ARSENIC AND ANTIMONY BIOMETHYLATION BY *SCOPULARIOPSIS BREVICAULIS*

by

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ABSTRACT

Many organisms have been isolated that transform inorganic arsenic compounds to organoarsenic species. In the case of antimony, simple alkylantimony species have been detected in the environment, but only a few organisms are known that produce these species. The fungus *Scopulariopsis brevicaulis* was selected as a model microorganism to study antimony biomethylation, because it is known for its ability to biomethylate the chemically similar element, arsenic.

Cultures of *S. brevicaulis* were incubated with inorganic antimony compounds. Volatile antimony biomethylation products, in headspace gases, were determined by using gas chromatography inductively coupled plasma mass spectrometry, and nonvolatile species, in media samples, were determined by using hydride generation gas chromatography atomic absorption spectroscopy. Potassium antimony tartrate and antimony trioxide were biomethylated. The major biomethylation products (~95%) were nonvolatile dimethylantimony and trimethylantimony species, found in the medium. Volatile trimethylstibine (Me_3Sb) was produced as a minor biomethylation product (~5%).

The methyl source for antimony biomethylation was investigated, by incubating *S. brevicaulis* with potassium antimony tartrate and CD_3-D-methionine, or $^{13}CD_3$-L-methionine. There was significant, and comparable, incorporation of the methyl group from L-methionine into alkylantimony and alkylarsenic species. There was incorporation of the methyl group from D-methionine into alkylarsenic and alkylantimony species, but less than for L-methionine. These results suggest that

antimony biomethylation proceeds via a mechanism very similar to that of arsenic biomethylation.

Mixtures of antimony and arsenic compounds were incubated with *S. brevicaulis* and the biomethylation products determined. In these interaction studies, it was found that inorganic antimony(III) compounds inhibited arsenic biomethylation whereas inorganic arsenic(III) compounds enhanced antimony biomethylation.

Microorganisms, resistant to the germicide, 10, 10'-oxybisphenoxarsine (OBPA), were isolated. The OBPA-resistant microorganisms, and *S. brevicaulis,* were incubated with OBPA for one month and the medium analyzed. There was no evidence, in the form of OBPA break-down products or intermediates on the pathway to trimethylarsine, to suggest that any of these microorganisms might produce volatile arsines from OBPA. Furthermore, when headspace gases from *S. brevicaulis* cultures, grown in medium containing OBPA, were analyzed, no significant amounts of trimethylarsine were detected.

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ABBREVIATIONS

AA	atomic absorption
AAS	atomic absorption spectroscopy
ATCC	American Type Culture Collection
BC	British Columbia
CE	capillary electrophoresis
cps	counts per second
E	metalloid (As or Sb)
EI	electron impact/ionization
GC	gas chromatography
GF-AAS	graphite furnace atomic absorption spectroscopy
HG	hydride generation
HG-GC-AAS	hydride generation gas chromatography atomic absorption spectroscopy
HPLC	high performance (pressure) liquid chromatography
ICP	inductively coupled plasma
ICP-AES	inductively coupled plasma atomic emission spectroscopy
ICP-MS	inductively coupled plasma mass spectrometry
IMI	International Mycological Institute
IT	ion trap
mM	millimolar
MS	mass spectrometry
NA	nutrient agar
NB	nutrient broth
ng	nanogram
NIST	National Institute of Science and Technology
nm	nanometer
OBPA	10, 10'-oxybisphenoxarsine
o. d.	outer diameter
PAT	potassium antimony tartrate
pg	picogram
psi	pounds per square inch
PTFE	polytetrafluroethylene
PVC	polyvinylchloride
rpm	revolutions per minute
RSF	relative sensitivity factor
SAM	S-adenosylmethionine
SIDS	sudden infant death syndrome
SIC	selected ion chromatogram
S/N	signal to noise ratio
SPE	solid phase extraction
TIC	total ion chromatogram
US-EPA	United States Environmental Protection Agency
UV	ultraviolet
w/v	weight/volume
w/w	weight/weight

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CHAPTER 1

INTRODUCTION

The metalloids, arsenic (atomic number 33, atomic mass 74.9) and antimony (atomic number 51, atomic mass 121.8) belong to Group 15 of the periodic table. For all elements in Group 15 the ground-state electron configuration is ns^2np^3. The common oxidation states of arsenic and antimony species in the environment are +3 and +5.

A vast amount of information on the environmental chemistry of arsenic exists but the environmental chemistry of antimony is not so well developed. Arsenic and antimony are ubiquitous in the environment although in the earth's crust the concentration of antimony (~0.2 mg Sb/kg) is approximately an order of magnitude less than the concentration of arsenic (~1.8 mg As/kg)[1]. Antimony's environmental chemistry is often discussed with respect to that of arsenic because both elements occur in Group 15 of the periodic table, and are often found in association in the environment, but there are very significant differences in their chemistry[2]. For example, the arsenate anion is four coordinate, whereas the antimonate anion is six coordinate.

Humans have used (and misused!) arsenic and antimony for thousands of years; indeed, the history of these elements is fascinating. Arsenic[3] and antimony[4] compounds were used extensively in medicine, and still have limited use today. For example, potassium antimony tartrate was extensively used as an emetic, and some organoarsenicals were used in the treatment of syphilis.

Today, wood preservatives (mostly chromated copper arsenate) account for greater than 90% of arsenic use, and the rest is used primarily in metal-alloys, and in arsenical herbicides and germicides[5]. The predominant use of antimony is as antimony trioxide, added to plastics, rubber, adhesives and textiles as a flame retardant. This use accounted for 56% of total antimony consumption in the United States in 1997[6]. The remaining antimony is used mostly in metal-alloys, ceramics and glass[6].

Certainly, the usage of arsenic and antimony provides a means for these elements to enter the environment but the predominant anthropogenic releases occur during the smelting and refining of other metals such as copper, lead and gold. Natural releases of arsenic and antimony, such as from volcanic activity and weathering, are also significant.

A summary of the speciation of arsenic and antimony, in various environmental compartments is given in this chapter. Also described is the currently accepted mechanism, originally proposed by Challenger[7], for arsenic biomethylation. This mechanism, and an analogous one for antimony, can be used to explain the presence of some biologically produced organoarsenic and organoantimony species in the environment. Finally, an overview of analytical methods to determine these species is provided.

1.1 TERMINOLOGY

The compound Me_3Sb is commonly named trimethylstibine or trimethylantimony. The terms methylantimony species, dimethylantimony species and trimethylantimony species are often used to refer to any antimony compounds containing one, two and three

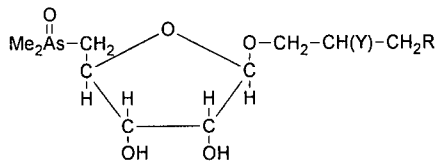
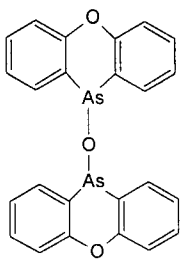
methyl groups, respectively. Therefore, to avoid confusion, in this thesis, SbH_3, $MeSbH_2$, Me_2SbH and Me_3Sb are referred to as stibine, methylstibine, dimethylstibine and trimethylstibine, respectively. The same nomenclature is used for arsenic i.e. AsH_3, $MeAsH_2$, Me_2AsH and Me_3As are referred to as arsine, methylarsine, dimethylarsine and trimethylarsine, respectively. In the general case of antimony species containing one or more methyl groups these are referred to as alkylantimony species, similarly arsenic species containing one or more methyl groups are referred to as alkylarsenic species. The term metalloid is used to mean "arsenic or antimony".

1.2 ARSENIC ENVIRONMENTAL CHEMISTRY

1.2.1 Introduction

Some arsenic compounds that are pertinent to this thesis are listed in Table 1.1. Inorganic arsenic compounds (e.g. Table 1.1, **1-5**), especially arsenic(III) compounds, are "highly toxic"; for instance, arsenic trioxide has been used many times as a poison[8]. Ingestion of inorganic arsenic compounds in drinking water is a cause of skin, bladder and lung cancers[9]. Some organoarsenicals are essentially nontoxic (e.g. Table 1.1, **13-15**)[8], while other organoarsenicals are "highly toxic"; for example, large quantities of some organoarsenicals (e.g. Lewisite and Adamsite) were manufactured for use in chemical warfare[10]. The mechanisms by which arsenic compounds exhibit toxicity vary greatly and are poorly understood[9].

Table 1.1. Some arsenic compounds.

	Name	Chemical Formula
1	Arsenous acid / arsenite (monobasic)	H_3AsO_3 / $[AsO_2]^-$
2	Arsenic trioxide	As_2O_3
3	Arsenic acid / arsenate (monobasic)	H_3AsO_4 / $[AsO_2(OH)_2]^-$
4	Arsenic pentoxide	As_2O_5
5	Arsine	AsH_3
6	Methylarsine	CH_3AsH_2
7	Dimethylarsine	$(CH_3)_2AsH$
8	Trimethylarsine	$(CH_3)_3As$
9	Methylarsonic acid	$CH_3AsO(OH)_2$
10	Dimethylarsinic acid	$(CH_3)_2AsO(OH)$
11	Trimethylarsine oxide	$(CH_3)_3AsO$
12	Tetramethylarsonium cation	$(CH_3)_4As^+$
13	Arsenocholine	$(CH_3)_3As^+CH_2CH_2OH$
14	Arsenobetaine	$(CH_3)_3As^+CH_2COO^-$
15	Arsenosugars Y=OH, NH_2; R=OH, SO_3H, OSO_3H, $OPO_3HCH_2CH(OH)CH_2OH$	
16	OBPA (10, 10'-oxybisphenoxarsine)	

The predominant arsenic species in the environment are inorganic arsenic species. The organoarsenic species in the environment primarily arise from biotransformation of inorganic arsenic species, although some organoarsenic species, such as methylarsonate, can have an anthropogenic source (for example when used as herbicides). Thermodynamic calculations predict that arsenic(V) species should be the only species in oxygenated waters, but in fact arsenic(III) species can constitute a significant proportion in oceanic surface waters, for example Andreae *et al.*[11] reported arsenite concentrations of 5-10% of total arsenic. The speciation of inorganic arsenic in waters is not the same as thermodynamically predicted because of the influence of biological activity[12]. Many microorganisms are known that oxidize arsenite[13] and many other microorganisms are known that reduce arsenate[14].

