $k = \frac{\pi}{\sqrt{2}}\sqrt{\delta v^2 - \delta v_e^2}$. Peak width was also used to independently calculate the rate, both methods yielded the same activation parameters.

The data give good linear Arrhenius fits for all data above 3˚C with only slight deviations being found for the temperatures just above the freezing point of water. The activation energy (E_a) is 14 kJ mol-1, indicating that very little energy is required to cause the coalescence. In terms of the activation parameters from the Eyring equation, $\Delta G\ddagger$ = 73 $kJmol^{-1}$, $\Delta H\ddagger$ = 11 $kJmol^{-1}$ and $\Delta S\ddagger$ = -190 $Jmol^{-1}K^{-1}$. The relatively small $\Delta G\ddagger$ suggests that As-S bond dissociation is an unlikely mechanism as the bond enthalpy[15] of the As-S bond is around 380 $kJmol^{-1}$. In addition, the $\Delta S\ddagger$ is negative indicating a markedly more ordered transition state, this suggests that there might be an associative mechanism for this exchange.

The zwitterionic ionization of the amine and carboxylic acid groups in **DMGSH** and **DMCYS** play an important role in the mechanism of the exchange. Exchange kinetics as a function of pH and substrate concentration are shown in **Figure 18**. The slowest methyl site exchange kinetics correspond to a singly protonated species. This is in accord with a prior potentiometric titration result for **DMGSH** which was suggested to be particularly labile at pH 7 and greater[7]. While at higher pH there be significant dissociation of Me_2As+ through an associative nucleophilic hydrolysis, for the pH used in these studies there is no substantial buildup of side products or other indications of competing side reactions.

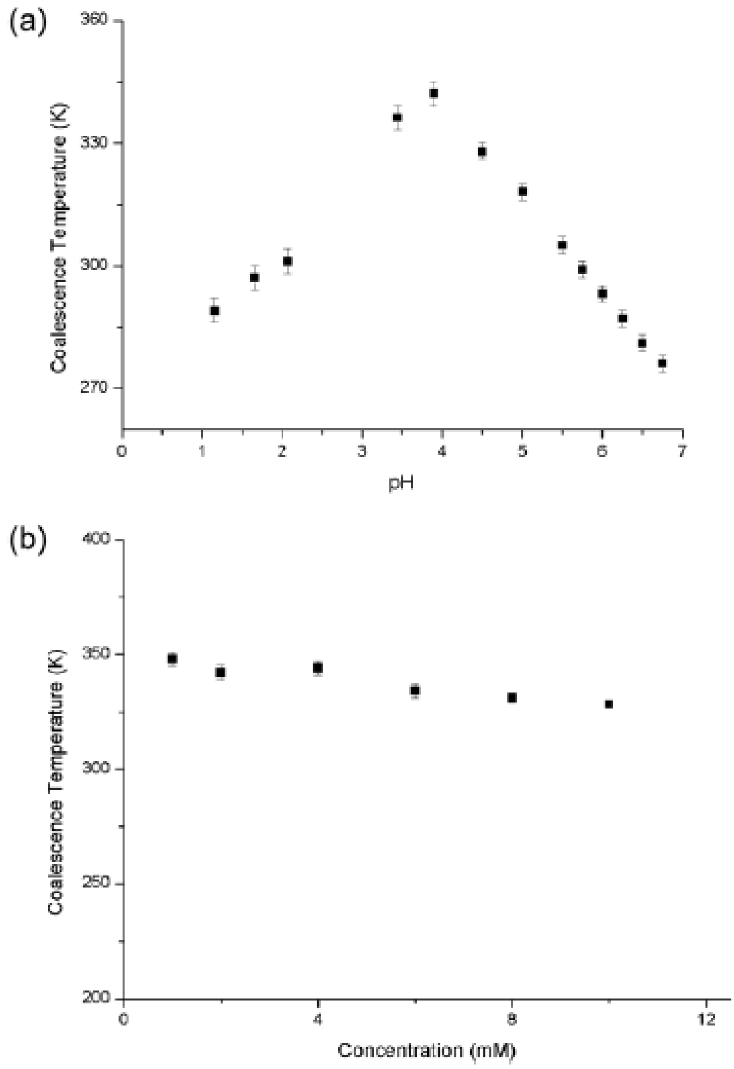


Figure 18: Dependence of coalescence temperature upon (a) the pH of a 5 mM solution of DMCYS and (b) concentration of DMCYS in 5 mM phosphate buffer.

The concentration dependence for the methyl site exchange, as reflected in its coalescence temperature, is shown in **Figure 18**b. The marginal decrease in coalescence temperature with increased concentration suggest that in addition to a rapid intramolecular mechanism there is second intermolecular, bimolecular, pathway. This second pathway is consistent with the 1/2 exchange results shown in Figure 1. However, the rate and thus contribution this second pathway makes to methyl site exchange is minor compared to basal unimolecular rate of site exchange. There are several mechanisms for methyl site exchange, with most obvious, a formal inversion of the arsenic geometry, being unlikely. Experimentally, arsenic (III) pyramidal inversion through a trigonal planar transition state has a high barrier, 176 kJ mol-1 for PhEtMeAs[16]. Theoretical calculations[17,17b] also suggest these transition states should be in excess of 150 kJ mol-1, which is much higher than our experimentally determined barrier of 80 kJ mol-1. Surprisingly facile racemisation at arsenic of the diastereomeric methylphenylarsinic acid adduct with glutathione was observed by Edmonds et al., and interpreted in terms of an unexpected and unaccountably low inversion barrier[18].

