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Investigations into the role of methylcobalamin and glutathione for the methylation of antimony using isotopically enriched antimony(V)†

Silvia Wehmeier, Andrea Raab and Jörg Feldmann*

University of Aberdeen, Department of Chemistry, Meston Walk, Aberdeen AB24 3UE, Scotland, UK

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Glutathione (γ-Glu–Cys–Gly, GSH) and methylcobalamin (CH_3-B_{12}) may play a role in the biomethylation process of antimony. To understand better the transformation of antimony in biological systems, we studied abiotic and biomethylation processes and the influence of GSH in the methylation.

CH_3-B_{12}, acting as a possible methylating agent for antimony, was studied with GSH and in the absence of GSH. The most abundant product of this reaction was monomethylantimony, with a small concentration of the dimethylantimony species, as identified by hydride generation cryotrapping gas chromatography inductively coupled plasma mass spectrometry (HG-CT-GC-ICP-MS). In the same experiments we found that tris(γ-Glu–Cys–Gly)trithioantimonite [$Sb(GS)_3$] and di(γ-Glu–Cys–Gly)methyldithioantimonite [$(CH_3)Sb(GS)_2$] complexes were present using flow-injection electrospray ionization MS. Both complexes were also identified in a fermented sewage sample, suggesting that these complexes may play a role as intermediates in the biomethylation of antimony.

However, CH_3-B_{12} is not the sole methylation agent, since it does not produce any trimethylantimony species as identified in anaerobic sewage sludge cultures inoculated with enriched $^{123}Sb(V)$. Species-specific $^{123/121}Sb$ isotope ratio measurements of the different methylantimony species suggest a stepwise methylation of antimony according to the Challenger mechanism.

KEYWORDS: antimony; biomethylation; methylcobalamin; glutathione; electrospray mass spectrometry; GC-ICP-MS; speciation

INTRODUCTION

In contrast to arsenic methylation, there is less information known about antimony methylation. Nevertheless, methylantimony species have been detected in pure[1] and mixed[2] bacteria cultures, fungi cultures,[3] soil,[4] geothermal waters,[5] plant material[6] and sewage environments.[7] It has been proposed that the biotransformation of inorganic antimony and arsenic involves successive reduction and oxidative methylation steps.[8] The reduction of antimonate (antimony(V)), to antimonite (antimony(III)), is followed by oxidative methylation to form pentavalent monomethylantimony species. The monomethylantimony species is reduced to its trivalent form, and further methylation forms dimethylantimony species. After reduction of dimethylantimony species, the last methylation step leads to trimethylantimony species, the precursor for the volatile end-product trimethylstibine. The mechanism of the biological pathway has not been solved. The role of *S*-adenosylmethionine (SAM) in the methylation process of antimony, in which the methyl group is transferred as CH_3^+ to the nucleophilic antimony, has been accepted.[9] This mechanism is not believed to operate for the methyl

*Correspondence to: Jörg Feldmann, University of Aberdeen, Department of Chemistry, Meston Walk, Aberdeen AB24 3UE, Scotland, UK.
E-mail: j.feldmann@abdn.ac.uk
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donor methylcobalamin (CH_3-B_{12}). Wood *et al*.[10] showed that CH_3-B_{12} is the methyl donor to mercury in sewage bacteria. The abiotic methylation reaction of CH_3-B_{12} with mercury first forms methylmercury and then dimethylmercury.[11,12] There have been a number of reports suggesting a connection between CH_3-B_{12} and arsenite methylation. McBride and Wolfe[13] studied whether CH_3-B_{12} functioned as the methyl donor in the biosynthesis of dimethylarsine from arsenate or arsenite in cell extracts of a certain methanogenic Archaea. Buchet and Lauwerys[14] indicated that CH_3-B_{12} can methylate arsenite at a low rate in the presence and absence of rat liver cytosol.

The biomethylation of arsenic is enzymatically controlled[15,16] and sulfur-containing molecules like glutathione (γ-Glu–Cys–Gly, GSH) are believed to be involved in the reduction, formation and stabilization of the trivalent intermediates.[17–20] It has also been demonstrated that arsenite methylation by CH_3-B_{12} and glutathione *in vitro* does not require an enzyme.[20,21] These groups also reported the enhancement of the abiotic arsenic methylation by the addition of selenium(IV).

The interaction of antimony(III) tartrate with GSH has been studied by Sun *et al*.[22] This showed that antimony(III) can react with thiolates as a soft metal, but little is known about its mode of binding to GSH, its stability or occurrence in nature. After antimony was intravenously injected into Wistar rats, increased biliary excretion of GSH was detected. It has been demonstrated that a glutathione-dependent hepato biliary transport system exists for antimony(III).[23] The standard treatment of human leishmaniasis involves the use of pentavalent antimony compounds. The mode of action of these compounds has not been fully elucidated, but the possibility that antimony(III) is involved has been suggested.[24] The bio-molecule for the conversion of antimony(V) to antimony(III) has not been identified, but GSH does promote the reduction of antimony(V) into antimony(III).[25]

In this paper, we present the abiotic methylation of antimony using CH_3-B_{12} as the methylating agent and discuss the role of GSH and addition of selenium in the methylation process. In addition, we compare this with the methylation and GSH interaction of antimony in a biological sample.