A number of comprehensive reviews and books describe the environmental chemistry of arsenic[8,9,12,15-17] and for this reason only organoarsenic species, and their possible origins, are discussed briefly in the following section. The organoarsenic species discussed herein are those of relevance to this thesis.

1.2.2 Organoarsenic Species in the Environment

Volatile alkylarsenic species (**6-8**) have been detected in gases from landfills, sewage treatment plants and hotsprings[18-20]. These species probably arise from microbiological activity although an abiotic origin, such as transmethylation by alkylmetal species, is possible in some cases.

Alkylarsenic(V) species, predominantly dimethylarsenic(V) species, can constitute, depending on the extent of biological activity, ~10% of the total arsenic in marine surface-waters[12]. The alkylarsenic(V) species arise from the action of algae. Marine macroalgae contain high concentrations of organoarsenic species; the predominant species being arsenosugars (**15**)[21].

Marine animals contain high concentrations of organoarsenicals as well. However, the predominant organoarsenical found in marine animals is arsenobetaine (**14**)[21].

The organoarsenic species present in marine organisms was, and is, subject to much interest because of concerns about the movement of large amounts of arsenic through the food chain to humans. Studies of arsenic speciation in the terrestrial environment are not so common because arsenic is typically found in much lower concentrations in freshwater and terrestrial organisms. Nevertheless, organoarsenic species were detected in, for example, edible mushrooms[22], freshwater algal species[23] and earthworms[24].

When humans, and other mammals, ingest inorganic arsenic species, their urine contains significant quantities of methylarsenic and dimethylarsenic species; current evidence suggests that the necessary arsenic biomethylation occurs in the liver. The biomethylation of arsenic in humans is currently a "popular" topic, especially in relation to the causes of cancer[9].

To summarize, organoarsenic species are ubiquitous in the marine environment and it appears that this might also be the case for the freshwater and terrestrial

environment. Various organoarsenic species are detected, ranging from simple alkylarsenicals to the more complex arsenobetaine and arsenosugars. The exact mechanisms by which these species form are still being elucidated but it is generally accepted that the key step, the formation of the arsenic-carbon bond, is the same in all cases. That is, the addition of a carbocation, derived from S-adenosylmethionine, to nucleophilic arsenic(III) species (this process is further discussed in Section 1.4).

1.3 ANTIMONY ENVIRONMENTAL CHEMISTRY

1.3.1 Introduction

The environmental chemistry of arsenic is well developed because of the availability of appropriate analytical techniques. In contrast, few techniques exist for the reliable determination of antimony, especially organoantimony, species. However, our knowledge of organoantimony species in the environment has significantly increased during the last two decades. This increase is attributed to chemical curiosity, in particular to the examination of the 'toxic gas hypothesis' that has been advanced to account for sudden infant death syndrome (SIDS, Section 1.5), and the application of recently developed analytical methodology. The toxic gas hypothesis provided considerable impetus to investigate the production of organoantimony compounds by microorganisms. New analytical instrumentation, especially inductively coupled plasma mass spectrometry (ICP-MS), provided the sensitivity and element specificity to detect organoantimony compounds. In addition, the multi-element capability of ICP-MS

allowed the detection of organoantimony compounds during investigations that may not have been directed specifically towards determining these species.

Some antimony compounds pertinent to this thesis are listed in Table 1.2. Antimony(III) species are more toxic than antimony(V) species, and the most serious effects usually involve the liver or heart[25]. The toxicological properties of antimony species are thought to arise from the high affinity of antimony for sulphydryl groups but the evidence for this is minimal. The mechanistic toxicology of antimony has been reviewed in comparison to that of arsenic[16]. Little attempt has been made to evaluate the toxicity of organoantimony compounds. In 1939, Seifter[26] performed experiments to determine the acute toxicity of trimethylstibine to animals and he concluded that: 'Trimethylstibine possesses no great or pronounced acute toxicity to animals.'

In this introduction, only the environmental chemistry of organoantimony species is discussed. However, it is worthwhile to note that the oxidation state of inorganic antimony species is important with respect to the possible formation of organoantimony compounds, because the formation of antimony-carbon bonds probably involves oxidative addition reactions to antimony(III) species. This is the situation for arsenic[12]. Thus, biomethylation of antimony(V) would occur only after reduction of antimony(V) to antimony(III). In the case of arsenic, microbial reduction of arsenic(V) to arsenic(III), with or without further methylation is commonly found[12]. The biological reduction of inorganic antimony(V) species has not been demonstrated. As expected from the thermodynamic data, antimony(V) predominates in oxygenated marine and fresh waters, and the concentrations reported ranged from 0.025 to 0.29 μg Sb/L[11,27-30].

Alkylantimony species are currently the most studied organoantimony compounds in the environment, largely because they are expected to be formed and because analytical methods are available for their determination. Organoantimony species, analogous to those of arsenic (i.e. stibiobetaine and stibiosugars) probably exist but the analytical chemistry of antimony is not sufficient to allow their detection.

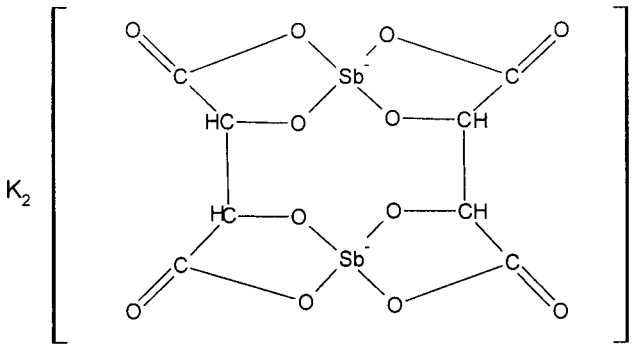
1.3.2 Volatile Organoantimony Compounds in the Environment

Volatile organoantimony compounds have been detected in a variety of environments, often in association with a miscellany of other volatile organometallic compounds.

Trimethylstibine (**24**) was detected in landfill and sewage gas by using GC-ICP-MS[19,20]. The initial identification of trimethylstibine was based on retention time. Subsequently, confirmation was made by using GC-MS (ion trap) to analyze landfill and sewage gas from Canadian sites[31]. The concentration ranges of trimethylstibine in German fermentation gases were estimated as 0.62-15 μg Sb/m^3 and 23.9-71.6 μg Sb/m^3 for sewage and landfill gas, respectively. Similar concentrations were found in Canadian fermentation gases.

Trimethylstibine and dimethylstibine (**23**) were detected, by using GC-ICP-MS analysis, above (and within) algal mats growing in the Meager Creek hot-springs (BC, Canada)[18]. Alkylantimony species have been detected in geothermal waters (Section 1.3.3).

Table 1.2. Some antimony compounds.

	Name	Formula
17	Hexahydroxyantimonate	$Sb(OH)_6^-$
18	Antimony trioxide	Sb_2O_3
19	Potassium antimony tartrate	
20	Trimethylantimony dichloride	$(CH_3)_3SbCl_2$
21	Stibine	SbH_3
22	Methylstibine	CH_3SbH_2
23	Dimethylstibine	$(CH_3)_2SbH$
24	Trimethylstibine	$(CH_3)_3Sb$

1.3.3 Nonvolatile Organoantimony Species in Waters and Sediment

The first organoantimony compounds to be detected in the environment were found in natural waters. Andreae *et al.*[27] derivatized antimony compounds in natural waters by using sodium borohydride. The derivative compounds: stibine, methylstibine and dimethylstibine were detected by using GC-AAS and it was unjustifiably concluded that the waters contain *methylstibonic* and *dimethylstibinic* acid. Although their work indicated that alkylantimony species are present in natural waters, the inorganic functional groups in the species remain unknown.

It is now known that the experimental conditions (30 mM HCl) used by Andreae and coworkers[27] are likely to produce artifacts (methyl groups redistribute during the hydride generation process so that SbH_3, $MeSbH_2$, Me_2SbH and Me_3Sb can form from a pure alkylantimony compound)[32,33]. Nevertheless, the generation of alkylstibines from pure inorganic antimony compounds, during hydride generation is very unlikely, so the work of Andreae and coworkers indicates that these natural waters must contain some alkylantimony species of indeterminate structure.

The following 'quantitative' results were reported by Andreae *et al.*[27] for river and sea waters and similar results were obtained by Bertine and Lee[28] (using the methods of Andreae *et al.*[27] on sea water from Saanich Inlet, BC, Canada). However, these results should be treated with caution in view of the aforementioned problems. Methylantimony species were the only alkylantimony species detected in rivers (0.5-1.8 ng Sb/L). Methylantimony (0.8-12.6 ng Sb/L) and dimethylantimony (0.6-3.2 ng Sb/L) species were detected in seawater. No trimethylantimony species were detected during these

studies. The alkylantimony compounds typically comprised 10% of the total antimony present. Because there is no known anthropogenic discharge of these types of compounds into natural waters they most probably arise from biological methylation. It was also reported, by Andreae *et al.*[27], that alkylantimony species were present in pure cultures of marine planktonic algae (details on the methodology used were not given) but, in a subsequent paper, Andreae and Froelich[11] state that alkylantimony compounds were not detected in marine phytoplankton (extracts were prepared by grinding the algae with 2 M HCl and analyzed by using HG-GC-AAS).

In 1984, Andreae and Froelich[11] presented an impressive survey of arsenic, antimony and germanium speciation in the Baltic Sea. The Baltic Sea is one of the most polluted marine areas on the planet. Deep marine basins that are often anoxic are characteristic of the Baltic Sea. Samples were collected on a cruise in 1981 from five points along the axis of the Baltic Sea and the Gulf of Finland. Samples were taken from 10 to 235 m depth mostly in 10 m increments. The samples were analyzed by using HG-GC-AAS. Methylantimony species were detected throughout the water column and were more abundant than dimethylantimony species that were only detected occasionally[11]. Most interesting was the observation that there was a trend of slightly higher levels of alkylantimony species in the surface layers for all five sample-points. Also, for two sample-points, slightly higher levels of alkylantimony species in the anoxic zone was observed. Andreae and Froelich suggested that the alkylantimony species were produced by bacteria, rather than algae, because no alkylantimony species were detected in marine phytoplankton[11] or macro-algae[34], and production of alkylantimony species is not closely tied to the eutrophic zone (as it is for arsenic).