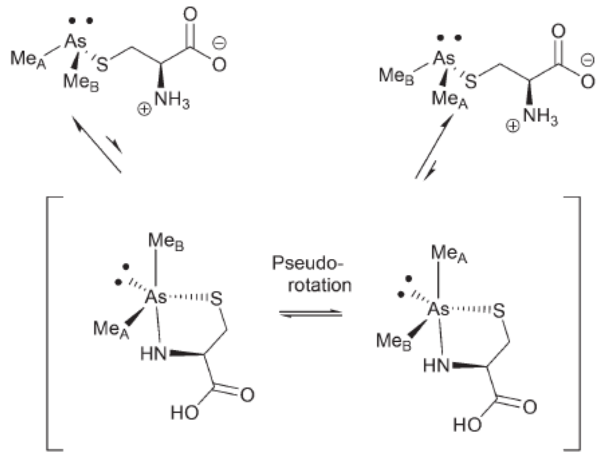


Figure 19: Proposed fluxionality in DMCYS.

To account for the rapid methyl site exchange in **DMGSH** and **DMCYS** we note that As(III) species are of course ambiphilic being potent nucleophiles and ligands as well being as metalloids with latent Lewis acidity. It is this latter character which would allow for an associative or chelation of the amine to the arsenic to give a net five coordinate intermediate with four substitutents and a stereochemically active lone pair, Scheme 3. For this geometry Berry pseudorotation barriers will be very low, and their action will lead to rapid methyl site exchange. This mechanism is in accord with the near zero slightly negative entropy of activation and the rate enhancement at higher pH. The increase in rate at lower pH may be due to a separate acid catalyzed exchange, but the generally low of solubility of these species limits a more extensive study under these conditions.

In conclusion, we have shown that the As-S bond is kinetically labile and can be interact with other thiols in aqueous solutions. Despite being more stable to oxidation, **DMGSH** in the equilibrium system is only 1.4 kJ mol-1 more stable than **DMCYS** in aqueous solutions. An associative intramolecular self-exchange mechanism as one of the mechanism responsible for this lability. This type of facile thio exchange has important implications of how methylarsenic species are active cells and within proteins. In what may be a helpful analogy, the facile dimethylarsenium transfer reactions discovered here have many parallels with the trans-nitrosylation chemistry of the nitrosylated thiolates, RSNO, which have been more extensively studied[19,20].

2.2 Supplementary material

The following supplementary material details the methodology and calculations used in the chapter 2.1.

Preparation of S-(dimethylarseno)cysteine

The synthetic preparation was adapted from the Cullen method[12], with the main change being nitrogen gas is used instead of carbon dioxide to produce an inert atmosphere. Cacodylic acid (0.445g) of and 0.929g of L-Cystine was mixed in distilled water under nitrogen for 16 hours. The precipitate was filtered dried under reduced pressure without heating. ^{1}H NMR (500 MHz, D_2O) δ 3.99 – 3.91 (m, 1H), 3.24 – 3.13 (m, 1H), 1.37 (s, 1H), 1.36 (s, 1H).

Preparation of S-(dimethylarseno) cysteine Solution.

DMCYS (0.0115g) was dissolved in 1 ml of Phosphate buffer at pH 4.2 (10% potassium phosphate buffer was prepared by the dissolution of potassium phosphate into water and subsequent adjustment of pH using NaOH. The solution was deuterated by drying the mixture and re-hydrating it with D2O). This solution was diluted to form a desired concentration of 6.40 mM by a 1/5 dilution using 200ul of the solution and 800 ul of buffer. To ensure the temperature is changing constantly and as expected, a plot of the solvent chemical shift against temperature. This indicates minimal changes to the pH or random errors in the temperature readings, as the R^2 is 0.999. This however does not rule out the possibility of systematic errors to the temperature readings. This could be remedied by using a temperature reference.

NMR data was acquired with a Varian 500 MHz instrument. All NMR acquisitions were made with 16 scans, transform size of 32k and shims were done with a gradient shimming on each measurement. Data was processed using Mnova 6.1.1 FID processing software.

To determine the rate constant of the reaction, the separation of the methyl peaks were used to calculate the rate constant using the following equation[21]:

$$k = \frac{\pi}{\sqrt{2}}\sqrt{\delta v^2 - \delta {v_e}^2}$$

This equation is applicable in this situation as δv and k are much larger than the bandwidth in absence of exchange (0.6 Hz). As it is not possible to lower the sample below 0 degrees due to the freezing point of water, δv was estimated using the lowest experimentally obtained separation of 7.8 Hz (solution of 1mM sample at 10 °C).

