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Chapter 3 Introduction

In Chapter 2 I 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 Fig 14.



**Figure 14: Possible mechanism for the observed coalescence of the dimethylarsonium peaks on 1H NMR.**

Fluxional dynamics of the methyl by Berry pseudorotation for example, would exchange the methyl sites and lead to NMR signal coalescence. If this is the case, chemical substitution of electron withdrawing or donation substituents on the nitrogen would cause changes in the rate of reaction.

Dimethyl arseno 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 demethylated 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 dimethyl arseno cysteine involves the formation an intermediate chelated 5 ringed species. The method of chemical substitution was chosen to validate this mechanism.



**Figure 15, proposed derivatives of dimethylarsenocysteine.**

The first synthesis target was dimethylarseno-N-acetyl cysteine (DMNAC)where the nitrogen is acetylate, 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 dimethylarseno-penicillamine (DMPEN) which contains 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 (DMNAC)

DMNAC is a new species that has not been previous synthesized. A synthetic procedure was adopted from the synthesis of dimethylarseno-cysteine involving the reduction of cacodylic acid the by N-acytl cysteine. A proof of concept for the reaction was done by adding 5 equivalents of N-acetyl cysteine to a solution of cacodylic acid in D2­­O. 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. 0.3579 g of cacodylic acid 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, resulting in a white powder. 1H NMR (400 MHz, D2O) 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 dissulfide 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, however 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 5 cacodylic acid as a source of arsenic, dimethylarsenoiodide(III) was used. This would give a clean reaction with a 1:1 ratio of arsenic and NAC.



Figure 15: New scheme for the preparation of DMNAC (py.HI)

*Preparation of Dimethylarsenoiodide.*

Me2AsI was prepared using the Burrows method[32](#_ENREF_4_1). Potassium iodide, 15g, and 5g of Me2AsOOH are 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 around 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. 1H NMR (400 MHz, CDCl3) δ 2.01.

*Preparation of dimethylarseno-N-acetyl cysteine*

0.5g of NAC was dissolved in dimethoxyethane and 1 ml of Me2AsI was added by syringe. 1 ml of pyridine 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 the filtrate was dried. NMR revealed the filtrand to be pyridinium iodide. NMR of the fitrate 1H 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 DNMAC with the expectation of the 1.15 ppm peak which was assigned MeAsOOH. This is most likely formed by the air oxidation of DNMAC, a reaction that is known to happen with the cysteine derivative. Recrystallization with various solvent mixtures was attempted but was unsuccessful.

A pure product is extremely important in our case because our experiments with the other derivatives have shown that the coalescence is sensitive to cysteine impurities.

3.1.2 Preparation of Dimethylarseno-penicillamine DMPEN

Dimethylarseno-penicillamine has not been previously synthesized. The synthetic scheme for the synthesis of DMNAC was adopted for the synthesis.



**Figure 16: Synthetic scheme for dimethylarseno-penicillamine**

Penicillamine, 0.5g, was suspended in dimethoxyethane. 1 ml of Me2AsI was added by syringe causing the full dissolution was penicillamine. 1 ml of pyridine 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 the filtrate was dried. Note this compound has an extremely unpleasant smell, and that Schlenk apparatus and proper fume hood containment methods are required. NMR revealed the filtrand to be pyridinium iodide. 1H NMR (400 MHz, d2o) 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). 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 proved unsuccessful.

3.2 Monomethylated derivatives

In Chapter 2 we showed that facile dimethylarsenic exchange occurred in the dimethylarsenic adducts of cysteine and glutathione. In this chapter we will take a look at its closely related cousin, the mono methylarsenous adducts. Monomethylarsonous acid is a key metabolite of the ingested inorganic arsenic though methyltransferase enzymes. 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, thus allowing it to bind very strongly to vicinal dicysteine residues. This chapter aims at examining if MMA species share similar reactivity with DMA and have labile arsenic-sulfur bonds.

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 literature methods.

*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*.

MeAsO(ONa)2 was dissolved in 50 ml of H2O. 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 SO2, 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 solution was dried and (MeAsO)x was extracted with benzene. Removing the benzene *in vacuo* resulting in a white solid (70% yield).