Alkylantimony species were identified in sediment from a West German river, by using HG-GC-ICP-MS[35]. The methodology used for hydride derivatization could have resulted in demethylation. Rough quantification was performed on the observed species by using interelement calibration; the concentration ranges reported were 0.2-9.8, 0.1-1.2 and 0.1-0.9 μg Sb/kg for $MeSbH_2$, Me_2SbH and Me_3Sb, respectively. In some samples, an additional peak was observed in the gas chromatogram. This peak was attributed (based on boiling point calculations) to triethylstibine.

Hirner *et al.*[18] measured alkylantimony species in geothermal waters obtained from various New Zealand locations by using HG-GC-ICP-MS. Methylantimony, dimethylantimony and trimethylantimony species were detected as the corresponding stibines. The highest concentration of 31 ng Sb/kg was for a dimethylantimony species present in Frying Pan Lake, Waimangu, New Zealand. However, the reliability of these numbers is questionable because: (1) the possibility of demethylation in the analytical system was not examined; (2) alkylantimony species in the blanks had to be corrected for; and (3) rough quantification was made by using interelement calibration.

1.3.4 Nonvolatile Organoantimony Species in Plants and Animals

Samples of pondweed (*Potamogetan pectinatus)* from antimony impacted lakes near Yellowknife, Canada, were extracted with acetic acid (0.2 M) and the extracts were analyzed by using HG-GC-MS (quadrupole)[36]. The extracts contained methylantimony, dimethylantimony and trimethylantimony species. When a pure standard of $Me_3Sb(OH)_2$ was analyzed by using this analytical system only a single peak was observed (Me_3Sb)

indicating that the analytical conditions used did not cause disproportionation. The pondweed samples contained between 48 and 68 mg total Sb/kg, dry weight; however, quantification of the organoantimony species was not done.

Moss samples (all identified as *Drepanocladus* sp.) and snails (*Stagnicola* sp.), from Yellowknife, were shown to contain alkylantimony species. The samples were freeze-dried, ground, and then sonicated in methanol/water (50:50); the extracts were analyzed by using HG-GC-AAS and HG-GC-MS (ion trap). It should be noted that these samples contained between 6 and 60 mg total Sb/kg, dry weight, but extraction efficiencies were less than 25%[37].

Plant samples (liverwort and moss) from the Louisa antimony mine, Eskdale, Scotland, were extracted with acetic acid (0.2 M) and the extracts were analyzed by using HG-GC-AAS[38]. The extracts contained methylantimony and dimethylantimony species, but trimethylantimony species were not detected. It was roughly estimated that the plant samples contained between 101 and 186 ng extractable dimethylantimony/g, dry weight. The total antimony content of the plant material was between 8.9 and 57.3 μg Sb/g, dry weight.

1.4 BIOLOGICAL TRANSFORMATION OF ARSENIC AND ANTIMONY

1.4.1 Introduction

An overview of the types and distributions of arsenic and antimony compounds found in the environment was provided in sections 1.2 and 1.3. The types and distribution of organoarsenic and organoantimony compounds in the environment can be reconciled in terms of anthropogenic inputs (inorganic and organometalloid species), and natural inputs (inorganic species), and biological transformation of species.

The research presented in this thesis is primarily concerned with the biological transformation of inorganic arsenic and antimony species. The simplest transformations likely to occur to these species are oxidation and reduction. Another biotransformation that may occur is the production of organometalloid species from inorganic species. This process may be as elaborate as the biological production of arsenosugars. A simpler process, biomethylation, is the production of alkylarsenic and alkylantimony species from inorganic arsenic and antimony species, respectively. The first organism in which this process was identified was the filamentous fungus *Scopulariopsis brevicaulis*. Consequently, this microorganism has been used extensively in biomethylation research, and was chosen for the studies presented in this thesis. A number of organisms are capable of metalloid biomethylation.

1.4.2 The Mechanism of Arsenic Biomethylation

Some of the earliest work on arsenic speciation was related to the gas trimethylarsine. Around 1815 some severe cases of arsenic poisoning occurred in Germany, attributed to the use of arsenic containing pigments (Scheele's green and Schweinfürter green) in wallpaper[7]. It was deduced that microorganisms growing on the wallpapers produced a toxic gas containing arsenic.

Gosio[39] isolated the filamentous fungus *Penicillium brevicaule* (now classified as *Scopulariopsis brevicaulis*) from wallpapers and demonstrated that it produces large amounts of a gas with a garlic-like odor, when cultured in media containing inorganic arsenic compounds. Gosio[39] incorrectly identified this gas, by using classical techniques, as diethylarsine.

Later, in 1932, Challenger[40] used classical techniques to correctly identify the gas as trimethylarsine. Challenger also demonstrated that *S. brevicaulis* biomethylates selenium and tellurium to dimethylselenide and dimethyltelluride, respectively. He then performed a variety of experiments to probe the mechanism of arsenic biomethylation by *S. brevicaulis* and the pathway that he proposed is shown in Figure 1.1[7].

Challenger proposed that the biological methylation of arsenate begins with its reduction to arsenite. Arsenic(III) has a lone pair of electrons, and so is nucleophilic, and readily forms a bond with a methyl carbocation. The resulting oxidative addition yields methylarsonic acid. Further cycles of reduction and oxidative methylation produce trimethylarsine. Challenger used the following evidence to support the mechanism:

(1) *S. brevicaulis* readily transforms methylarsonic acid and dimethylarsinic acid to trimethylarsine.

(2) *S. brevicaulis* readily transforms RAs(V) and RR'As(V) species to RMe_2As and RR'MeAs, respectively.

(3) The oxidative addition of a methyl carbocation to arsenic(III) is a well known chemical reaction (i.e. the Meyer reaction: $CH_3I + NaH_2AsO_3 \rightarrow NaI + CH_3AsO(OH)_2$).

The source of the methyl cation Challenger postulated to be 'active' methionine. Subsequently, active methionine was identified as S-adenosylmethionine (Figure 1.1).

Since Challenger's initial studies further work has been performed which supports the Challenger mechanism[12]. Intermediates on the pathway (dimethylarsenic(V) and trimethylarsenic(V) species) were identified in the medium of *S. brevicaulis* and *Cryptococcus humicolus* (a yeast) cultures[41,42]. The mechanism has also been refined, by taking into account the relative mobility of species across cell membranes[42].

In higher organisms, arsenic biomethylation is thought to proceed by the same mechanism. Biomethylation appears to terminate at dimethylarsinic acid in humans (i.e. methylarsenic and dimethylarsenic species are excreted in urine but not trimethylarsenic species)[9]. In some organisms (e.g. clams), biomethylation proceeds further to produce the tetramethylarsonium ion (Me_4As^+)[21].

Methyltransferase enzymes for mammalian systems have been isolated[43]. Biomethylation is also likely to be enzyme catalyzed in *S. brevicaulis*.

1.4.3 The Challenger Mechanism and Complex Organoarsenicals

The Challenger mechanism is used to explain the formation of more complex organoarsenicals (**13-15**) in the environment. Clearly, the most important step in the formation of organoarsenicals is the formation of arsenic-carbon bonds. The most likely way that this would occur in a biological system is by electrophilic attack of a carbocation on arsenic(III) species (Figure 1.1). Instead of the methyl group, the other substituent groups of S-adenosylmethionine can act as the carbocation. Thus, a combination of methylation steps, addition of an adenosyl group, and some further chemistry, which does not involve the arsenic-carbon bond, may lead to arsenosugars, arsenobetaine, etc[21].

1.4.4 The Mechanism of Antimony Biomethylation

Alkylantimony species have been detected in few environments (Section 1.3) and it has only recently been demonstrated that *S. brevicaulis*, can biomethylate antimony(III) species[44-46]. Indeed, the first demonstration of antimony biomethylation by a culture of microorganisms was made only four years ago[47]. When the present work was initiated, nothing was known about the mechanism of antimony biomethylation although it was thought to proceed via a mechanism analogous to that of arsenic. For arsenic, *S. brevicaulis*, a model microorganism, was used to perform biomethylation mechanistic studies. It is worthwhile to note that antimony(III) is not as nucleophilic as arsenic(III) so the formation of an antimony-carbon bond by oxidative addition is less likely although an enzymatic system should be able to overcome this barrier. The mechanism of antimony biomethylation by *S. brevicaulis* is examined in this work.