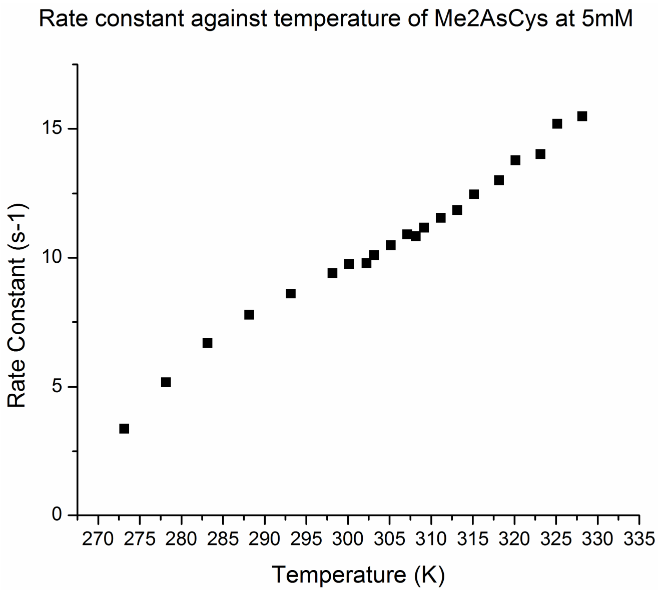


Figure 20: Plot of rate constant against temperature of a 5mM solution of DMCYS

The NMR dynamic exchange was modeled using DNMR3 with Spinworks 3 software[22]. The experimentally determined variables (low temperature limit) of the system at 5°C with a concentration of 1mM was used as the input parameter for the simulation (two spins at 1.3832 and 1.3698 with 0.6 line width). Other parameters include Permutation vector set as 2,1 (mutual exchange of the system), the relaxation time 1 sec (same as acquisition), populations were 0 as this is a case of mutual exchange and maximum iterations = 30. The rate constant RC (1,2) was gradually increased by 1 s^{-1} until the simulated system exhibited coalescence. It was found that a rate constant of 16 s^{-1} gave coalescence for the two peaks.

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Chapter 3

Rapid exchange in related arsenic derivatives.

Introduction

The previous chapter showed that facile dimethylarsenic exchange occurs in the dimethylarsenic adducts of cysteine and glutathione. One of the proposed mechanisms involves the intramolecular nucleophilic attack of the amine on the arsenic which leads to a 5 membered ring as outlined in **Figure 21**.

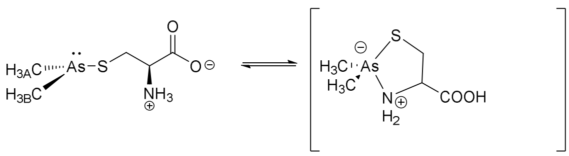


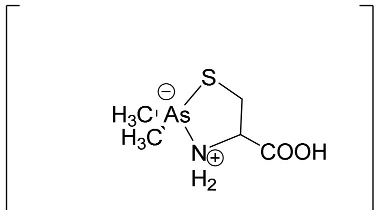
Figure 21: Possible mechanism for the observed coalescence of the dimethylarsonium peaks.

Fluxional dynamics of the methyl by Berry pseudorotation for example, would exchange the methyl sites and lead to the observed NMR signal coalescence. If this is the case, the addition of electron withdrawing or donating substituents on the amino group would cause changes in the rate of reaction.

Dimethylarseno species are demethylated in the body as outlined in section 1.1.1. The monomethylated species is not only biologically relevant, but might interact in a similar way to the dimethylated species. In section 3.2.1, monomethylated species was synthesized and its interaction with cysteine in solution was investigated.

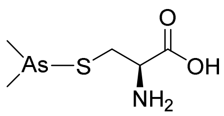
3.1 Synthetic analogues to Dimethylarsenocysteine

One of the mechanisms proposed for the exchange of the methyl peaks in aqueous **DMCYS** involves the formation an intermediate chelated 5-membered ringed species. The method of chemical substitution was chosen to validate this mechanism. Two synthetic targets, dimethylarseno-N-acetyl cysteine (**DMNAC**) and dimethylarseno-penicillamine (**DMPEN**) were chosen as shown in Figure 15.

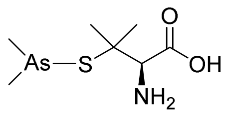


Proposed Intermediate

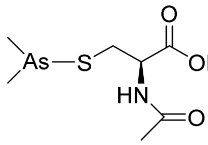
Synthetic analogues



dimethyl arseno cysteine
DMCYS



dimethylarsenopenicillamine
DMPEN



dimethylarsenoN-actylcystei
DMNAC

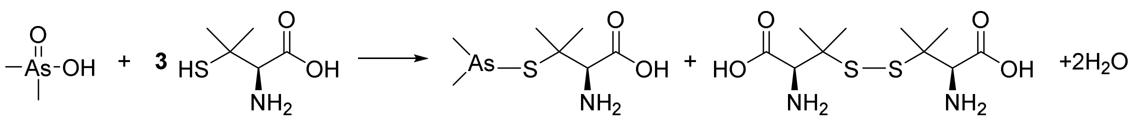
Figure 15, proposed derivatives of dimethylarsenocysteine.