EXPERIMENTAL

Reagents

Deionized water (18 MΩ Elga, UK) was used throughout the experiments. Antimony(III) potassiumtartrate (GPA; BDH, Poole, UK), Potassium antimonate ($KSb(OH)_6$; AnalaR, BDH, Poole, UK), enriched ^{123}Sb as antimonate (98.7% ^{123}Sb and 1.3% ^{121}Sb) magnesium chloride ($MgCl_2 \cdot 4H_2O$; AnalaR, BDH, UK), sodium selenite ($Na_2SeO_3 \cdot 5H_2O$; AnalaR, BDH, UK), CH_3-B_{12} (Sigma Aldrich GmbH, Germany) and GSH (Sigma Aldrich, Germany) were used for the preparation of reaction solutions and for the synthesis of the complexes. Tris (AnalaR, BDH, UK) and methanol (BDH, UK) were used for the precipitation of the complexes. Complexes were dissolved in 1% formic acid (100% p.a. BDH, UK) for (ESI-MS) injections. $(CH_3)_3Sb(OH)_2$ solution was used as methyl antimony standard and 6% sodium borohydride ($NaBH_4$; FIA, Baker) solution was prepared fresh daily for the hydride-generation (HG) methodology.

Sample preparation

Abiotic methylation reaction

CH_3B_{12} and GSH stock solutions were prepared under nitrogen. For the antimony(III) methylation, 0.3 μM antimony(III), with 0, 3 or 30 μM GSH, and with 10 mM $MgCl_2$, 0.1 mM $Na_2SeO_3 \cdot 5H_2O$ and 0.2 mM CH_3-B_{12} in a final volume of 10 ml Tris buffer (pH 7.8; abiotic methylation experiment) or H_2O (influence of GSH experiment) were left to react at 37 °C in the dark for 30 min.

For the antimony(V) methylation experiment, 0.2 μM antimony(V), with 0, 10 μM GSH, and with 0.1 mM $Na_2SeO_3 \cdot 5H_2O$ and 0.2 mM CH_3-B_{12} in a final volume of 10 ml H_2O were left to react at 37 °C in the dark for 30 min.

Synthesis of antimony–GSH complexes

Tris(γ-Glu–Cys–Gly)trithioantimonite [$Sb(GS)_3$] was synthesized by dissolving 0.15 g antimony(III) tartrate and 0.15 g $KSb(V)(OH)_6$ with 0.45 g and 0.75 g GSH respectively in 1 ml water. After 24 h reaction time at room temperature, in the dark, the resulting complex was precipitated in methanol and dried under nitrogen.

Synthesis of methyl antimony–GSH complexes

Di(γ-Glu–Cys–Gly)methyldithioantimonite [$(CH_3)Sb(GS)_2$] was synthesized by dissolving crystals of the previously synthesized [$Sb(GS)_3$] complexes in 100 μl 2 mM CH_3-B_{12} solution; after 24 h reaction time at room temperature in the dark, the complex was precipitated in methanol and dried under nitrogen.

Biological sample

Small fermenter bottles (100 ml) were set up with 60 g digested sewage sludge (as the bacterial medium) from a waste-water treatment plant in northeast Scotland. To encourage the growth of methanogenic bacteria, 10 ml acetate solution (20 g l^{-1} calcium acetate, BDH, UK) was added. Additionally, each fermenter bottle contained 1.5 g l^{-1} antimony (antimony(III) tartrate, BDH, UK) and 1.5 mg l^{-1} $^{123}Sb(V)$ isotopically enriched (98.7%) potassium antimonate solution.

The fermenter bottles were incubated at 37 °C in the dark, on a horizontal shaking plate, for 14 days. Sterile control fermenters produced methane, hence indicating biological activity, which also resulted in the production of traces of trimethylstibine.

Non-volatile methylantimony species were determined in the sludge filtrate (filtered sludge) and methanol–water-extracted sludge. After the incubation time, the sludge was vacuum filtered on a 0.45 μm cellulose nitrate filter (Whatmann Laboratory Division, UK). Sludge was extracted with methanol/water (80 : 20, v/v). The methanol (BDH, GPR grade 99.5%)–water (18 MΩ water, Elga UHQ II, Bucks, England) extraction procedure was as follows: approximately 10 g sludge (weighed) were extracted with 10 ml methanol/water (80 : 20, v/v). The extraction included a vortex mixing step, followed by 10 min ultrasonic agitation, and another vortex mixing step. Finally, the sample was centrifuged for 5 min. The extract was collected and the remaining sludge was extracted a second time. Both extracts were combined and the volume evaporated at room temperature using nitrogen to 3–5 ml. The methanol–water extract is referred to as cell lyses (Note: the extracted sludge medium has methylantimony species from the cell and from the medium.)

Fermenter gas samples were collected for volatile antimony compounds in Tedlar® bags (5 l bag, Supleco, Belafonte, USA) by purging the fermenter with 500 ml nitrogen.

Solid phase extraction (SPE)

Andrewes and co-workers[9,26] separated inorganic antimony from di- and tri-methylantimony using ammonium carbonate buffer (pH 12), and Smith *et al*.[3] reduced the level of inorganic antimony substrate prior to analysis by using a potassium acetate buffer (pH 9.6).

SPE columns for reaction mixture clean up were prepared using basic alumina (aluminium oxide). Into a 2.5 ml syringe were placed 2 g of alumina and a small glass-wool plug to hold the alumina in place. The SPE column was primed with 4 ml water or buffer solution. For sample elution, 1 ml water or the following different buffer systems were tested: 50 mM ammonium carbonate buffer (pH 12), 0.1 M potassium acetate buffer (pH 7.5 and pH 9.6), 50 mM citrate buffer (pH 5.4). The sample eluent was subjected to analysis using HG-gas chromatography (GC)–inductively coupled plasma (ICP)-MS.