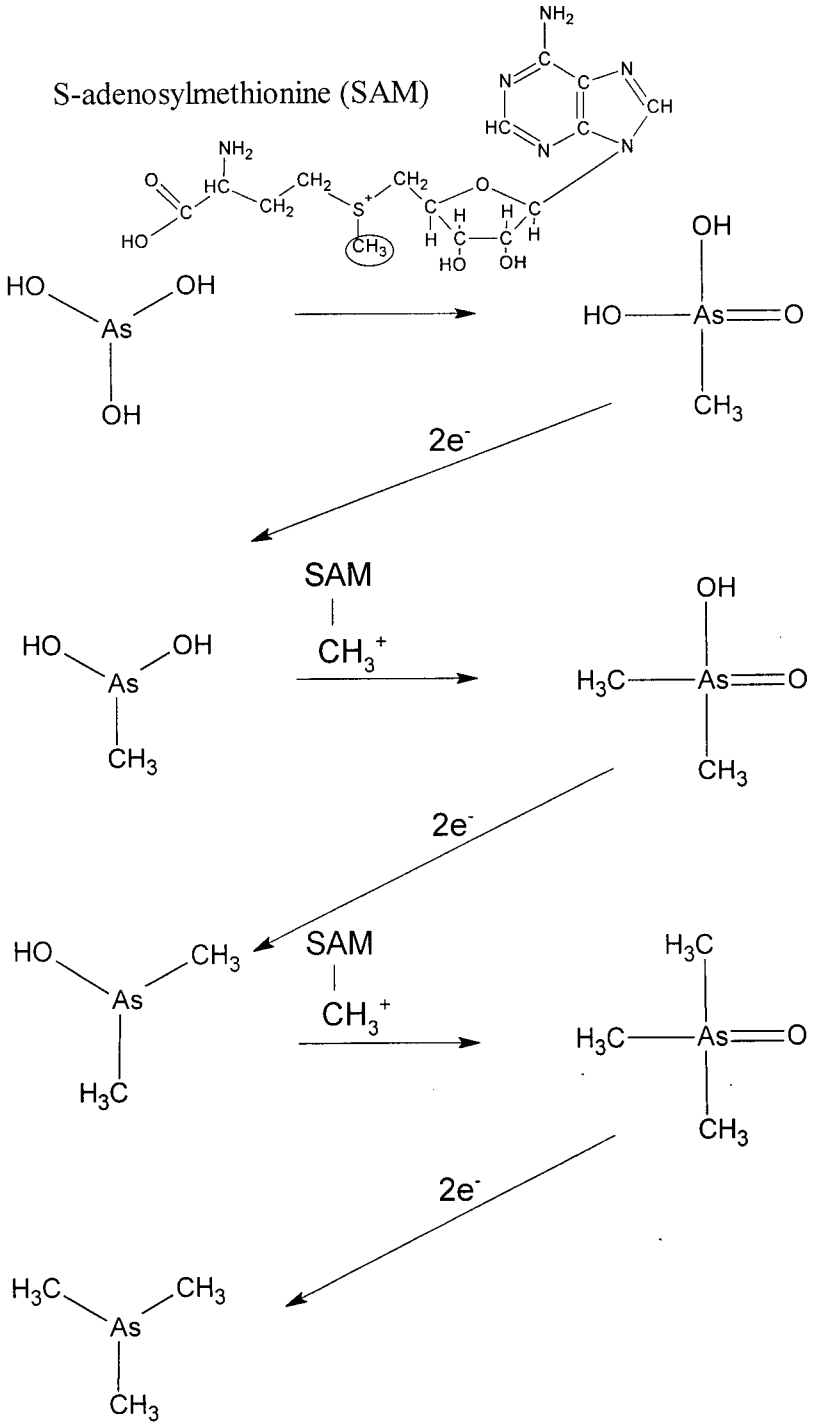


Figure 1.1. Mechanism of Arsenic Biomethylation. The circled group in SAM is the methyl source for biomethylation.

1.5 BIOMETHYLATION OF ARSENIC AND ANTIMONY BY MICROORGANISMS AND SUDDEN INFANT DEATH SYNDROME (SIDS)

Sudden infant death syndrome (SIDS), also known as cot death, is defined as 'unexpected deaths in sleeping quarters of apparently healthy infants'. Richardson[48] has proposed a 'toxic gas hypothesis' as a possible explanation of SIDS. The hypothesis is that microorganisms growing on infants' cot bedding material, that contain phosphorous, arsenic or particularly antimony (used a fire retardant) may transform these elements to toxic volatile species. The resulting phosphines, arsines and stibines, could be inhaled by infants and cause chronic poisoning. Research concerning this hypothesis has involved:

(1) Epidemiology.

(2) Toxicology.

(3) Analysis of arsenic and antimony content in SIDS victims.

(4) Analysis of leachable arsenic and antimony in bedding materials.

(5) Characterization of microorganisms on bedding materials.

(6) Studies on the production of trimethylarsine and trimethylstibine by cultures of microorganisms in the laboratory (a large amount of research in this area has specifically focused on the production of trimethylstibine by *S. brevicaulis*).

Aspects (1) - (5) are beyond the scope of this study. An extensive summary of the literature on the toxic gas hypothesis is provided in reference 49. Some of the results

presented in this thesis are related to the toxic-gas hypothesis in terms of (6). Results concerning (6) are now discussed.

Richardson used colorimetric methods to identify gases produced by microorganisms growing on pieces of cot mattresses within agar medium. He concluded that phosphines, arsines or stibines or mixtures of gases were produced[48]. But the observed color changes could have been the result of the generation of other gases[50] such as sulfides[51].

The use of a more specific detection method, involving the trapping of arsines and stibines on silver nitrate impregnated paper followed by analysis using HG-AAS, failed to show that cultures of *S. brevicaulis* or *Phaeolus schweinitzii* (a wood rotting fungus that is known for its ability to biomethylate arsenic) could biovolatilize potassium antimony tartrate or antimony trioxide, even though arsenic trioxide was readily biovolatilized[52].

Gates and coworkers[51] incubated various aerobic and anaerobic microorganisms, including *S. brevicaulis* and six *Bacillus* sp., with antimony(III) compounds. The volatile species were trapped on 5% mercuric chloride impregnated papers and the papers were analyzed by using mass spectrometry. No trimethylstibine was produced under aerobic conditions. Also, trimethylstibine was not produced by oral facultative-anaerobes, or anaerobes cultured from cot mattresses. However, some anaerobic cultures from a pond-sediment did produce trimethylstibine.

1.6 ANALYTICAL METHODS FOR THE SPECIATION OF ARSENIC AND ANTIMONY

1.6.1 Introduction

Analytical methods for the determination of arsenic species are well developed. Hydride generation, usually combined with gas chromatography, coupled to a sufficiently sensitive arsenic specific detector, is used to determine simple arsenic species[53]. The next-generation of techniques combine HPLC with an arsenic specific detector[54]. The state-of-the-art involves the use of high-resolution separation techniques (e.g. HPLC or CE) in combination with sensitive MS techniques that provide additional information; e.g. Pergantis *et al.*[55] detected arsenosugars at the picogram level and obtained structural information by using nanoelectrospray quadrupole time-of-flight MS.

A variety of techniques for performing inorganic antimony speciation, i.e. the determination of antimony(III) and antimony(V), have been described. For instance, a recent review by Smichowski *et al.*[56] describes methods for antimony speciation in waters. Only a few techniques exist for determining organoantimony species, the most popular being hydride generation gas chromatography (HG-GC) for solutions and GC for gases. The main reasons for the paucity of analytical methods for organoantimony speciation are:

1. The total antimony content in environmental matrices is relatively low.

2. There is a lack of accessible, well characterized compounds that could be used as standards to develop and validate new (and in some cases, old) analytical methods.

In this section, an overview is provided of analytical methods, for arsenic and antimony speciation, that were used in the research described in this thesis.

1.6.2 Hydride Generation-Gas Chromatography-Atomic Absorption Spectroscopy (HG-GC-AAS)

The reduction of inorganic metalloid species, such as arsenic and antimony, to volatile hydrides, which can be collected and determined by a range of techniques (including AAS, ICP-AES, ICP-MS and EI-MS), is an established method in analytical chemistry[53]. It is especially useful in trace analysis because it is a much more selective and efficient way (compared to the best nebulizer) to get metalloids into a plasma, flame, or ion source.

When a sample contains a mixture of inorganic species in different oxidation states, e.g. arsenic(III) and arsenic(V), the proportion of species in each oxidation state can be determined by preferentially reducing low oxidation state species at high pH (pH > 4). At low pH (pH < 2), all species are reduced. Some simple alkylmetalloid species can also be reduced to volatile hydrides that can be separated by using gas chromatography[57].

Hydride generation techniques, for arsenic analysis, are discussed in a large number of papers[53], especially for the analysis of urine[9], which contains predominantly

alkylarsenic and inorganic arsenic species. However, HPLC techniques have generally superseded hydride generation techniques for arsenic speciation[54].

In the case of antimony, few reliable HPLC techniques exist[58-65], and so hydride generation is still the method of choice[11,27,28,32,35,36,38,66]. Hydride generation for antimony species, especially alkylantimony species, has only recently been developed[27,57] and is still undergoing refinement[32,38]. Andreae, and coworkers[27], inserted a GC column between a reaction vessel and an AAS detector (i.e. HG-GC-AAS) and showed that it is possible, in principle, to determine alkylstibines that are formed from unknown precursors as a result of hydride-forming reactions. Results from hydride generation, in the determination of alkylantimony species, need to be interpreted carefully, because artifact peaks can be formed during hydride generation. For example, when solutions of pure trimethylantimony dichloride, trimethylantimony dihydroxide, or dimethylantimony dihydroperoxychloride were reacted with $NaBH_4$ all compounds formed four stibines (**21-24**)[33]. Dodd *et al.* claimed that preconditioning the analytical system with the reagents $NaBH_4$ and 4 M acetic acid eliminated demethylation[36], but this could not be reproduced by other workers[32]. The production of artifact peaks is enhanced as the pH, in both batch and semi-continuous hydride generation systems, is decreased, and is enhanced in some analytical matrices[32]. However, Craig *et al.*[38] report that rigorous exclusion of oxygen, when performing HG-GC-AAS, minimizes artifact peak production, even at pH 1.

Separation of arsines (i.e. **5-8**), and stibines (i.e. **21-24**), is simple because of their wide range of boiling points (Table 1.3). Capillary columns or packed columns may be

used; the separation is best at low temperatures. Packing materials used include Tenax-TA, Porapak-PS and Supelcoport SP-2100 (10% on Chromosorb).

Table 1.3. Boiling points of arsines and stibines.

Compound	Boiling Point (°C)	Compound	Boiling Point (°C)
Arsine	- 55	Stibine	- 17
Methylarsine	2	Methylstibine	41
Dimethylarsine	36	Dimethylstibine	61
Trimethylarsine	50	Trimethylstibine	81

One general disadvantage of hydride generation methodology is that information on the original species, from which the hydrides are formed, is lost[67]. Despite these problems, hydride generation has proven useful for revealing that alkylantimony and alkylarsenic species exist in the environment. Hydride generation provides a convenient means of determining alkylarsenic[42] and alkylantimony species in cultures of *S. brevicaulis* (Chapter 2).