The first synthesis target was **DMNAC** (section 3.1.1) where the nitrogen is acetylated, thus delocalizing its lone pairs and preventing it from nucleophillically attacking the arsenic. If the formation of the 5 membered species is responsible for the observed rapid methyl exchange, the N-acetyl cysteine derivative is not expected to exhibit coalescing methyl peaks.

Another synthetic target was **DMPEN** (section 3.1.2) which replaces the cysteine with penicillamine, a cysteine derivative which contains two additional methyls between the thiol and the β-carbon. The additional of two methyls substituents is expected to stabilize the 5-membered intermediate and thus increase the rate of chelation. If the observed dimethyl exchange involves intramolecular attack, a lower coalescence temperature is expected for this species with respect to dimethyl arsenic cysteine.

3.1.1 Preparation of Dimethylarseno-N-acetyl cysteine

Dimethylarseno-N-acetyl cysteine is a new species that has not been previous synthesized. A synthetic procedure was adopted from the synthesis of **DMCYS** involving the reduction of cacodylic acid the by N-acetyl cysteine[1] (**NAC**). A proof of concept for the reaction was done by adding 5 equivalents of N-acetyl cysteine to a solution of cacodylic acid in D_2O. The reaction was followed by NMR over a 1 hour period. Over this time the cacodylic acid peak at 1.15 ppm disappears and a new peak at 1.35ppm, assigned to **DMNAC**, grows in.



Synthetic scheme 1 for **DMNAC**

This preparation is performed under nitrogen to prevent the oxidation of the final product. Cacodylic acid, 0.3579 g, was placed in a round bottom flask and dissolved in 10 ml of degassed water. 0.9724 g of N-acetyl cysteine was added and the solution was left stirring under nitrogen for 16 hours. Unfortunately, unlike the synthesis of the cysteine derivative, the disulfide side product did not precipitate out of solution. Water was removed leaving a white powder. ^1H NMR (400 MHz, D_2O) δ 4.76 – 4.68 (m, 1H), 4.66 – 4.56 (m, 2H), 3.31 (m, J = 14.3, 4.5 Hz, 1H), 3.20 (m, 2H), 3.04 – 2.90 (m, 6H), 2.15 (d, J = 4.9

Hz, 3H), 2.06 (d, J = 4.9 Hz, 6H), 1.35 (d, J = 2.8 Hz, 6H). Peaks at 2.06, 3.04 and 4.66 ppm could be assigned to N, acetyl cysteine disulfide and the resonance at 1.35 ppm was assigned to the methyls on the As. This NMR demonstrated that the reaction has gone to completion, however the target product has yet to be separated from the disulfide side product. Extraction with various solvents was unsuccessful at extracting **DMNAC** from the mixture. Recrystallization was attempted with various solvent mixtures, but did not result in a purified product. Chromatography was not possible due to the sensitive nature of the product.

As it was not possible to obtain a clean product with this method, an alternative reaction scheme was proposed that didn't involve the production of n-acetyl cysteine disulfide. Instead of using the oxidation state (V) cacodylic acid as a source of arsenic, dimethylarsenoiodide(III)[1,2,3] was used. This would give a clean reaction with a 1:1 ratio of arsenic and **NAC**.

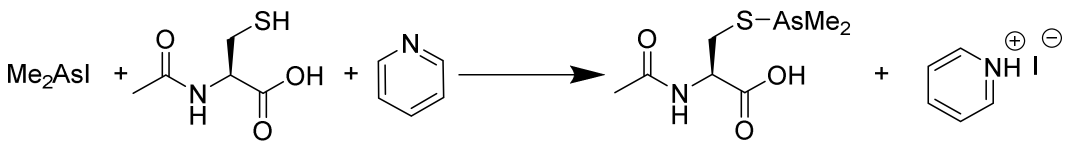


Figure 22: New scheme for the preparation of DMNAC (py.HI)

Preparation of Dimethylarsenoiodide.

Me_2AsI was prepared using the Burrows method[2]. It is important to note that this compound (and its derivatives) is extremely toxic and has a pungent unpleasant smell, hence Schlenk apparatus and proper fume hood containment methods are required. Potassium iodide, 15g, and 5g of Me_2AsO_2H were dissolved in 45ml of distilled water. Concentrated HCl, 5ml, is added to make a clear colorless solution. Sulfur dioxide is bubbled for 15 minutes through the solution at which point the solution turned to light yellow. After 5 minutes of bubbling the solution darkened to an opaque black, followed by the formation of a bottom layer which was clear yellow. The bottom layer was extracted and distilled under reduced pressure of 16 mm at 401K giving a clean yellow liquid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.01.

