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Pentavalent Arsenic Can Bind to Biomolecules**

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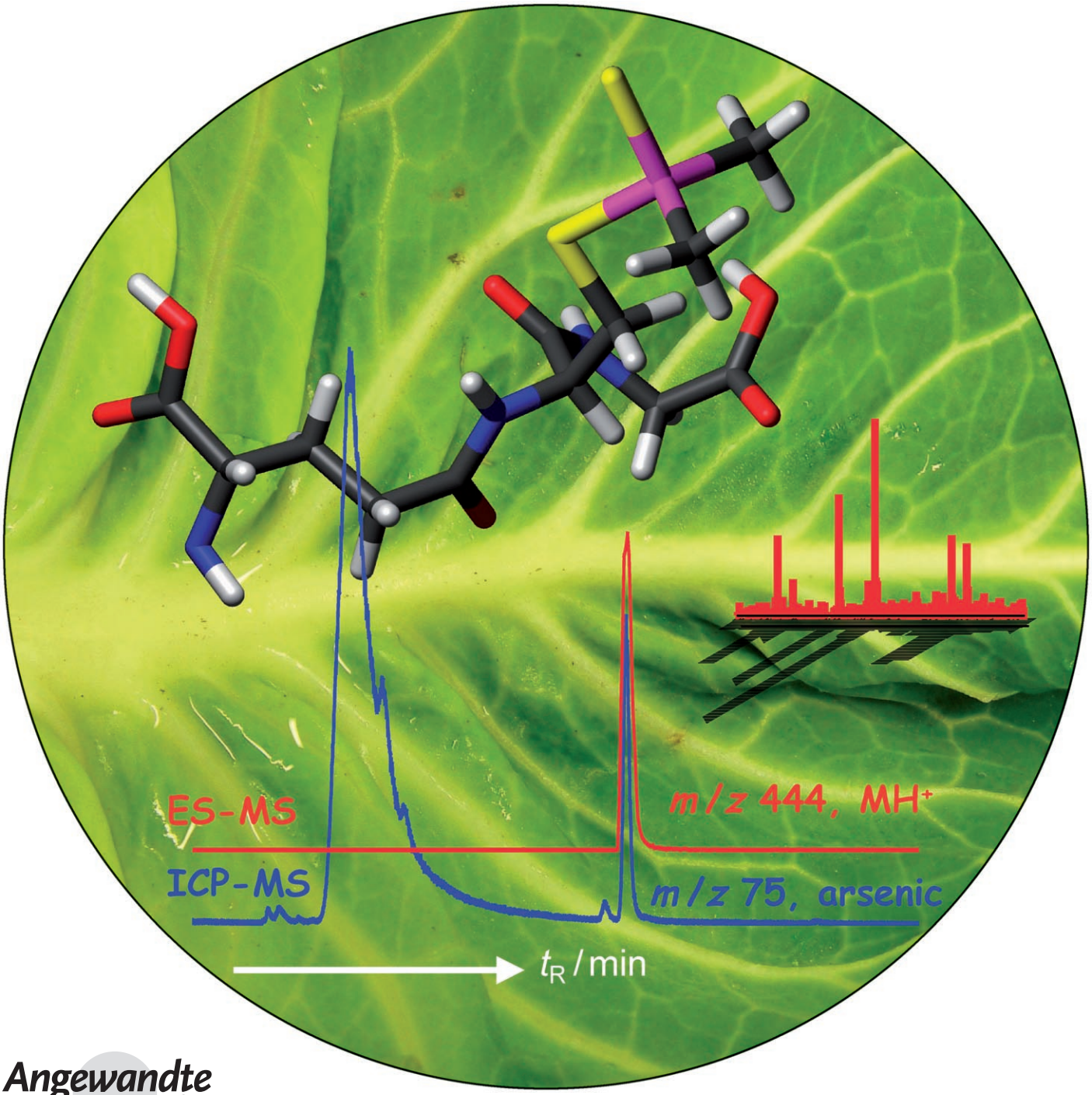
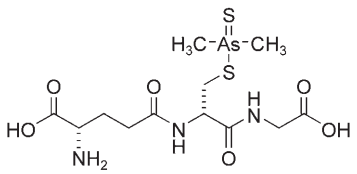