1H NMR (500 MHz, CDCl3) 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[33](#_ENREF_4_2)) of (CDCl3): δ 1.58/1.59 (5.0%), 1.50/1.51 (26.8%), 1.48 (60.0%), 1.43 (8.1%). 1H NMR (400 MHz, D2O) δ 1.17 (s, 1H). 1H NMR (500 MHz, C6D6) δ 1.21 (s, 1H). 1H NMR (500 MHz, CD3OD) δ 1.25 (dd, *J* = 11.1, 7.1 Hz, 1H). ESI of the compound did not reveal any tetramer peaks as NMR later indicated that it possibly hydrolyses.

Monomethyl arsinous acid takes the form of cyclic and linear oligomers, 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 CDCl3 NMR which contains 4 sets of multiplets in CDCl3.

3.2.2 Examining the nature of Monomethyl arsinous acid

Monomethyl arsinous acid exists in different oligomers with complicated and poorly understood equilibria and dynamics. . Marsmann and Wazer[34](#_ENREF_4_3" \o "Marsmann, 1970 #196) proposed the possibility of the species olgiomerizes 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 did not observe a hydrolysis with diphenyl ether.

To validate the possible presence of oligomers, a temperature dependent NMR experiment was performed with the sample in CDCl3. For this experiment 0.0975g of (MeAsO)x was dissolved in 1000 ul of CDCl3. The sample was initially cooled down to 273.15K and the temperature was slowly brought up in 10° increments.



**Figure 17:** 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, we notice a shoulder peak appearing at 1.48 ppm. We 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 of the peaks stay constant during the heating and the mixture returns to its original composition upon cooling to room temperature. Clearly under these condition there are no new species or alteration of the concentrations of the solution components. There remains open questions about different solvents, in particular the use of different solvents (such as diphenylether, the solvent used by Marsmann et al). Concentration variation, especially that of high concentrations of (MeAsO)x may confirm the oligiomerization. In addition it is difficult to heat the solvent to higher temperatures inside the NMR and this experiment could be extended by heating the compounds separately in other solvents. The oligomerization might not be a reversible process in which cause this would not cause a coalescence of the peaks in the NMR.

The molecular weight has to be experimentally determined. A calibration curve was made correlated using both the expected ratio of the two compounds based on volume calculations and the ratio based on the integrals of the corresponding peaks in the NMR spectrum.

The scaling co-efficient of 0.85 indicates we have only 85% of what we would have if we assumed that the molecular weight of (MeAsO)x was 116. This is expected as aliphatic isomers of (MeAsO)x would contain sodium and have a higher MW per unit.

3.2.3 Interaction of MeAs(OH)2 with cysteine

Previously in the project we 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.



*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 D2O (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 D2O. All solutions were deoxygenated by bubbling N2 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 mixing. 1H NMR (500 MHz, D2O) 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 corresponds to the following species, 1.76 ppm peak corresponds to MeAs(Cys)2 , 1.64 ppm peak to MeAs(OH)(Cys) and 1.358 ppm peak to MeAs(OH)2.

It is expected that this system undergoes the following equilibrium:



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 visulalize the species in terms of molar ratios:

**Figure 18: NMR titration of Cysteine against MeAsOH in D2O**

3.2.4 Temperature sensitivity of the methyl peak

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 we observe. 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 Me2AsCys case, we might also observe the coalescence of all the methyl peaks.

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.



The results indicate that there is minimal change to the peaks of the arsenic bound methyls and the coalescence of these peaks was not observed.

3.3 Summary

It has been demonstrated that methylarsenic and dimethylarsenic 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, we also saw dimethylarsenic transfer between cysteine and glutathione groups. Whilst it was not possible to directly model this interaction, it was qualitatively shown that arsenic readily transfer between various thiols. In addition it was shown that monomethyl arsenic species also have bond lability by titrating cysteine to a solution of monomethylarsenious acid. I have identified the formation of both MeAs(Cys)(OH) and MeAs(Cys)2, and these concentrations would change depending on the amount of cysteine added. 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. 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.

3.4 References

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