Instrumentation

ESI-MS

The HP1100 series liquid chromatography/mass-specific detector (LC/MSD) instrument (Agilent Technologies, USA) was used as a mass detector for the detection of Sb–GSH and methylantimony–GSH complexes by their molecular peaks $(M + nH)^{n+}$. The electrospray settings were as follows: capillary voltage 4000 V, nebulizer pressure 40 psi, drying gas flow 12 l min^{-1} at 350 °C, quadrupole temperature 100 °C, fragmentor voltage 100 V for positive ionization mode, and the MSD was run in scanning mode (m/z 120–1400). 100 μl sample solution was injected for ESI-MS using the flow injection analysis (FIA) mode and 1% formic acid and nitrogen as carrier solution and carrier gas respectively.

HG-GC–ICP-MS

Abiotic methylation reaction mixtures, fermenter sludge filtrates and methanol extracts were analysed for monomethylantimony, dimethylantimony, and trimethylantimony species as the volatile hydride derivates with 6% $NaBH_4$, using HG-GC–ICP-MS. The HG procedure was carried out in a pH 7 or pH 1 system for antimony(III) sludge extracts and medium. The set-up consisted of a reaction vessel, which was connected to a U-shaped glass tube (6 mm OD, $L = 25$ cm, filled with adsorbing material of 10% SP 2100 on 80/100 *Supelcoport* (Supelco Inc., USA), and wrapped with nichrome wire. The U-tube was connected via a transfer-line (Chrompack Ultimetall, methyldeactivated, 0.53 mm ID, $L = 90$ cm) to the ICP-MS torch. All lines were heated (80 °C) continuously to prevent condensation.

The helium flow was directed with a six-port stainless-steel switching valve (rotor material: polyarylethylketone–PTFE composite; Valco, 4C6WE, 1/16″, Valco Europe, Schenkon, Switzerland). After adding the sample/standard to a final 10 ml aqueous solution (pH 7 or pH 1), the closed system was purged of oxygen with 120 ml min^{-1} helium. After this initial phase, 1 ml 6% $NaBH_4$ was injected slowly with a syringe via the septum (white silicone/PTFE septa, 75 mm thick, Supelco Inc., USA) of the reaction vessel side neck-port. During a 5 min reaction time the antimony species were reduced and formed volatile stibines, which were purged out continuously, with a 120 ml min^{-1} helium flow, onto the U-tube submerged in liquid nitrogen. After the reaction, the trapped stibine species were released by removing the liquid nitrogen and heating the U-tube to 170 °C. The volatile antimony species were transported through the transfer-line into the torch where they were mixed with the additional argon flow, which carried a nebulized internal standard aerosol (generated in the spray chamber) into the plasma. An ICP time-of-flight (TOF) mass spectrometer (Renaissance Leco, USA) with 1350 W forward power, 40.68 MHz, 20 kHz spectral frequency for data collection, 0.89 l min^{-1} nebulizer flow, and a Meinhard, Wu-Hieftje spray chamber (Leco) nebulizer was used with a 170 ms integration time; a 10 μg l^{-1} rhodium solution was used as a continuous internal standard to monitor the plasma stability.

Cryotrapping-cryofocusing GC–ICP-MS (CT-CF-GG–ICP-MS)

For the determination of the stibine species, sub-samples were taken from the Tedlar® bags and analysed with an in-house-prepared trimethylstibine gas standard. The preparation of a gas standard by HG and the analysis by CT-CF-GC–ICP-MS is described elsewhere.[27]

Gas-samples were analysed using a quadrupole-ICP mass spectrometer (Spectromass 2000, Spectro Analytical).

RESULTS AND DISCUSSION

Abiotic methylation of antimony by CH_3-B_{12}

Identification of [Sb(GS)$_3$] and [(CH$_3$)Sb(GS)$_2$] complexes using ESI-MS

[Sb(GS)$_3$] and [(CH$_3$)Sb(GS)$_2$] were identified in the abiotic reaction and biological samples. The molecular peak identification using ESI-MS for the synthesized Sb–GS complexes and fermenter samples is summarized in Tables 1–3.

Our attempts to separate Sb–GS complexes and monomethylantimony species prior to ESI-MS analysis by high-performance LC (HPLC) were not successful. The chromatographic recoveries of ion exchange (using conditions as detailed in the literature[28–30]) and also reverse phase (which has been used to successfully separate As–GS complexes[31]) indicated that Sb–GS complexes are irreversibly retained on the column. Antimony speciation by HPLC has, so far, only been reported for antimony(III) and antimony(V) and trimethylantimony oxide.[28,29,32]

The complexes were, however, characterized as their protonated molecular masses by m/z signals in flow-injection ESI-MS. Antimony has two natural isotopes, m/z 121 (57.2%) and 123 (42.7%). Therefore, the isotope abundance can be used for identification because the mass ratios of molecules containing only one antimony correspond to the isotopic pattern. [Sb(GS)$_3$], $[M+H]^+$, showed a molecular mass peak at 1040/1042. Peaks at 520.5/522.5 and 347/349 were attributed to the doubly and triply protonated structures respectively. The [(CH$_3$)Sb(GS)$_2$] complex showed a signal at m/z 749/751, and at m/z 375, 377 $[(CH_3)Sb+2H]^{2+}$ and 250, 252 $[(CH_3)Sb+3H]^{3+}$. $[(CH_3)SbO(OH)_2+H]$ showed a signal at m/z 186/188. This confirms the study from Yan *et al.*,[33] that antimony(III) is strongly coordinated to the sulfur atoms.