Arsenic has a strong affinity for sulfur and the formation of arsenic–sulfur compounds. Since the first report of the biological formation of As–S compounds, numerous As–S compounds with pentavalent As have been identified in biological samples;[1,2] however, all such compounds comprise small covalently bound molecules that do not contain amino acids. That the interaction between arsenic, in the form of arsenite (As^{III}), and sulfur plays a major role in the metabolic pathway of arsenic was recognized early on,[3] as was the ability of As^{III} to bind to thiol groups of peptides and proteins such as glutathione (GSH), phytochelatins (PC), metallothioneins, and others in vitro. So far, only a few arsenic–peptide species have been identified in vivo, such as a series of arsenic phytochelatin complexes in plants and arsenic glutathione complexes in rat's bile, all of which contain trivalent arsenic.[4,5] In all these cases, As behaves as predicted by Pearson's hard/soft acid–base (HSAB) concept.[6] Here we show for the first time that pentavalent arsenic can also bind to biomolecules when it is activated by sulfide, in contrast to the HSAB concept, by identifying the dimethylarsinothioyl glutathione complex (**1**) in cabbage (*Brassica oleracea*).



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An unknown dimethylated arsenic species was found during the study of the metabolism of inorganic and methylated arsenic species in *B. oleracea*. The plants were exposed to dimethylarsinic acid (DMA^v) through their roots, and no arsenic species other than DMA^v was found in the hydroponic solution before and after exposure. The shoot was extracted with 1% formic acid at +4 °C, as these conditions conserve arsenic glutathione and phytochelatin complexes.[4,7] The extract was analyzed immediately by reverse-phase (RP)-HPLC coupled simultaneously to electrospray mass spectrometry (ES-MS/MS) to gain molecular information from the mass fragments, and to inductively coupled plasma mass spectrometry (ICP-MS) to gain quantitative information about the different arsenic species in the extract. This method has previously been used successfully to identify and quantify unstable trivalent arsenic complexes.[4,7] Shoots of *B. oleracea* exposed to DMA^v contained three arsenic species (Figure 1), whereas other plants exposed to DMA^v

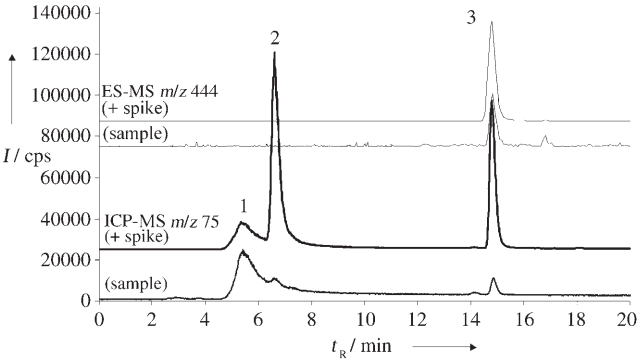

Figure 1. RP-HPLC chromatograms of *B. oleacera* shoot extract and the extract spiked with synthetic $DMAS^v$-GS: Top: using ion-trap ES-MS (Agilent XCT) extracted ion chromatograms of the protonated molecular ion (*m*/*z* 444); bottom: using ICP-MS (Agilent 7500c) for arsenic (*m*/*z* 75). Peak 1: dimethylarsinic acid (DMA^v); peak 2: dimethylarsinothioic acid ($DMAS^v$); peak 3: dimethylthioarsinoyl glutathione ($DMAS^v$-GS, **1**).

under similar conditions contained only DMA^v in their shoots (data not shown). The first two As species in the extract of *B. oleracea* revealed similar retention times and mass spectra to DMA^v (peak 1, *m*/*z* 139 $[M+H^+]$, MS^2 *m*/*z* 107 and 121) and its thio form $DMAS^v$ (peak 2, *m*/*z* 155 $[M+H^+]$, MS^2 *m*/*z* 109 and 137) and were isolated in quantities of 4.5 and 1.9 mg(As) kg^{-1}(dry weight), respectively (mass spectra not shown). The third arsenic-containing compound (0.2 mg(As) kg^{-1}(dry weight)) revealed by ICP-MS showed a signal at *m*/*z* 444 in the ES mass spectrum (Figure 1 and Figure 2a). None of the arsenic species identified so far fit to this compound. MS^2 spectra (Figure 2b) of this compound revealed an abundant species at *m*/*z* 315 corresponding to the loss of glutamic acid, which is a typical loss for GSH. The main fragment in MS^3 spectra of the peak at *m*/*z* 315 corresponded to a peak at *m*/*z* 177 ($[Cys-Gly]^+$), which confirmed the presence of GSH (Figure 2c).