1.6.3 Hydride Generation-Gas Chromatography-Mass Spectrometry (HG-GC-MS)

In HG-GC-AAS methods alkylarsenic and alkylantimony species are identified by matching GC retention times of hydrides, generated from samples, against those generated from standards. However, there is the possibility of misidentification because some alkylarsines, or alkylstibines, have similar retention times (e.g. dimethylarsine and ethylarsine). Another problem that makes identification of antimony species difficult is

that dimethylantimony and methylantimony species are not available to use as standards. Combining mass spectrometry with hydride generation provides a means of unambiguously identifying alkylarsenic and alkylantimony species (Chapter 2). Secondly, HG-GC-MS methods prove to be very useful in mechanistic studies (Chapter 4) for measuring the incorporation of an isotopic label.

Antimony has two naturally occurring isotopes, ^{121}Sb and ^{123}Sb, with natural abundances of 57.3% and 42.7%, respectively, and these characterize the fragmentation pattern of antimony compounds. Arsenic has only the one isotope, ^{75}As, and so arsenic compounds have less distinct fragmentation patterns. For example, the quadrupole mass spectra (NIST) of trimethylstibine and trimethylarsine are plotted in Figure 1.2.

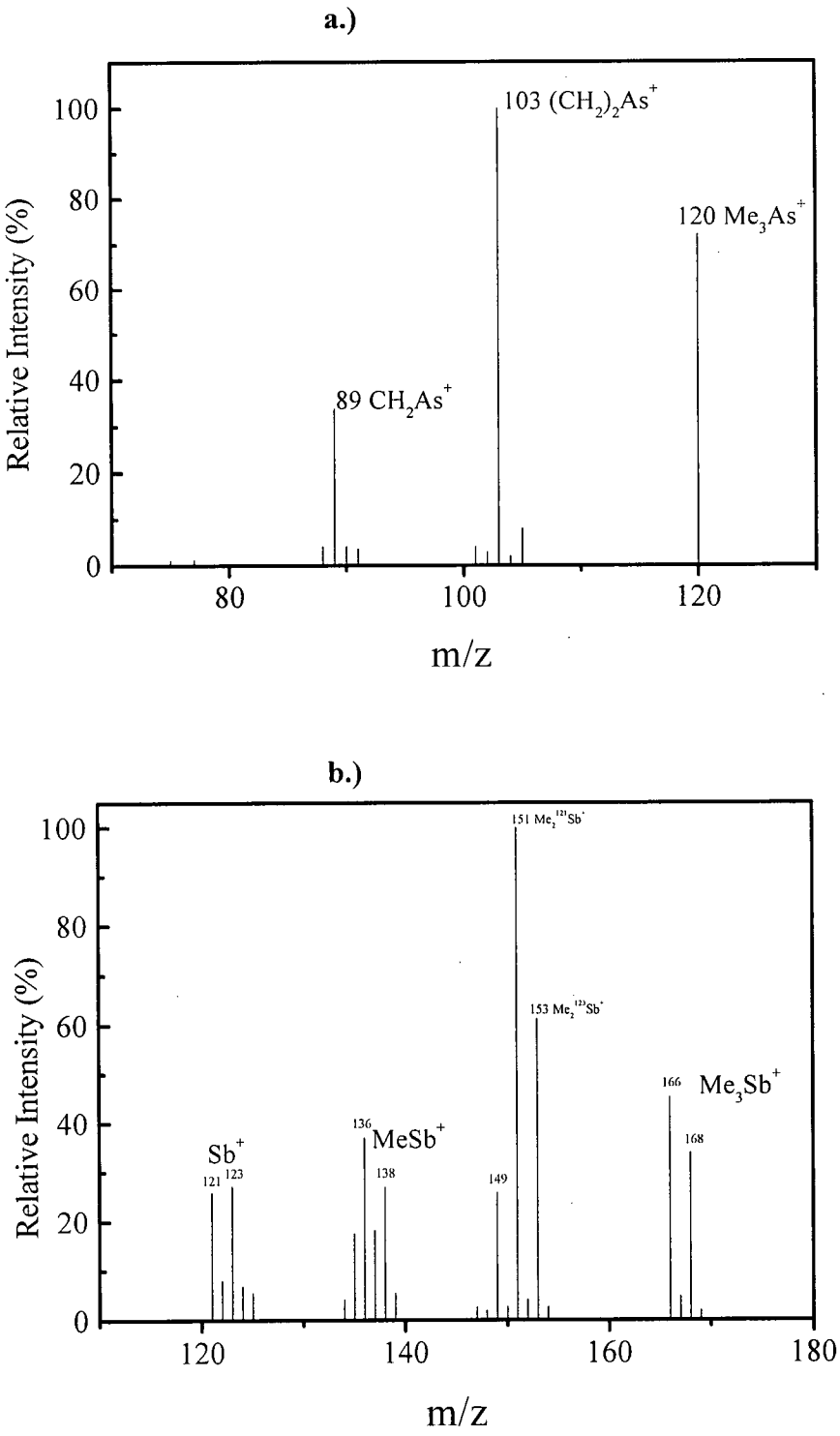


Figure 1.2. Quadrupole mass spectra of **a.)** trimethylarsine and **b.)** trimethylstibine (NIST).

1.6.4 Gas Chromatography-Inductively Coupled Plasma-Mass Spectrometry (GC-ICP-MS)

Volatile arsines, or stibines, in the headspace of cultures, can be collected in U-shaped traps, packed with an appropriate chromatographic material. Although arsines and stibines are retained on some packing materials at room temperature, it is preferable to trap at low temperature (- 78 °C) to increase trapping efficiency and avoid problems due to instability. The trap is connected to a detector then slowly heated. The arsines, or stibines, slowly desorb from the trap in order of increasing boiling point (Table 1.3), so that separation occurs. If this separation is not sufficient (usually because rapid sampling resulted in a broad distribution of hydrides on the trap) the hydrides can be cryo-focused onto a second GC column[18].

Quantification of arsines and stibines, at the concentrations relevant to this thesis, is problematic because it is difficult to prepare gaseous standards of known concentration because of adsorption effects and the low stability of some of the compounds. Another problem is that arsines and stibines are not available as standards. Feldmann[68] described a method for semi-quantification (reproducibility ± 30%) of organometal(loid) gases by using GC-ICP-MS. During gas analysis, an aqueous internal standard (usually rhodium) is continuously aspirated. This procedure has been applied to determining the concentration of stibines, arsines, and other organometal(loid) compounds in gases from landfills, sewage treatment plants, and hot-springs[18].

1.7 SCOPE AND OBJECTIVES

This research was commenced with the objective of showing that *S. brevicaulis* can biomethylate antimony. To do this both media and headspace gas samples of *S. brevicaulis* cultures, grown in the presence of antimony compounds, were analyzed for nonvolatile and volatile alkylantimony species, respectively, under normal aerobic growth conditions. A second major objective was to establish if the mechanism of antimony biomethylation is similar to the mechanism of arsenic biomethylation. To facilitate these investigations it was necessary to develop, or modify, appropriate analytical techniques. For example, it was known that intermediates on the pathway to trimethylarsine are found in the medium and can be identified by using HG-GC-AAS[42]. Thus, assuming this is the case for antimony, appropriate techniques to determine alkylantimony compounds in the medium were developed.

In Chapter 2 results of speciation of the medium from cultures of *S. brevicaulis*, which were incubated with inorganic antimony compounds, are described.

Cultures of *S. brevicaulis* were incubated with potassium antimony tartrate and headspace gases collected in U-tube traps. The content of the U-tube traps was determined by using GC-ICP-MS. The results are described in Chapter 3.

In Chapter 4, the use of mass spectrometry to show that the isotopically labeled methyl group of $^{13}CD_3$-L-methionine is incorporated intact into alkylantimony species is described. A parallel study was also performed with arsenic so that the amounts of label incorporated could be compared. The incorporation of label from CD_3-D-methionine into alkylarsenic and alkylantimony species was determined to provide evidence that the biomethylation of arsenic and antimony is enzymatic.

Because arsenic and antimony are often found as co-contaminants in the environment, it is useful to determine the interaction of these two elements with respect to biomethylation, such research is described in Chapter 5.

The research described in this thesis was commenced in response to concerns about the volatilization of arsenic and antimony by microorganisms growing on infants' bedding materials. Thus, in Chapter 6 the interaction of *S. brevicaulis*, and some other microorganisms, with 10, 10'-oxybisphenoxarsine (OBPA), a fungicide commonly used in consumer plastics, is reported.

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CHAPTER 2

ANTIMONY SPECIATION IN THE GROWTH MEDIUM AND BIOMASS OF *SCOPULARIOPSIS BREVICAULIS*

2.1 INTRODUCTION

Various organoarsenic species have been detected in the environment[1]. These include simple alkylarsenic species and more complex species such as arsenobetaine and arsenosugars (Table 1.1). Antimony, positioned below phosphorous and arsenic in Group 15 of the periodic table, has similar chemical properties to arsenic, thus analogous organoantimony species might also exist in the environment. The detection of such species is challenging because the concentration of antimony in the environment is an order of magnitude less than that of arsenic[2] and few organoantimony standards are available for method development. Nevertheless, a few alkylantimony analogues of the arsenic species have been found in the environment.

In anaerobic systems, trimethylstibine commonly occurs along with trimethylarsine. Trimethylstibine, in association with trimethylarsine, was detected in landfill and sewerage gases, and in algal mats from hot springs[3-6]. Trimethylstibine was also detected in headspace gas samples of mixed anaerobic cultures of microorganisms[7-9]. Nonvolatile alkylantimony species were found in plant extracts[10-12], seawater[13], and sediments[14]. In contrast to arsenic[1,15,16], only a few studies have linked specific organisms with antimony biomethylation[10,11,17-19].