Abiotic methylation of antimony by CH_3-B_{12}

Identification of methylated antimony species

The demonstration that ESI-MS could be used to identify Sb–GS complexes led to the investigation of abiotic methylation of antimony using CH_3-B_{12} with and without GSH. The reaction solution was subjected to HG-CT-GC–ICP-MS. The chromatogram shows the occurrence

Table 1. Molecule identification[a] in the ESI-MS analysis of abiotic methylation reaction mixture. HP1100 series LC/MSD instrument (Agilent Technologies, USA), positive ionization mode from *m/z* 120 to *m/z* 1400, capillary voltage 4000 V, nebulizer pressure 40 psi, drying gas flow 12 l min^{-1} at 350 °C, quadrupole temperature 100 °C and fragmentor voltage 100 V, 100 µl sample solution was injected for ESI-MS using the FIA mode and 1% formic acid and nitrogen were used as carrier solution and carrier gas respectively

m/z	Identity	Sb(III)+ GSH + CH_3-B_{12}	Sb(V) + GSH + CH_3-B_{12}
1040, 1042	$[Sb(GS)_3+H]^+$	+	+
520.5, 522.5	$[Sb(GS)_3+2H]^{2+}$	+	+
347, 349	$[Sb(GS)_3+3H]^{3+}$	−	−
749, 751	$[(CH_3)Sb(GS)_2+H]^+$	+	+
375, 377	$[(CH_3)Sb(GS)_2+2H]^{2+}$	+	+
250, 252	$[(CH_3)Sb(GS)_2+3H]^{3+}$	−	−
186, 188	$[H_3C-Sb-O(OH)_2+H]^+$	−	−

[a] +: detected; −: not detected.

Table 3. Molecule identification[a] in the ESI-MS analysis of fermenter samples (methanol–water-extracted sludge) spiked with antimony(III) tartrate or isotopically enriched antimonate, ^{123}Sb(V). For conditions, see Table 1

m/z	Identity	Sb(III)	^{123}Sb(V)
1040, 1042	$[Sb(GS)_3+H]^+$	+	+
520.5, 522.5	$[Sb(GS)_3+2H]^{2+}$	+	+
347, 349	$[Sb(GS)_3+3H]^{3+}$	−	+
749, 751	$[(CH_3)Sb(GS)_2+H]^+$	+	+
375, 377	$[(CH_3)Sb(GS)_2+2H]^{2+}$	+	+
250, 252	$[(CH_3)Sb(GS)_2+3H]^{3+}$	−	+
186, 188	$[H_3C-Sb-O(OH)_2+H]^+$	−	+

[a] +: detected; −: not detected.

Table 2. Molecule identification[a] in the ESI-MS analysis of synthesized [Sb(GS)$_3$] and [(CH$_3$)Sb(GS)$_2$] complexes. For conditions, see Table 1

m/z	Identity	Sb(III)+ GSH	Sb(V)+ GSH	Sb(III) + GSH + CH_3-B_{12}	Sb(V) + GSH + CH_3-B_{12}
1040, 1042	$[Sb(GS)_3+H]^+$	−	−	+	+
520.5, 522.5	$[Sb(GS)_3+2H]^{2+}$	−	−	−	−
347, 349	$[Sb(GS)_3+3H]^{3+}$	+	+	+	+
749, 751	$[(CH_3)Sb(GS)_2+H]^+$	−	−	+	+
375, 377	$[(CH_3)Sb(GS)_2+2H]^{2+}$	−	−	−	−
250, 252	$[(CH_3)Sb(GS)_2+3H]^{3+}$	−	−	−	−
186, 188	$[H_3C-Sb-O(OH)_2+H]^+$	−	−	−	−

[a] +: detected; −: not detected.

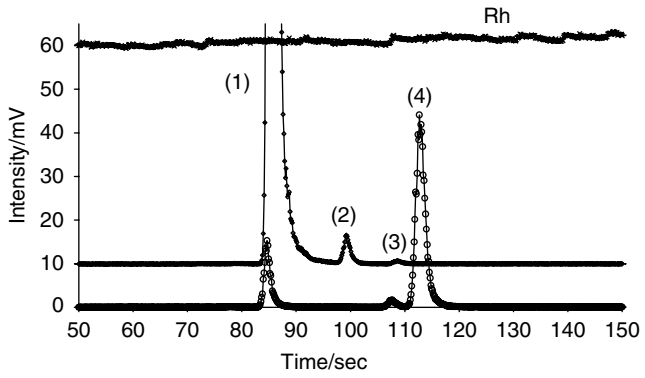

Figure 1. HG-CT-GC–ICP-MS chromatogram of abiotic methylation of antimony(III) by CH_3-B_{12} (diamonds). Open circles represent $(CH_3)_3Sb(OH)_2$ standard (1 ng). Plasma conditions were monitored by an internal rhodium (10 ppb) standard (top trace). Peaks are volatile derivatives of non-volatile methylantimony species: (1) SbH_3; (2) $(CH_3)SbH_2$; (3) $(CH_3)_2SbH$; (4) $(CH_3)_3Sb$.

of inorganic antimony (as SbH_3), and small amounts of monomethylantimony (as CH_3SbH_2) and dimethylantimony species (as $(CH_3)_2SbH$) (Fig. 1). Although the enormous amounts of stibine do not affect the plasma conditions and is well separated from methylstibine, it was decided to separate the inorganic antimony from the organic antimony species by SPE. SPE was investigated to separate the methylantimony compounds from the large amounts of antimony(III) tatrate in the abiotic reaction mixture. Water, ammonium carbonate, potassium acetate and citric acid buffer systems were used for eluting the methylantimony species. However, none of the buffer systems separated mono- and di-methylantimony species from the inorganic antimony in the abiotic reaction mixture. Inorganic antimony was retained on the SPE column, but so were the methylantimony species.