Preparation of Dimethylarseno-N-acetyl cysteine

N-Acetyl cysteine, 0.5g, was dissolved in dimethoxyethane and 1 ml of Me_2AsI was added by syringe. Pyridine, 1 ml, was added and precipitation immediately occurred. The solution was refluxed for 15 minutes and left to stir for 2 hours. The solution was filtered and NMR revealed the filtrand to be pyridinium iodide. Solvent was removed from the filtrate leaving a white solid, ^{1}H NMR (400 MHz, D2O) δ 4.76 – 4.68 (m, 1H), 4.66 – 4.56 (m, 1H), 3.31 (dd, J = 14.3, 4.5 Hz, 1H), 3.20 (dd, J = 14.1, 4.6 Hz, 1H), 3.04 – 2.90 (m, 2H), 2.06 (d, J = 4.9 Hz, 3H), 1.35 (d, J = 2.8 Hz, 6H), 1.15 (s, 1H). This spectrum could be assigned to that of **DMNAC** with the exception of the 1.15 ppm peak which was assigned to the methyls on cacodylic acid. From the integrals, we estimate a 14% cacodylic acid contamination. This is most likely formed by the air oxidation of **DMNAC**, a reaction that is known to happen with the cysteine derivative. This was confirmed by taking the NMR of a sample of **DMNAC** solution (D_2O) kept at room temperature for 24 hours, which showed significant growth in the cacodylic acid peak. Purification by recrystallization with various solvent mixtures was attempted but was unsuccessful.

A pure product is extremely important for the variable temperature experiments because previous experiments have shown that methyl peak coalescence is sensitive to cysteine impurities. One possible improvement to the synthetic procedure could be the inclusion of a reductant in solution which would prevent the oxidation of the product.

3.1.2 Preparation of Dimethylarseno-penicillamine

There are not reported sythesies of Dimethylarseno-penicillamine. The synthetic scheme for **DMNAC** was adopted for the synthesis.

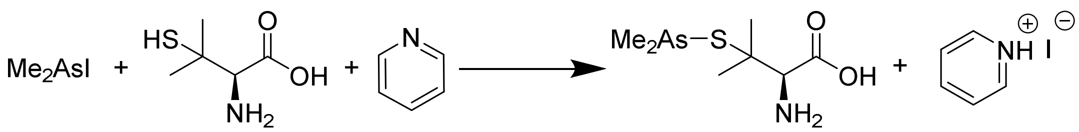


Figure 23: Synthetic scheme for DMPEN

Penicillamine, 0.5g, was suspended in dimethoxyethane. 1 ml of Me_2AsI was added by syringe causing the full dissolution was penicillamine. Pyridine, 1ml, was added and a white precipitate immediately appeared. The solution was refluxed for 15 minutes and left to stir for 2 hours. The solution was filtered and the filtrate was dried. NMR revealed the filtrand to be pyridinium iodide. NMR of the filtrate: ^{1}H NMR (400 MHz, d_2o) δ 3.86 (d, J = 2.3 Hz, 1H), 3.60 (s, 1H), 3.36 (s, 1H), 2.02 (d, J = 1.1 Hz, 1H), 1.60 (s, 4H), 1.44 (s, 4H), 1.38 (s, 3H), 1.35 – 1.28 (m, 6H) 1.15 (s, 0.7 H). The NMR revealed additional unexpected peaks that could be attributed to cacodylic acid and the disulfide adduct of penicillamine. Attempts to further purify the product using recrystallization and extraction methods proved unsuccessful. Samples of **DMPEN** dissolved in D_2O and kept at 24℃ for 24 hours showed significant growth in the relative integral of the 1.15ppm peak. This shows that the product when dissolved in solution is vulnerable to aerial oxidation and represents a significant challenge to the synthesis of **DMPEN**.

3.1.3 Section conclusion

One of the main difficulties to the synthesis of both **DMPEN** and **DMNAC** is the susceptibility of both these compounds to oxidation. This is not a problem with **DMCYS** because it could be purified by recrystallization. Both the synthesis of **DMPEN** and **DMNAC** were done under nitrogen however around 10% contamination of cacodylic acid still occurred. One possible way around the problem is to perform the reactions in the presence of a reduction to prevent the oxidation of the product. Different extraction methods could also be tried to separate the product from the impurities.

3.2 Monomethylated derivatives

The previous chapter showed facile dimethylarsenic exchange occurred in the dimethylarsenous adducts of cysteine and glutathione. In this chapter its closely related cousin - monomethylarsenous adducts are examined. Monomethylarsonous acid (**MMA**) is a key metabolite of the ingested inorganic arsenic though methyltransferase enzymes[4]. These species are immensely interesting because like dimethylated species, they are also have a high affinity for thiol groups. One particularly interesting property is the ability of monomethyl arsenic derivatives to bind to two thiols[5,6] thus allowing the arsenic chelation by two vicinal dicysteine residues. One aim of this chapter is to detemine if **MMA** species share similar reactivity with **DMA** and have labile arsenic-sulfur bonds.

3.2.1 Methylarsine Oxide

In solution, **MMA** behaves very differently from **DMA** – it forms oligomers through arsenic-arsenic bonds. The starting material for the monomethyl derivatives is $(MeAsO)_x$ which was synthesised by the Cullen Method[7].

Preparation of Methylarsenate(V) acid sodium salt.

Arsenic trioxide, 3g, was dissolved in 10 ml of 10M NaOH. 15 ml of MeI was added, forming a bilayer solution. The solution mixture was heated to reflux for 16 hours, which resulted in a white precipitate of methylarsenate(V) acid sodium salt in 72% yield.

Preparation of Methyl arsonious acid sodium salt.