The filamentous fungus, *Scopulariopsis brevicaulis*, is common in the environment; it is an opportunistic pathogen and is well known for its ability to volatilize arsenic as trimethylarsine (Gosio gas, Section 1.4.2)[1]. Indeed this was one of the organisms that was isolated during the early studies of the toxic Gosio gas[20] and, as part of the "toxic gas hypothesis", it is postulated that *S. brevicaulis* is the microorganism in bedding most likely to produce toxic gases[21] (Section 1.5). Consequently, *S. brevicaulis* has received attention by researchers interested in finding antimony analogues of organoarsenic species in the environment and those specifically interested in the "toxic gas hypothesis".

Gates, and coworkers[9], incubated cultures of *S. brevicaulis* (two strains) with potassium antimony tartrate (0.2% w/v), or antimony trioxide, and sampled headspace gases by using chemofocusing; a filter paper, soaked in 5% mercuric chloride, was suspended over the cultures. The filter paper was analyzed by using mass spectrometry. There was no evidence for the production of trimethylstibine by *S. brevicaulis* even though growth was vigorous. Some anaerobic microorganisms, isolated from pond sediment, produced trimethylstibine. The detection limit of their technique was ~5000 ng Sb.

Pearce *et al.*[22] incubated two strains of *S. brevicaulis* with antimony trioxide, or potassium antimony tartrate (30 or 300 mg Sb/L), and analyzed headspace gases by using chemofocusing/HG-GC-AAS. No volatile antimony species were detected. The detection limit of their methodology was 50 ng Sb.

Pearce *et al.*, and Gates *et al.*, stated that although they did not detect trimethylstibine in headspace gases of *S. brevicaulis* cultures their results do not preclude antimony biomethylation by *S. brevicaulis*; Gates *et al.* suggested that trimethylstibine might be produced by *S. brevicaulis* but oxidizes before it can be detected.

The biomethylation of arsenic does not always produce volatile trimethylarsine, in fact when *S. brevicaulis* was incubated with 1 mg As/L as sodium arsenite, or sodium arsenate, the predominant product of biomethylation was nonvolatile trimethylarsine oxide[23], which is an intermediate on the Challenger pathway[20] (Section 1.4.2). Thus, monitoring the liquid phase of *S. brevicaulis* cultures, which have been incubated with inorganic antimony compounds, is desirable because nonvolatile antimony biomethylation products, or products of trimethylstibine oxidation might be detected.

In the experiments described in this chapter, a solid phase extraction (SPE) technique was developed for the cleanup of media samples that contain high concentrations of inorganic antimony, or arsenic, species. Because the SPE technique removes interfering inorganic metalloid species, large sample volumes can be used in semi-continuous HG-GC-AAS analysis, which results in sufficiently low detection limits for the species of interest in these studies.

In this chapter, experiments are described where cultures of *S. brevicaulis* were grown in media containing either inorganic antimony compounds or in some cases, for comparison, inorganic arsenic compounds. Some of the results presented here were obtained by analysis of medium from experiments described in other chapters, such as those where trimethylstibine was also measured; reference to the appropriate chapters is given where necessary.

The alkylantimony, or on occasion alkylarsenic, species present in media, after a period of incubation, were determined by using SPE and HG-GC-AAS. In some experiments, the speciation of the inorganic arsenic, or antimony, compounds in the medium was also determined by using HG-GC-AAS. Biomass was freeze-dried at the end of incubation, extracted with methanol/water and the contents of the extracts determined.

Also described in this chapter are experiments that were done to determine if the dimethylantimony species detected is directly produced by *S. brevicaulis*. This was done because it was suggested that trimethylstibine oxidation might produce dimethylantimony species[19]. Thus, to determine the origin of the dimethylantimony species the products of trimethylstibine oxidation were determined. Furthermore, it is possible that the detection of dimethylantimony species could be due to analytical artifacts forming during hydride generation. Various strategies were used to examine this possibility.

The material presented here was published in references 24, 25 and 26.

2.2 EXPERIMENTAL

2.2.1 Materials

All reagents were of analytical grade or better and obtained from common distributors, except where noted. Purified water was prepared by using ion exchange.

Ammonium carbonate buffer (50 mM) was prepared by dissolving the appropriate amount of ammonium carbonate in water and adjusting the pH with potassium hydroxide (20% w/v) to pH 12.0. Sep-Pak Plus SPE cartridges (long body) were obtained from Waters Millipore.

Trimethylantimony dichloride[27] and trimethylarsine oxide[28] were synthesized in-house by using literature methods and an appropriate amount of solid was dissolved in water to give a stock solution of 1000 mg metalloid/L. Working solutions were made by diluting the stock solutions by the appropriate amount. Sodium borohydride (2% w/v) was prepared fresh daily by dissolving an appropriate amount of solid in deionized water. Citrate buffer (50 mM) was prepared by dissolving the appropriate amount of citric acid in distilled water and adjusting to pH 6 using potassium hydroxide (20% w/v).

Chemically defined minimal-salts/glucose medium (Table 2.1)[29] was prepared in batches of 4 L as required.

Table 2.1. Composition of minimal-salts/glucose medium[29].

Nutrient	Concentration (g/L)
$(NH_4)_2SO_4$	2
KH_2PO_4	0.1
$MgSO_4.7H_2O$	0.05
$FeSO_4.7H_2O$	0.0018
Thiamine hydrochloride	0.01
D-Glucose	10.0
Succinic acid	5.9
KOH	Adjust pH to 5.0

2.2.2 Description of *S. brevicaulis* Cultures

Scopulariopsis brevicaulis was obtained from the UBC culture collection. The original deposit of *S. brevicaulis* was obtained from the American type culture collection (ATCC 7903). *It should be noted that S. brevicaulis is a Class II biohazard.* This strain is equivalent to IMI 40026 and CBS 467.48 and can biomethylate arsenic[23]. Submerged cultures of *S. brevicaulis* mycelial balls were maintained in 1 L Erlenmeyer flasks. Each flask contained 400 mL of minimal-salts/glucose medium (Table 2.1).

The medium was inoculated with *S. brevicaulis*. The antimony or arsenic substrate compound and media supplements (such as D-methionine or L-methionine) were added immediately after inoculation, to give the culture compositions that are detailed in Table 2.2.

Antimony trioxide was directly added to the cultures in the solid form. All other antimony and arsenic compounds were added as solutions after filter sterilization (0.2 μm). Solutions of D-methionine and L-methionine were added, where appropriate, via 0.2 μm syringe filters as described in Chapter 4.

Erlenmeyer flasks were shaken horizontally (~135 rpm, 1.75 inch displacement) and maintained at 26 °C. The fungi grew in the form of regular balls of mycelium, which visibly increased in size throughout the experiments.

Nonliving-biomass controls consisted of autoclaved cultures of *S. brevicaulis*, previously incubated in 400 mL of minimal-salts/glucose medium for one month. The appropriate antimony compounds were added (Table 2.2) and these controls were then treated in the same manner as live cultures.

In the time-course experiments (**2** and **5**), media was sampled at appropriate times (Table 2.2) aseptically, in a biological safety cabinet, and sterilized before analysis, by passing it through a 0.2 μm filter.

After a period of incubation (usually 1 month), the cultures and controls were autoclaved (121 °C, 19 psi, 20 min). The concentrations of alkylantimony, or alkylarsenic, species in the sterilized media were then determined by using SPE and HG-GC-AAS (Section 2.2.5). In some cases, the speciation of inorganic arsenic or antimony

was determined by diluting sterile medium by an appropriate amount and analyzing by using HG-GC-AAS (Section 2.2.5.2).

Biomass was separated from medium by using suction-filtration. The biomass was washed with deionized water and freeze-dried. The freeze-dried biomass was ground to a fine powder by using a mortar and pestle. Extraction was performed by repeating the following steps three times:

1. 10 mL of 50/50 methanol/water was added to the ground sample in a 20 mL polycarbonate centrifuge tube and sonicated for 10 minutes.
2. The sample was centrifuged for 10 minutes.
3. The supernatant solution was drawn off with a Pasteur pipette and placed in a 50 mL round bottom flask.

After the three extracts from above were combined in a 50 mL round bottom flask, the samples were rotary evaporated to dryness and the residue was dissolved in 5 mL of water. The biomass extract was analyzed for alkylantimony species by using SPE and HG-GC-AAS (Section 2.2.5).

Table 2.2. Summary of experiments performed to examine the biomethylation of antimony compounds.

Expt.	Culture	Contents of Culture	Length of Incubation	Comments
1	**1 – 2**	0.2 g antimony trioxide + *S. brevicaulis*	1 month	2 replicates
	3 – 4	0.2 g antimony trioxide + Nonliving biomass[a]	1 month	2 Nonliving-cell controls
	5	0.2 g antimony trioxide	1 month	Medium-only control
2	**1 – 2**	10 mg Sb/L as PAT[b] + *S. brevicaulis*	1 month	Alkylantimony Species Time-course: Sampled every 7 days (20 mL)
	3 – 4	10 mg Sb/L as PAT + nonliving biomass	1 month	2 Nonliving-cell controls
	5	10 mg Sb/L as PAT	1 month	Medium-only control
3	**1 – 2**	1000 mg Sb/L as PAT + *S. brevicaulis*	2 months	2 replicates
	3 – 4	1000 mg Sb/L as PAT + nonliving cells	2 months	2 Nonliving-cell controls
4	**1 – 2**	10 mg Sb/L as potassium hexahydroxyantimonate + *S. brevicaulis*	1 month	2 replicates
	3 – 4	10 mg Sb/L as potassium hexahydroxyantimonate + nonliving cells	1 month	2 nonliving-cell controls
	5	10 mg Sb/L as potassium hexahydroxyantimonate	1 month	Medium-only control
5	**1 – 2**	10 mg Sb/L as PAT + *S. brevicaulis*	1 month	Inorganic antimony species time course: Sampled on days 5, 13, 20, 28 and 35
	3	10 mg Sb/L as PAT + nonliving cells	1 month	Inorganic antimony species time course: Sampled on days 5, 13, 20, 28 and 35

[a] – Non living biomass was obtained by incubating *S. brevicaulis* for one month in minimal-salts/glucose medium, the culture was then autoclaved and the medium decanted off.