Several solutions used to elute antimony from basic alumina are described by Smichowski *et al*.[34] Andrewes and co-workers[9,26] separated inorganic antimony from di- and tri-methylantimony in cultures of *Scopulariopsis brevicaulis* using ammonium carbonate buffer (pH 12), and Smith *et al*.[3] reduced the level of inorganic antimony substrate from *Clostridum* spp. culture media prior to analysis by using a potassium acetate buffer (pH 9.6). The biologically produced non-volatile mono-, di- and tri-methylantimony species were detected using HG-GC atomic absorption spectrometry.

However, when the abiotic methylation reaction mixture was spiked with trimethylantimonyhydroxide, $(CH_3)_3Sb(OH)_2$, and passed through the SPE column, 80% of the trimethylantimony species was eluted with potassium acetate buffer, pH 7.5, while inorganic antimony, monomethyl- and dimethyl-antimoiny species were retained on the column. Additionally, inorganic antimony was also separated from mono-, di-, and tri-methylantimony species when a biological sample (methanol–water-extracted fermenter sludge) was passed through the SPE column and eluted with potassium acetate buffer.

In our abiotic reaction mixture, mono- and dimethyl-antimony species were retained on the SPE column, although the spiked standard was eluted almost quantitatively. This indicates that mono- and dimethyl-antimony species in this reaction mixture are possibly of a different nature than the biological samples, because the methylantimony species cannot be separated from inorganic antimony. Methylantimony species in biological systems may be bound to biomolecules that allow a separation of the methylantimony species from inorganic antimony using SPE.

These results confirm that antimony is methylated by CH_3-B_{12} in the abiotic reaction to mono- and dimethyl-antimony species, but the mechanism is not clear.

The species were identified by comparing the retention times of the sample with the volatile species obtained when a $(CH_3)_3Sb(OH)_2$ standard solution was treated with $NaBH_4$ under the same conditions to produce trimethylstibine, $(CH_3)_3Sb$. Monomethylantimony, and dimethylantimony species standard compounds are not available, but in HG methodology they are often generated as by-products. This means that HG of a trimethylated antimony compound can, in addition to trimethylstibine, result in the generation of stibine, monomethylstibine and dimethylstibine.[35–37] This has been found to be a particular problem with antimony speciation in environmental samples, and has been discussed extensively.[38,39] Concentrations of species in the samples were calculated using a one-point calibration of the hydride-generated $(CH_3)_3Sb(OH)_2$ standard and were based on the assumption that peak areas of the by-products of the antimony standard are proportional to the antimony concentration, since the ICP-MS response is independent of the metalloid species.

The monomethylantimony species yield was 2% in the abiotic antimony(III) methylation by CH_3-B_{12}, as shown

Table 4. Abiotic methylation of antimony(III) by CH_3-B_{12}. Values are means plus/minus standard deviation ($n = 3$). The control samples consists of Tris–HCl buffer pH 7.8, 0.3 μM antimony(III) tartrate, 0.1 mM selenium(IV), 30 μM GSH and 0.02 mM CH_3-B_{12} in a total volume of 10 ml. The other samples have either one compound omitted (−), or $MgCl_2$ (1 mM) was added (+). Samples were incubated at 37 °C in the dark for 30 min (361 ng antimony, as antimony(III) tartrate in 10 ml)

	Species mass (ng)	
	Monomethylantimony	Dimethylantimony
Control	8.2 ± 0.9	0.7 ± 0.2
Mg^{2+}(+)	3.5 ± 0.4	0.8 ± 0.4
Se (−)	1.9 ± 0.5	0.1 ± 0.3
GSH (−)	2.8 ± 0.2	0.5 ± 0.1
CH_3B_{12}(−)	0	0

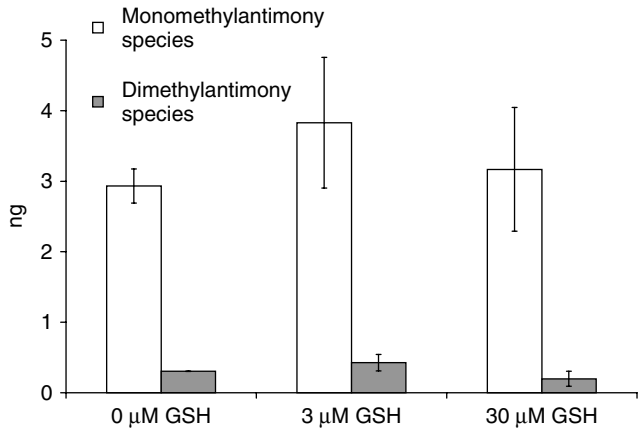

Figure 2. Influence of GSH (0, 3, 30 μM) in the abiotic methylation of antimony(III) tartrate by CH_3-B_{12}. Values are means plus/minus standard deviation ($n = 3$). Medium consists of 0.3 μM antimony(III), 1 mM $MgCl_2$, 0.1 mM selenium, 0.02 mM CH_3-B_{12}, 0, 3 or 30 μM GSH, in a total volume of 10 ml (water). Incubated at 37 °C in the dark for 30 min.