Separation of the oxidized plant extract (using hydrogen peroxide) showed that the only arsenic species present was DMA^v. We therefore attempted to synthesize this unknown compound from $DMAS^v$ and GSH. Mixing these two compounds in water resulted in the formation of a molecule that displayed the same *m*/*z* ratio, MS^2 and MS^3 spectra, and retention time as the compound in the plant sample. Spiking of the plant extract with the synthesized compound showed excellent co-elution (Figure 1). The trivalent DMA^{III}-GS complex, synthesized according to Scott et al. and characterized by ^{1}H NMR spectroscopy,[8] eluted after 25 min under the chosen HPLC conditions.

As oxidation of arsenic during electrospray ionization cannot be excluded, the standard was further characterized by accurate mass spectrometry and ^{1}H NMR spectroscopy. The

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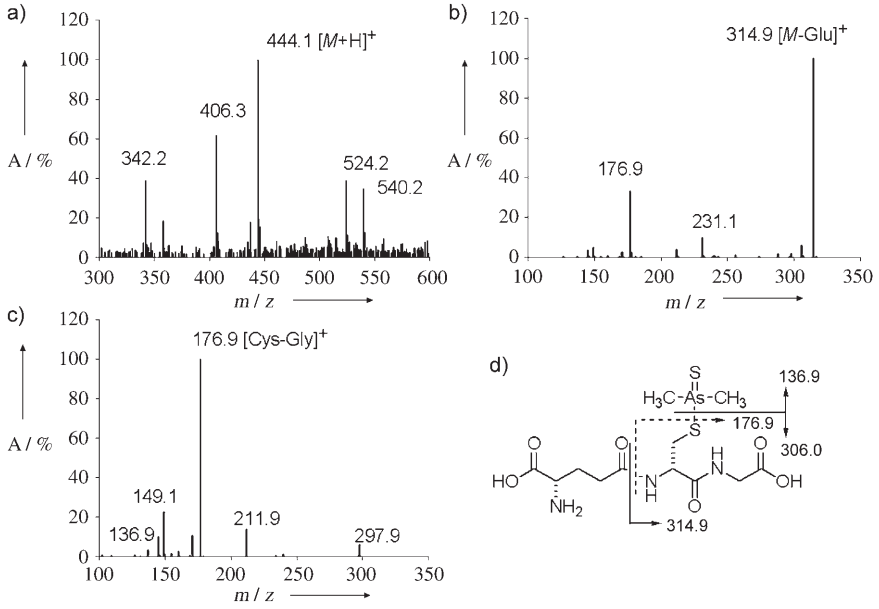

Figure 2. a) MS spectrum of the compound with a retention time of 14.5 min (see Figure 1) from the unspiked sample. b) MS^2 spectrum of the signal at m/z 444 in part (a). c) MS^3 spectrum of the signal m/z 314.9 in part (b). d) Structure of **1** showing the various fragments.

accurate mass was 444.02329 Da, which matches the protonated molecular formula $C_{12}H_{22}AsN_3O_6S_2$ with an error of −0.58953 mDa (−1.3 ppm).[9]

From the ES-MS data, the compound could be either pentavalent (GSH directly bound to As through its cysteine unit) or trivalent (GSH bound through a sulfur–sulfur bridge to As). The ^{1}H NMR chemical shift of the CH_3 groups binding to As in the complex appeared at $\delta = 1.99$ ppm, which was nearly identical to those for pentavalent DMA^V ($\delta =$ 1.97 ppm) and $DMAS^V$ ($\delta = 1.99$ ppm). This signal lies much further downfield than those for the trivalent arsenic species DMA^{III}-GS ($\delta = 1.34$ ppm) and DMA^{III} ($\delta =$ 1.36 ppm). This result confirms unequivocally that the compound present in *B. oleracea* is dimethylthioarsinoyl glutathione ($DMAS^v$-GS; **1**) and shows that pentavalent arsenic can occur in a biomolecule bound to the thiol groups of peptides.