[b] – PAT = potassium antimony tartrate

Table 2.2. Continued

Expt.	Culture	Contents of Culture	Length of Incubation	Comments
6	**1 – 2**	100 mg Sb/L as PAT + 0.1g $^{13}CD_3$-L-methionine + *S. brevicaulis*	1 month	Determined incorporation of $^{13}CD_3$ label into alkylantimony species by using GC-MS (Chapter 4)
	3 – 4	100 mg Sb/L as PAT + *S. brevicaulis*	1 month	See Chapter 4
	5 – 6	100 mg Sb/L as PAT + 0.1g CH_3-L-methionine + nonliving biomass	1 month	See Chapter 4
7	**1 – 2**	100 mg Sb/L as PAT +0.1g $^{13}CD_3$-L-methionine +*S. brevicaulis*	1 month	Determined incorporation of $^{13}CD_3$ label into alkylantimony species by using GC-MS (Chapter 4)
	3 – 4	100 mg Sb/L as PAT +0.1g CD_3-D-methionine + *S. brevicaulis*	1 month	Determined incorporation of CD_3 label into alkylantimony species by using GC-MS (Chapter 4)
8	**1 – 4**	10 mg Sb/L as PAT + 0 – 100 mg As/l as arsenite + *S. brevicaulis*	1 month	Interaction with sodium arsenite (Chapter 5)
9	**1 – 4**	10 mg Sb/L as PAT + 0 – 100 mg As/l as arsenite + *S. brevicaulis*	1 month	Interaction with sodium arsenite (Chapter 5)
10	**1 – 4**	100 mg Sb/L as PAT + 0 – 100 mg As/L as arsenite + *S. brevicaulis*	1 month	Interaction with sodium arsenite (Chapter 5)
16	**1 – 3**	1000 mg Sb/L as PAT + *S. brevicaulis*	20 days	Also analyzed headspace (Chapter 3)
	4	1000 mg Sb/L as PAT		Medium-only control. Also analyzed headspace (Chapter 3)

2.2.3 Determination of Trimethylstibine Oxidation Products

Trimethylstibine was generated in septa-capped vials (15 mL) as follows: An aqueous solution (50, 100, 200 or 500 μL) of trimethylantimony dichloride (100 mg Sb/L) was placed in a vial and water was added to give a total volume of 4 mL. Sodium borohydride (1 mL, 6% w/v) was then injected through the septa. Immediately after vigorously shaking the vial, the headspace gas (1 mL), containing trimethylstibine, was sampled with a syringe and injected, via a 0.2 μm filter, into another septa-capped vial (15 mL) containing 2 mL of sterile minimal-salts/glucose medium, and a headspace of laboratory air. During injection the needle of the gas-syringe was placed below the level of the medium so that the trimethylstibine bubbled through the medium. The vials were then placed on the shaker used for the incubation of *S. brevicaulis*, and left for a week. The medium was then analyzed by using SPE and HG-GC-AAS (Section 2.2.5).

2.2.4 Separation of Trimethylantimony and Dimethylantimony Species

Basic alumina (30 g, 80-200 mesh, Brockman activity I, Fisher Scientific) was placed into a 60 mL syringe; a small glass-wool plug was used to hold the alumina in place. The alumina was rinsed with 50 mL of water. Then 50 mL of medium containing dimethylantimony and trimethylantimony species (from a culture of *S. brevicaulis* that had been incubated with potassium antimony tartrate for one month) was passed through the alumina, and the eluate was collected (Eluate 1). The alumina was then further rinsed with 50 mL of distilled water. Finally, the alumina was rinsed with 50 mL of ammonium carbonate (50 mM, pH 12), and the eluate collected (Eluate 2). The two samples of eluate were analyzed by using HG-GC-AAS (Section 2.2.5.2).

2.2.5 Details of Analysis

Hydride generation-gas chromatography-atomic absorption spectroscopy (HG-GC-AAS) was used to determine the concentrations of inorganic metalloid species, or alkylmetalloid species. In the case of the later, solid phase extraction was used to remove the inorganic species and the eluate was analyzed.

2.2.5.1 Solid Phase Extraction (SPE)

In-house SPE columns were prepared in the following manner: basic alumina (80-200 mesh, Brockman activity I, Fisher Scientific) was placed in a syringe barrel; a small glass-wool plug held the alumina in place. Two column sizes were used. Small columns were made from syringe barrels (10 mL) that contained 5 g of alumina and large columns were made from syringe barrels (60 mL) that contained 20 g of alumina.

The SPE column was rinsed with ammonium carbonate buffer; 10 mL of buffer per 5 g of alumina. Then the sample was passed through the column, and the eluate collected for analysis. No attempt was made to force the sample through the column but rather it was allowed to drip through under gravity. No attempt was made to rinse the column after the sample had eluted.

2.2.5.2 Hydride Generation-Gas Chromatography-Atomic Absorption Spectroscopy (HG-GC-AAS)

HG-GC-AAS may be used to determine alkylarsenic or alkylantimony species and the procedure described herein is directly applicable after removing inorganic metalloid species by using SPE. However, HG-GC-AAS may also be used to determine the speciation of the inorganic metalloid species, i.e. metalloid(III) and metalloid(V), and this is done by performing HG-GC-AAS twice, obviously no SPE is done. In the first run, the concentration of antimony(III), or arsenic(III), species is determined by using 50 mM citrate, or 4 M acetic acid, respectively, as the buffer. In the second run, the total concentration of inorganic metalloid (i.e. III and V) is determined by using 1M HCl as the "buffer"; the purge gas flow rate is 30 mL/min and the sample uptake rate is 3 mL/min. The concentration of metalloid(V) is determined by difference.

Semi-continuous HG-GC-AAS methodology (Figure 2.1), based on a design described by Cullen *et al.*[23], was used. First, the 6-port valve is switched to the trap position and the U-shaped trap is immersed in liquid nitrogen. An appropriate volume of sample is pumped through the reaction coil, where it mixes with sodium borohydride, buffer and helium purge gas. The reaction of acetic acid with sodium borohydride produces a large volume of hydrogen, so lower purge gas flow rates and sample uptake rates are used for arsenic analysis. After the reaction coil, the evolved hydrides and helium gas are separated from liquid waste in the gas-liquid separator. The evolved hydrides pass through a dry-ice/acetone trap (that removes water vapor) and are collected in the U-shaped trap immersed in liquid nitrogen.

After all sample has passed through the reaction coil and the system thoroughly rinsed, the 6-port valve is switched to the inject position. The liquid nitrogen is removed from under the U-shaped trap, and replaced by hot water (~90 °C), which results in rapid injection of hydrides onto the GC column. Simultaneously the recorder and GC temperature program are started. A hydrogen/air flame within a quartz tube cuvette is used to atomize hydrides that elute from the GC column. The cuvette is mounted in the light path of the AAS. Details of the instrumentation are summarized in Table 2.3.

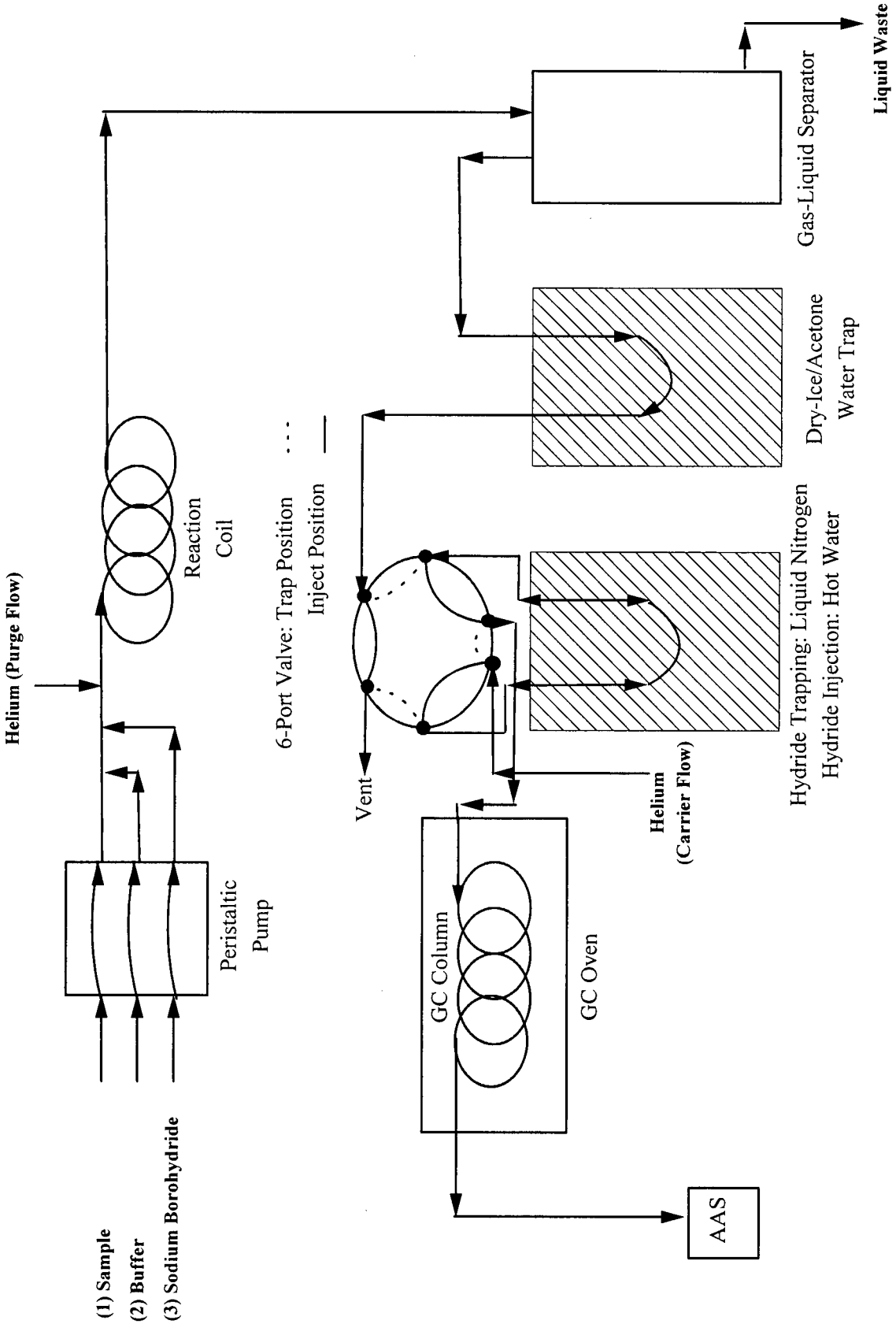

Figure 2.1. HG-GC-AAS apparatus.