in Table 4. The methyl group is transferred from CH_3-B_{12} to antimony(III). GSH was not required in the abiotic methylation process (Fig. 2). Sodium selenite did enhance the abiotic methylation ($P = 0.01$). The addition of Mg^{2+} to the reaction mixture decreased the methylation yield of monomethylantimony species significantly ($P < 0.01$). The abiotic methylation is not yet fully understood, because antimony(III) as $Sb(OH)_3$, or possibly $Sb(OH_4)^-$ in solution, is a centre for nucleophilic attack and it is likely that the methyl group is transferred as a carbonium ion (CH_3^+). A potent methyl carbonium ion donor is SAM, and this reaction is referred to as the Challenger mechanism, which was formulated for the methylation of arsenic.[8] SAM has been shown to be the methylating agent for the metal(loid)s selenium, tellurium, phosphorus and antimony.[40] For the abiotic methylation of arsenic by CH_3-B_{12}, it was suggested[14,21] that, under the highly reducing conditions of this reaction, methylation occurs via nucleophilic attack of an arsenite–GS complex on the Co–C bond of CH_3-B_{12}. Abiotic methylation by CH_3-B_{12} has been shown for mercury,[11,12] arsenic,[13] and platinum.[41] Research into abiotic methylation presented by Zakharyan and Aposhian[42] showed that arsenite methylation by CH_3-B_{12} and GSH does not require an enzyme. Inorganic arsenite was methylated by CH_3-B_{12} in a simple abiotic system to produce mainly methylarsonic acid, MA(V), and small amounts of dimethylarsinic acid, DMA(V). It was also shown that, in such a system, a reducing environment (such as GSH or sodium selenite) was required; moreover, selenite enhanced the methylation in an additive manner.[42] Recently, the investigation into the abiotic methylation of arsenic, which had been carried out as a radioactive-labelled experiment, was confirmed using HPLC–ICP-MS.[21]

The abiotic methylation of antimony by CH_3-B_{12}, on the contrary, did not require GSH, but selenite did enhance the methylation. Zakharyan and Aposhian[20] reported 2.1% and 12% methylation yields of the arsenite by CH_3-B_{12} in the presence of GSH and the addition of selenite respectively. Pergantis *et al.*[21] reported a comparable methylation yield (to Zakharyan and Aposhian[20]), and observed that the methylating efficiency was greater with higher GSH concentration. In our experiment, the yield of the abiotic methylation of antimony(III) with Mg^{2+}, in the absence of Mg^{2+} and selenite was 1%, 2% and 0.5% respectively (Table 4).

Influence of GSH concentration in the abiotic methylation of antimony(III) and antimony(V)

Figure 2 shows that the concentration of the mono- and di-methylantimony produced was not significantly ($P = 0.18$) different in the abiotic reaction mixtures with varying GSH concentrations. The 0, 3 and 30 μM GSH concentrations resulted in 2.9 ± 0.2 ng, 3.8 ± 0.9 ng, and 3.2 ± 0.9 ng monomethylantimony species respectively and 0.33 ± 0.07 ng, 0.43 ± 0.12 ng, and 0.20 ± 0.11 ng dimethylantimony species respectively from 361 ng antimony, as antimony(III) tartrate. A 30 times higher GSH concentration did not alter the methylation yield. There was no difference in the abiotic methylation reaction when it was carried out in water or in Tris buffer (pH 7.8), as shown in Fig. 2 and in Table 4 respectively.

GSH is present in many cells at millimolar concentration and generally is the most abundant non-protein thiol.[43] Sun *et al.*[22] identified a complex of antimony and GSH, *in vitro*, with the stoichiometry 1 : 3, as $[Sb(GS)_3]$.

A relationship between antimony and GSH excretion in the bile has been shown,[23] and the transport of the metalloid as an unstable GSH complex was suggested. However, whether antimony methylation is altered by the GSH concentration has never been investigated. Buchet and Lauwerys[17] studied the role of thiols in the *in vitro* methylation of inorganic arsenic by rat liver cytosol and reported the stimulation of GSH for methylation activity, especially on DMA(V) production. They showed a decrease of methylation of arsenite to MA(V) in rat-liver cytosol when the GSH concentration was too high. This suggests a large excess of thiol groups may also block the methylation reaction, possibly by decreasing the amount of free trivalent arsenic and leading to a possible increased biliary excretion of arsenite.[17] The study described above, however, investigated the important role of GSH in the biological system of rat liver cytosol. GSH may react through different mechanisms: protection of labile thiol groups, activation of methylating enzymes, and regulation of free trivalent arsenite concentration. No MA(V) was detected in the abiotic methylation of arsenic by CH_3-B_{12} when GSH was omitted in the study conducted by Zakharyan and Aposhian.[42] Antimony, on the contrary, was abiotically methylated in the absence of GSH. A 2% methylantimony yield was obtained by the abiotic CH_3-B_{12} methylation of antimony(III) tartrate, which is larger than the generally

reported methylation yields of antimony in biological systems (<0.05%,[3,7] and 0.4% methylation produced by a pure culture of the wood-rotting fungus *Phaeolus schweinitzii*[44]). This might suggest that limiting effect for methylation of antimony is the transport into the cell. Hartmann *et al*.[45] reported antimony biomethylation as a fortuitous process, but catalysed at least in part by enzymes responsible for arsenic methylation.