Pearson's HSAB concept states that pentavalent arsenic is a hard acid not likely to bind to sulfur-containing biomolecules.[6] Arsenic seems to become softer when it is present as its sulfide so that it can establish stable bonds to thiols, such as cysteines of peptides and proteins. The presence of two methyl groups probably helps as well to stabilize the molecule, whereas the stability of the trivalent arsenic glutathione species decreases with decreasing number of thiol groups from As^{III}-$(GS)_3$ to MA^{III}-$(GS)_2$ to DMA^{III}-GS.[10,11] Exposure of plants to MA^v or As^v produced only trivalent arsenicals such as MA^{III}-$(PC)_2$ and As^{III}-$(PC)_3$. Interestingly, exposure to DMA^v forms only pentavalent arsinothioyl metabolites (DMA^v, $DMAS^v$, and $DMAS^v$-GS) and not the trivalent or pentavalent oxo forms of arsenic glutathione species (DMA^v-GS).

So far, the only hint that pentavalent As–S compounds bound to proteins/peptides might exist comes from a scheme proposed by Rosen and co-workers.[12] They suggested that such a molecule might be formed as an intermediate during the reduction of As^v to As^{III} by the ArsC arsenate reductase of *E. coli*, in which As is bound between a thiol group of the enzyme and a thiol group from GSH and which initiates the reduction of As^v. As yet, they have not been able to isolate this intermediate. $DMAS^v$-GS found in *B. oleracea* is similar to their intermediate in that it contains pentavalent As bound to a peptide, but $DMAS^v$-GS is relatively stable and present in *B. oleracea* in relatively high concentrations.

This result indicates the importance of sulfur metabolism when arsenic is taken up and metabolized by plants. Arsenic shows a different physiological behavior when bound to biomolecules, and it seems that this behavior has a significant influence on the translocation and accumulation of arsenic in plants.[13] The finding that pentavalent sulfidic arsenic binds to glutathione in $DMAS^v$-GS changes the view of how arsenic may interact with other sulfur-rich biomolecules such as proteins, and it highlights the fact that sulfide reactions may have a key role in the reactivity of arsenic intermediates and the metabolic pathway of arsenic in any organism.

Experimental Section

Brassica oleracea plants were grown from seed for 12 weeks (two plants per pot) in Vermiculite and fertilized once a week except in the last week, when no fertilizer was used. The roots were freed of Vermiculite before the plants were exposed to arsenic in the form of DMA^v for 24 h. After the incubation period, the plants were separated into root and shoot. Each part of the plant was ground separately under liquid nitrogen and extracted with 1% formic acid (solid/liquid ≈ 1:3) for 90 min at 4 °C. After that, the extract was filtered (0.45 μm) and injected onto the HPLC column. The separation and detection conditions were similar to those described previously.[4,10] Briefly, the arsenic species were separated by a C_{18} reversed phase column (Spherisorb ODS2, 250 mm × 4.6 mm; Waters, USA); eluent A was 0.5% formic acid in water and eluent B was 0.5% formic acid in methanol (0–20% methanol). A linear gradient to 20% methanol within 20 min and then 10 min at 20% methanol was used at a flow rate of 1 mL min^{-1}. The flow from the column was split with one part of the eluent going into the ICP-MS (Agilent 7500c, Agilent, USA) and four parts going into the ES-MS (Agilent XCT, Agilent USA). The ES-MS was used in positive mode. The ICP-MS data were used for calculations of the As concentrations in the different species. DMA^v was used as calibration species for the quantification of all arsenic species in the chromatograms (peak areas versus concentration). ^{1}H NMR (400 MHz) spectra were recorded in 1:1 D_2O/H_2O on a Varian Unity INOVA spectrophotometer using a standard presaturation pulse sequence, and the resonances were referenced on an internal standard (methanol, $\delta = 3.34$ ppm). For the accurate mass measurement, the sample was diluted 1:1 in 1% formic

acid in methanol. Glutathione was used as an internal standard and measured with an error of 0.16267 mDa (0.53 ppm).

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