Methylarsenate(V) acid sodium salt was dissolved in 50 ml of H_2O. Dissolution of the initial salt was promoted by gradual heating of the solution. Once dissolved the solution is treated with sulfur dioxide which is bubbled through the solution. The solution quickly becomes clear (suggesting acid sensitivity) then light yellow after 2 minutes. After saturating with SO_2, the solution was quickly boiled for 2 minutes then cooled for 15 minutes. Neutralisation with sodium carbonate turned the solution from light yellow to clear. The solvent was removed and $(MeAsO)_x$ was extracted with benzene. Removing the benzene *in vacuo* resulting in a white solid (70% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.58 (d, J = 6.8 Hz, 5.9%), 1.52 – 1.49 (m, 27%), 1.48 (d, J = 2.1 Hz, 58%), 1.44 – 1.42 (m, 8%). This corresponds to the literature reference (Aposhian et al[8]) of ($CDCl_3$): δ 1.58/1.59 (5.0%), 1.50/1.51 (26.8%), 1.48 (60.0%), 1.43 (8.1%). ^{1}H NMR (400 MHz, D_2O) δ 1.17 (s, 1H). ^{1}H NMR (500 MHz, C_6D_6) δ 1.21 (s, 1H). ^{1}H NMR (500 MHz, CD_3OD) δ 1.25 (dd, J = 11.1, 7.1 Hz, 1H). ESI of the compound did not reveal any tetramer peaks, possibly because the ionisation process would break apart the tetramer.

Methylarsine oxide takes the form of cyclic and linear oligomers[9], hence resulting in the formula $(MeAsO)_x$, where the exact number of oligomers depends on the concentration of the solution. This is shown in the $CDCl_3$ NMR which contains 4 sets of multiplets in $CDCl_3$. As the equilibria and dynamics[7,9] of methylarsine oxide is not well understood, I chose to look at the equilibria in more detail before carrying out additional reactions. Marsmann and Wazer[10] proposed the possibility of the species oligiomerizing at higher concentrations and temperatures, to give a cyclic anhydride, in particular with a preference for a tetrameric form. For example at 48% wt concentrations of arsenosomethane at 120°C (in diphenyl ether) it is tetrameric. It is interesting to note that the Wazer[10] did not observe hydrolysis with diphenyl ether.

To validate the possible presence of oligomers, a temperature dependent NMR experiment was performed with the sample in $CDCl_3$. For this experiment 0.0975g of $(MeAsO)_x$ was dissolved in 1000 ul of $CDCl_3$. The sample was initially cooled down to 273K and the temperature was slowly brought up in 10 degree increments.

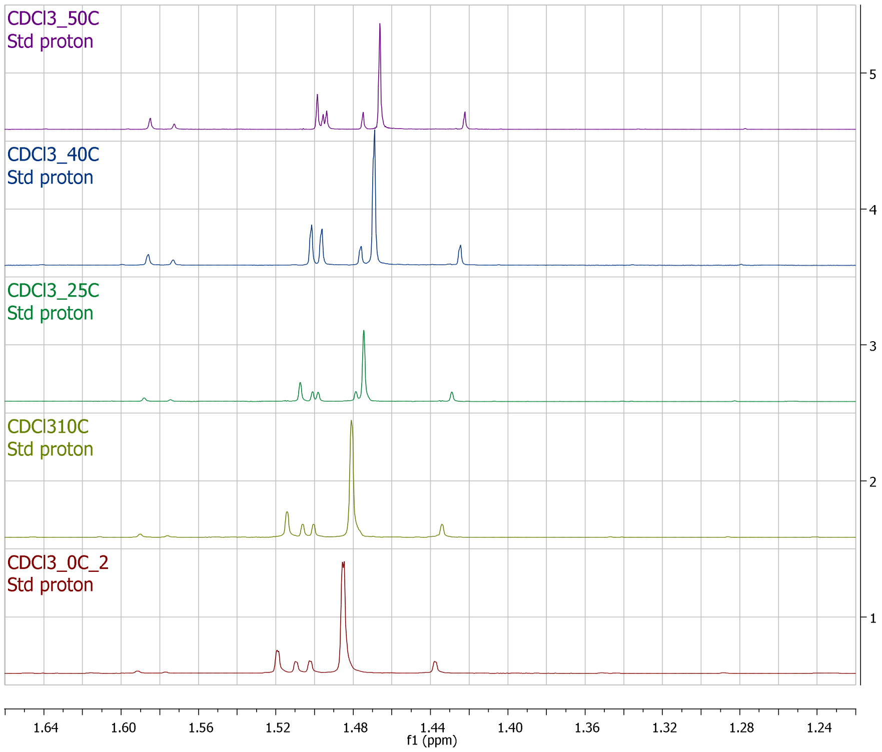


Figure 24: Temperature variation on the sample of (MeAsO)x dissolved in CDCl3, 1) 273.15 K, 2) 283.15, 3)298.15 K, 4)313.15K, 5) 323.15K.