As mentioned earlier, our attempts to separate Sb–GS complexes and monomethylantimony species prior to ESI-MS analysis by HPLC were not successful. To tackle the question of which form of methylantimony species occurs, selective pH-dependent HG may give an answer as to whether antimony is trivalent or pentavalent in its methylated species. The traditional approach to distinguish between inorganic antimony in its trivalent and pentavalent oxidation states is achieved by altering the pH.[46] The reaction solution was analysed by hydride generation at pH 7 and pH 1. The yield of methylstibine from monomethylantimony species is significantly higher at low pH, as described earlier by Andreae *et al*.[39] The yield of antimony(V) methylation by CH_3-B_{12} in the absence of GSH and with 10 μM GSH at pH 7 was 0.014 ± 0.005 ng and 0.011 ± 0.002 ng antimony as methyhylstibine respectively. At pH 1 the amounts of methylstibine increased to 3.0 ± 0.7 ng and 1.6 ± 0.4 ng respectively. No di- and tri-methylantimony species were detected. However, the early study by Andreae *et al*.[39] has been the only published work regarding pH conditions in HG for inorganic antimony and methylantimony determination. The efficiency of the process for antimony(III) and antimony(V) depends strongly on the pH of the reaction medium. The reaction yield normally decreases sharply above the pH corresponding to the pK_{a1} of the species concerned. This has been shown in using HG with 4 M acetic acid for the determination for arsenite, MA(V), DMA(V), and trimethylarsine oxide. Thus arsenic(V) (arsenic acid, $pK_{a1} = 2.3$) can be separated from arsenic(III) (arsenous acid, $pK_a = 9.2$) by reducing the former at pH 1.5 and the latter at pH 7.[39] Andreae *et al*.[39] applied this approach to the speciation analysis of antimony, which shows similar differences in the pK_{a1} for the trivalent (2.7) and pentavalent (11.0) species. No antimony(V) reduction took place at pH 6–7 (Tris–HCl), hence permitting the selective reduction of antimony(III) at near-neutral pH; they found the best yields for the reduction of synthezised monomethylstibonic acid and dimethylstibinic acid were in a mildly acidic solution. Dodd *et al*.[47] used similar conditions for the determination of antimony(III) and methylantimony species in freshwater plant extracts. Therefore, our results show that the majority of monomethylantimony is in a form that cannot be reduced to methylstibine at neutral pH, whereas a decrease in pH results in a higher yield of this volatile antimony species, in particular when GSH is absent. This suggests that either GSH binds methylantimony strongly or antimony is mainly in a pentavalent oxidation state, which needs lower pH to be reduced to trivalent stibines. Determinations of bond lengths and the electronic environment of antimony using EXAFS and XANES would be necessary to answer these questions.

Inoculation of anaerobic sewage sludge culture with enriched ^{123}Sb(V)

The fermenter samples spiked with antimony(III) tartrate and enriched ^{123}Sb(V) antimonate showed $[Sb(GS)_3]$ and $[(CH_3)Sb(GS)_2]$ complexes and monomethylantimony species with an enriched ^{123}Sb(V) antimonate abundance. This was used to identify the complex formation and methylation of the enriched ^{123}Sb(V) in the bacterial medium in the fermenter.

The Sb–GSH complex was previously identified by Sun *et al*.,[22] who suggested that the complex is a monomer in solution. It is also possible to form an intermediate complex between two monomers and a free GSH. Strong binding of antimony(III) to the thiolate sulfur of intracellular GSH indicated that the major biological target for antimony(III) appears to be thiolate in proteins and enzymes. The sulfur-bonding was shown by Yan *et al*.[33] in complexation of antimony(III) by trypanothione (N^1, N^8-bis(glutathionyl)spermidine). GSH may serve as a transporter for antimony-species,[22,48] but it is also likely that the binding of methylantimony to GSH results in a stabilized complex. $[(CH_3)Sb(GS)_2]$ was the only methylantimony species identified under these conditions. Because the HPLC separation of methylantimony species was not successful, only the HG technique can be used for the detection and identification of methylantimony species formed in abiotic methylation of antimony.

Mono-, di- and tri-methylantimony species, however, were produced in a laboratory fermenter with sewage sludge enriched for the growth of methanogens, spiked with isotopically enriched antimonate, ^{123}Sb(V). The biological production of the methylantimony species of each substrate was identified using the isotope ratios of the individual stibines produced by HG at pH 7. The stibines were separated and detected by GC–ICP-MS. The $^{123/121}$Sb ratio of the methylantimony species was significantly higher (3.89 ± 1.29) than the IUPAC value (0.747 85[49]), indicating the incorporation of the isotopic enriched antimonate. This showed that antimony(V) can be methylated in an anaerobic culture. Antimony(V) methylation in aerobic cultures has been reported in the literature.[3,45,50] Monomethylantimony species was the most prominent methylantimony species found in the sludge medium. The concentrations of the mono-, di- and tri-methylantimony species were 2.2 ± 0.3 ng g^{-1}, 0.14 ± 0.2 ng g^{-1} and 0.96 ± 0.4 ng g^{-1} sludge (wet; $n = 3$) respectively. In the methanol extract of the sludge 9.7 ± 0.8 ng g^{-1}, 0.33 ± 0.05 ng g^{-1} and 0.2 ± 0.06 ng g^{-1} sludge (wet; $n = 3$) of each species were found respectively. That is, 0.6% of the isotopic enriched antimonate added, ^{123}Sb(V), was methylated, the highest antimony biomethylation yield reported in the literature so far. The distributions of the non-volatile methylantimony species in the sludge filtrate and sludge methanol extract were respectively 68% and 95% of monomethylantimony species, 4% and 3% of dimethylantimony and 28% and 2% of trimethylantimony. This shows that more monomethylantimony species is present in the cell and more trimethylantimony in the filtrate.

The antimonate source was reduced prior to methylation, in agreement with the Challenger methylation mechanism. Whether the antimony(V) has been reduced in the anaerobic system prior to uptake into the cells or by GSH in the cells is not clear. Research on antimonate reduction in the literature is mostly limited to antimony(V) drugs for human leishmaniasis, where Ferriara *et al*.[51] support the antimony(V) reduction to antimony(III) by GSH or other polypeptides and proteins.

A sulfur-bound antimony–peptide complex[33] and the formation of [Sb–GS] complexes have been reported. Therefore, it seems likely that GSH stabilizes trivalent methylantimony species. However, the structural form of the methylantimony species in aqueous solutions has only been studied for trimethylantimony oxide,[32,52] showing $[(CH_3)_3SbOH]^+$ as the characteristic molecule. In biological systems, methylantimony species may be stabilized by binding to biomolecules like GSH.

With the exception of abiotic methylation by CH_3-B_{12} the biomethylation is likely to be coupled to enzymes, as has been shown for arsenic methylation.[15,42] The results of Andrewes *et al*.[1] did not support the biomethylation of antimony in its pentavalent state by the fungus *S. brevicaulis*, whereas other groups[45,50,53] reported the methylation of an antimony(V) substrate.

Trimethylstibine, $(CH_3)_3Sb$, was the sole volatile methylated antimony compound detected in the headspace gas from the fermenter bottles during the course of the experiment. Figure 3a and b shows chromatograms of the gas standard and sample respectively, analysed using cryotrapping GC–quadrupole-ICP-MS. The sample chromatogram shows methylantimony species isotopically enriched in the mass 123. This is indicating again that the isotopically enriched antimonate, $^{123}Sb(V)$, source was methylated by the microorganisms in the sludge fermenter. The measured concentration of $(CH_3)_3^{123}Sb$ from the spiked fermenter was 0.23 ± 0.03 ng g^{-1} sludge (wet; $n_{biological} = 3$). This is 0.01% volatilization of the added isotopic enriched antimonate, $^{123}Sb(V)$, source.

The $^{123/121}Sb$ isotope ratios were 14, 8 and 4 for the non-volatile mono-, di- and tri-methylantimony species respectively. The solution already contained naturally occurring antimony; the incorporation of the isotopically enriched antimonate shifts the antimony isotope ratio of these species. This indicates that the methylation follows a stepwise methylation proposed by Challenger, because the highest ^{123}Sb incorporation is in the monomethylantimony species, which is further methylated in a stepwise fashion to dimethylantimony and trimethylantimony. The non-volatile end-product trimethylantimony showed the lowest ^{123}Sb incorporation of the different methylated antimony species, but it has the same isotope ratio as its volatile counterpart trimethylstibine. The isotope ratio of the trimethylstibine, $(CH_3)_3Sb$, was 3.64 ± 0.92 ($n_{biological} = 3$), and the $^{123/121}Sb$ isotope ratio in the trimethylantimony species, $(CH_3)_3SbO$, was 4.85 ± 1.03 and 3.89 ± 1.29 ($n_{biological} = 3$) determined for the sludge filtrate and the methanol–water-extracted sludges respectively. The isotope ratios of the $(CH_3)_3Sb$ and $(CH_3)_3SbO$ are not significantly different ($P = 0.4$) within the uncertainty of isotope ratio measurements in quadrupole-ICP-MS. This suggests that an equilibrium between the volatile trimethylstibine and trimethylantimony species occurs in the solution.

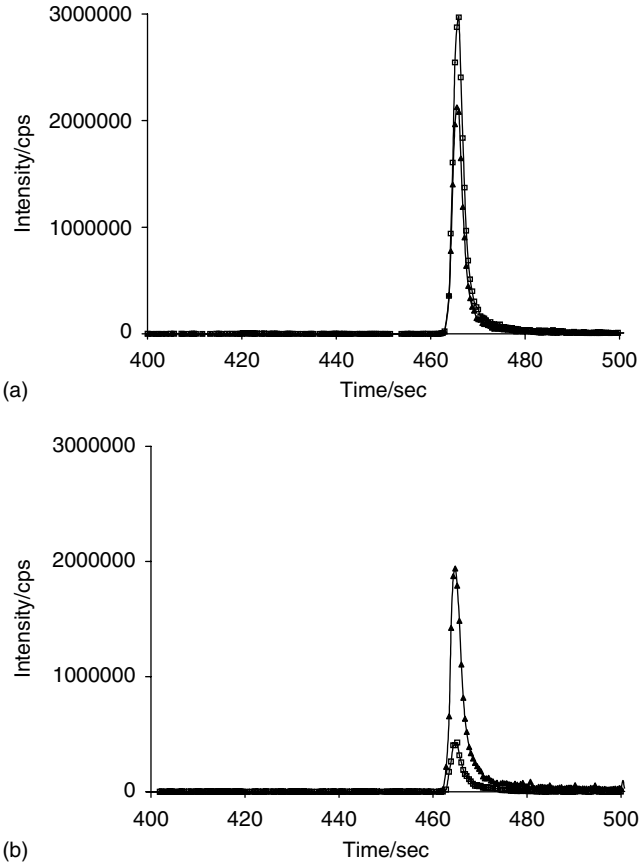

Figure 3. (a) $(CH_3)_3Sb$ standard (2 ng ^{121}Sb) analysed using cryotrapping-cryofocussing-GC–quadropole-ICP-MS. (b) Biologically produced $(CH_3)_3Sb$ after isotopic enriched antimonate, $^{123}Sb(V)$, addition. Squares are ^{121}Sb and triangles are ^{123}Sb signal intensities.

CONCLUSIONS

We have synthesized and identified biologically the $[Sb(GS)_3]$ and $[(CH_3)Sb(GS)_2]$ complexes. We believe that these complexes could be intermediates in the antimony biomethylation process. In an abiotic antimony methylation reaction, however, GSH was not required. For the first time CH_3-B_{12} was shown to react as a methylating agent in an abiotic methylation, forming monomethylantimony species and small amounts of dimethylantimony species. The methyl transfer mechanism from CH_3-B_{12} to antimony is not fully understood, but is likely to play a role in biological systems

together with GSH. However, CH_3-B_{12} cannot be the sole methylating agent, since the methylation of antimony stops at the intermediate and does not form trimethylantimony or the volatile trimethylstibine, which has been detected in an anaerobic sewage sludge cultures.

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