As the temperature increases, a shoulder peak appears at 1.48 ppm. I also notice in increase in the intensity of the peaks at 1.57 and 1.59. No coalescence of the peaks is observed suggesting that this process is slow on the NMR time scale. The integrals return to their original ratio upon cooling, showing that the oligiomerisation is reversible. The reversible oligiomerisation shows that the As-As bond is labile in $CDCl_3$ and could break to reform different oligiomeric species. Unfortunately, due to instrumental limitations it was not possible to extend the temperature range for this experiment. When $(MeAsO)_x$ is dissolved in D_2O, only one peak at 1.36ppm is observed corresponding to the hydrolyzed species $MeAs(OH)_2$. As this was the case, I chose to continue the investigation by looking into the interaction between monomethylarsenic species with cysteine in aqueous solutions.

This series of experiments could be extended by the use of different solvents, in particular diphenylether (the solvent used by Marsmann et al[10]). In addition concentration variation could be looked at especially high concentrations of $(MeAsO)_x$.

3.2.2 Interaction of $MeAs(OH)_2$ with cysteine

Previously in the project I characterised the interaction of dimethyl arsenicals with cysteine. This provided us with a new and expected insight into the lability and kinetics of the As-S bond. With monomethyl derivatives the situation is more complicated as the arsenic can bind with two cysteines.

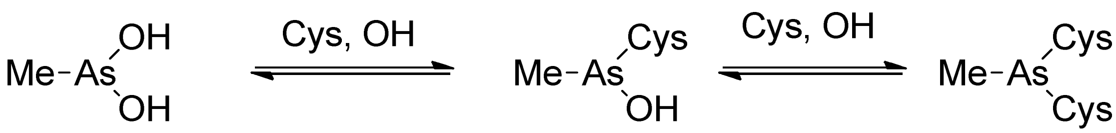


Figure 25: Interaction of Cysteine with DMA in aqueous solution

Preparation of Monomethyl arsenious acid solution.

A solution of $(MeAsO)_x$ was prepared by dissolving 0.0245 g of the compound in 1.0 ml of D_2O (buffered with 10% deuterated sodium phosphate). A 231 mM solution of cysteine was prepared by dissolving 0.0277g of cysteine in 2000 ul of the same buffered D_2O. All solutions were deoxygenated by bubbling N_2 for 10 minutes. For the NMR titration, 500ul of the stock $(MeAsO)_x$ solution placed in a NMR tube. The cysteine solution was titrated into the NMR tube at 100ul aliquots followed by 30 sec of intense vortex mixing followed by 5 minutes wait time. ^{1}H NMR (500 MHz, D_2O) δ 4.06 (dd, J = 11.1, 5.1 Hz, 10H), 3.54 – 3.24 (m, 21H), 1.76 (s, 13H), 1.63 (s, 4H), 1.36 (s, 16H).

The peaks have been assigned to the following species, 1.76 ppm peak corresponds to the methyls on $MeAs(Cys)_2$, 1.64 ppm peak to MeAs(OH)(Cys) and 1.358 ppm peak to $MeAs(OH)_2$. From the integrals on the NMR spectra of the system during the titrations it is possible to work out the concentration of each species after each addition. As the concentration of the species change due to the dilution caused by the titration, it is easier to visualize the species in terms of molar ratios:

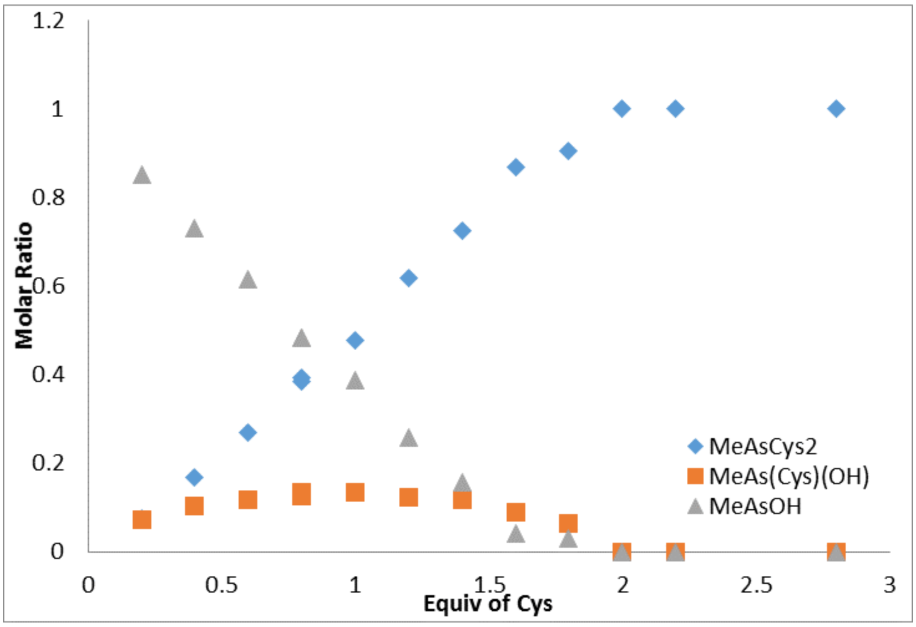


Figure 26: NMR titration of Cysteine against MeAsOH in D_2O

These results show that cysteine interacts with MMA in D_2O and does so at a rate slower than the NMR time scale as no line broadening was noticed. It is also shows that after an excess (3 equiv) of cysteine is added, that only $MeAs(Cys)_2$ remains in solution, indicating that the two cysteine bound species is more stable. What is interesting is that between 0 and 2 equivalents, the species all exist in solution. During a repeat of the experiment, the solution of 1 equivalent of cysteine was left to react for 2 hours and no additional change in peak integrals were observed, confirming that MeAs(OH)(Cys) is a stable intermediate. In addition I titrated MMA into a solution of $MeAs(Cys)_2$ to confirm that indeed the reaction was reversible and saw peaks at 1.64 ppm and 1.36 ppm appear, corresponding to MeAs(OH)(Cys) and $MeAs(OH)_2$ respectively.

I have shown that monomethylarsenic species exhibit arsenic sulfur and arsenic oxygen bond lability, similar to dimethylarsenic species. The next step is to see if this rate of this lability could be characterised using NMR techniques.

3.2.3 Temperature sensitivity of the methyl peak in MeAs(OH)(Cys)

As arsenic has a lone pair, the arsenic the species MeAs(OH)(Cys) is chiral and forms an overall diastereomer with the chiral α Carbon in the cysteine. This should result in the presence of two peaks for this species as opposed to the singlet that I observed. This suggests there might be dynamic exchanges interactions occurring that is causing the signal to average out. In addition, if the cysteines are labile like in the **DMCYS** case, I might also observe the coalescence of all the methyl peaks. However, this might not be observed if there is little chemical shift difference between the products or if the reaction is diastereoselective.

A preliminary NMR experiment was done with a system with 65 mM of $(MeAsO)_x$ and 77mM of Cysteine at 25°C and 40°C.

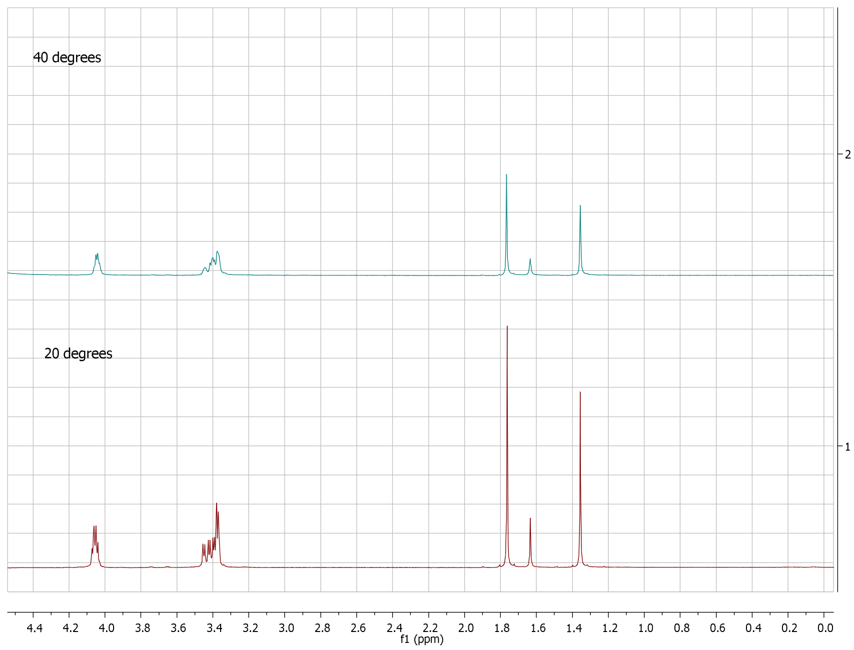


Figure 27: NMR of $(MeAsO)_x$ in D_2O at 40℃ (top) and 20℃ (bottom)

So far results indicate that there is minimal change to the peaks of the arsenic bound methyls as splitting of these peaks were not observed. This experiment could be extended by covering a larger temperature range to see if any detectable peak separation occurs. In addition, the different solvent systems could be used to allow for a larger temperature range.

3.3 Conclusion

To summarize, I have demonstrated that methylarsenic and dimethylarsenic adducts undergo facile could be exchange between different thiol adducts such as cysteine and glutathione. In the case of dimethylarsenic cysteine, the methyl exchange could be studied using DNMR – elucidating the entropy and enthalpy of the interaction. In addition to transfer between the thiols of cysteines, dimethylarsenic transfer between cysteine and glutathione groups was also observed. Whilst it was not possible to directly model this interaction, it qualitatively shows that arsenic readily transfers between various thiols.

In addition it was shown that $MeAs^{2+}$ species are also labile. By titrating cysteine into a solution of MMA, I have identified the formation of both MeAs(Cys)(OH) and $MeAs(Cys)_2$. The concentrations of these species would also change reversibly depending on the mole fraction. Unfortunately it was not possible to explore the kinetics of these species using the DNMR as line shapes did not change with temperature. Further kinetic studies of these systems could be done using stopped flow techniques.

The ability of arsenic to rapidly break and form new bonds has important biological implications[11]. This rapid exchange mechanism gives rise to the possibility that arsenic is transported via a shuttle mechanism where it hops into to various species and is carried around the body. In additional to binding to small molecules such as cysteine and glutathione, arsenic could also bond to viscinal cysteines of large proteins, disrupting its function. Understanding this interaction is key to understanding the mechanism of arsenic based drugs such as ATO and Darinaparsin[12].

3.4 References

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