Table 2.3. Instrumental parameters for HG-GC-AAS

	Arsenic analysis	Antimony analysis
HG parameters		
Helium purge gas flow rate	30 mL/min	300 mL/min
Sample uptake rate	3 mL/min	8 mL/min
"Buffer":		
Inorganic Metalloid(III)/organometalloid species	4 M Acetic acid	50 mM Citrate, pH 6.0
Inorganic Metalloid(III+V)	1 M HCl	1 M HCl
GC parameters (HP 5890)		
GC temperature program	50 – 150 °C, 15 °C/min	50 – 150 °C, 30 °C/min
Helium carrier gas flow rate	40 mL/min	40 mL/min
Column	3.5 m 1/8 inch o.d. PTFE tubing packed with Supelcoport SP-2100 (10% on Chromosorb, 45/60 mesh, Supelco)	
AAS parameters (Varian 1275)		
Lamp current	10 mA	10 mA
Monochromator wavelength	193.7 nm	217.6 nm
		231.2[a] nm
Monochromator slit width	1 nm	0.2 nm
Quartz Cuvette Atomizer (H_2/air Flame)		
Hydrogen flow rate	120 mL/min	120 mL/min
Air flow rate	120 mL/min	120 mL/min

[a] – Alternative wavelength for confirming compound contains antimony; sensitivity ~1/2 of 217.6 nm.

2.3 RESULTS

2.3.1 Solid Phase Extraction

A typical HG-GC-AAS chromatogram for a 50 μL sample of minimal-salts/glucose medium, containing a 10:1 ratio of inorganic antimony to trimethylantimony species, with no SPE cleanup (Figure 2.2a) contains a large broad stibine peak. The large tail of the stibine peak interferes with the trimethylstibine peak thus increasing the detection limit for trimethylantimony species. To solve this problem the approach adopted was to remove the inorganic antimony species before hydride derivatization, by using a SPE column.

In preliminary experiments, medium containing 10 mg Sb/L as PAT and 1 mg Sb/L as trimethylantimony dichloride, was passed through QMA, NH_2, and alumina B Sep-Pak plus SPE cartridges. These cartridges removed greater than 99% of the inorganic antimony(III) species and the trimethylantimony species was almost completely recovered from the alumina B cartridge and the NH_2 cartridge but not the QMA cartridge. Unfortunately, the commercial Sep-Pak cartridges are expensive, so an in-house prepared SPE column was tested. When a sample of medium was passed through the in-house prepared SPE column, packed with basic alumina, inorganic antimony(III) species were retained (>99%), whereas trimethylantimony dichloride eluted. When HG-GC-AAS was performed on this "clean" sample the trimethylantimony dichloride was more readily detected (Figure 2.2a).

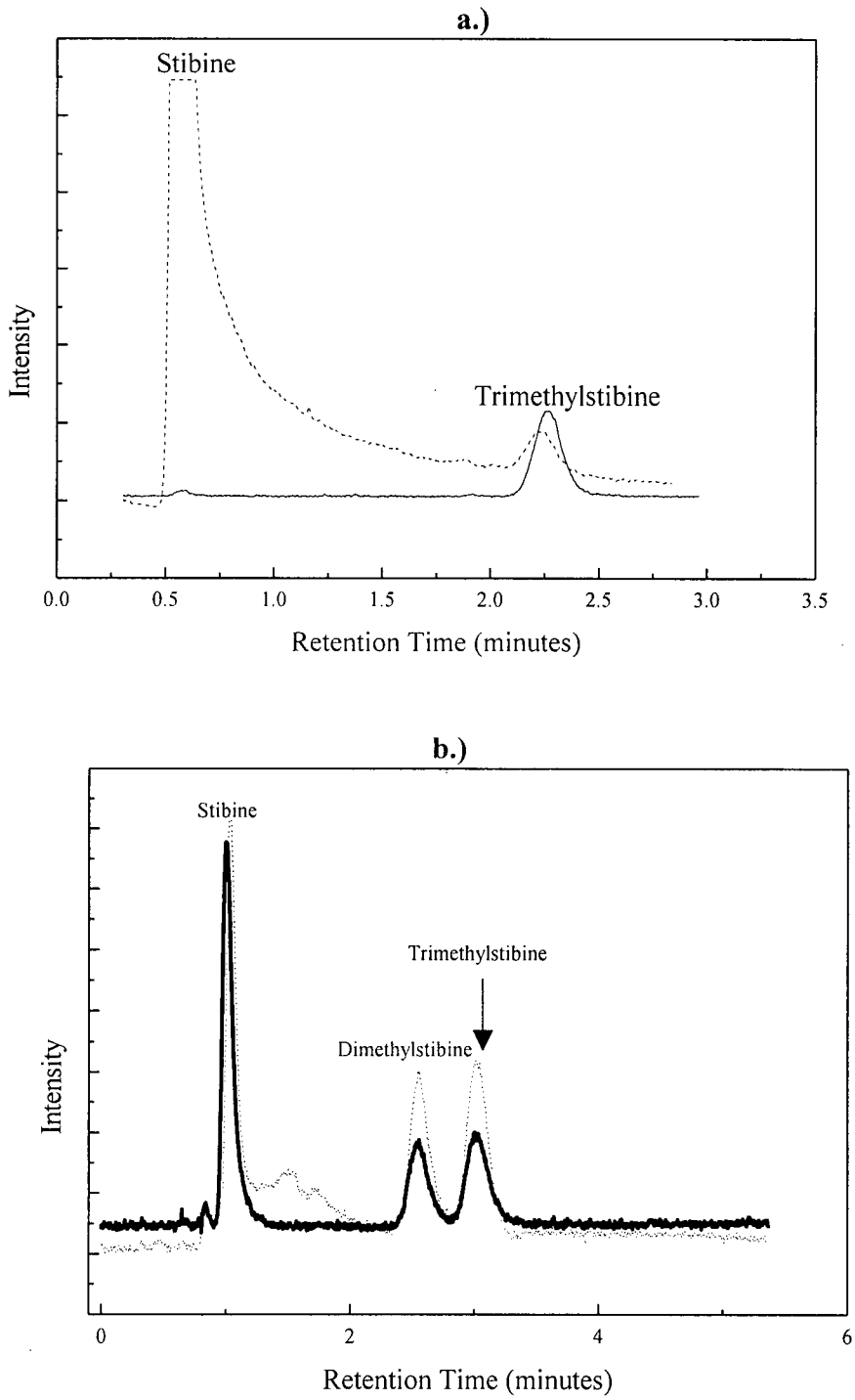

Figure 2.2. a.) HG-GC-AAS chromatograms: 50 μL of medium that contained 10 mg Sb/L as PAT and 1 mg Sb/L as trimethylantimony dichloride (---); 50 μL of the same medium after it has passed through the basic alumina SPE column (—).
b.) HG-GC-AAS chromatograms: 10 mL of medium that contained dimethylantimony and trimethylantimony species (···); 10 mL of the same medium after it has passed through an SPE column (–).

SPE method development was performed using trimethylantimony dichloride to monitor recovery of organoantimony analytes; however, no methylantimony or dimethylantimony compounds were available for method development, so it was necessary to assume that the trimethylantimony dichloride standard and alkylantimony species, formed by *S. brevicaulis*, would behave in the same manner on basic alumina. To further validate the SPE technique, a real sample of medium, from a culture of *S. brevicaulis* that had been incubated with potassium antimony tartrate for one month, was analyzed. The medium contained dimethylantimony and trimethylantimony species. This medium had been refrigerated for more than 6 months and over this time all the inorganic antimony(III) species had oxidized. Thus, large quantities of stibine did not form during hydride generation at pH 6.0. This medium was analyzed before and after passing it through the SPE cartridge (Figure 2.2b). The ratio of dimethylantimony species to trimethylantimony species did not change when the sample passed through the SPE cartridge although the peak areas did decrease. For the purposes of quantification it was assumed that the recovery of alkylantimony species when using the SPE method is 100% even though the recovery is clearly less than 100%. This assumption is not a significant source of error compared with the major assumption that the trimethylantimony dichloride standard and trimethylantimony analyte species have similar hydride derivatization efficiencies.

Oxidation of inorganic antimony(III) species (using hydrogen peroxide, for example) as an alternate method to prevent interference in HG-GC-AAS is not practical because oxidizing agents may change the alkylantimony species, and oxidizing agents

react with sodium borohydride, which decreases the sensitivity of the HG-GC-AAS method.

The dimethylantimony species was retained on the alumina SPE cartridge when the alumina was pre-rinsed with water rather than ammonium carbonate buffer, which was useful in allowing separation of the dimethylantimony and trimethylantimony species (Section 2.2.4). That is although pH changes have little effect on the ability of alumina to remove inorganic antimony(III) species, pH changes do significantly effect the recovery of analytes.

The SPE method is applicable without modification to the determination of trimethylarsenic species in a matrix containing large amounts of inorganic arsenic(III) species. When a sample of medium is passed through the SPE column inorganic arsenic(III) species are retained (>95%) but trimethylarsine oxide elutes (>80%).

2.3.2 Alkylantimony Species in Media

A summary of results is provided in Table 2.4. In all of the experiments described in this chapter, no retardation of fungal growth, due to the addition of inorganic antimony compounds, was visually observed. This is consistent with the work of Pearce *et al.*[22] who determined the EC_{50} (median effective concentration for inhibition of hyphal extension) values for two strains of *S. brevicaulis* to be greater than 300 μg Sb/mL as potassium antimony tartrate.

Table 2.4. Summary of results.

Expt.	Culture	Contents of Culture	Concentration of Antimony species (ng Sb/mL)		~ % biomethylation
			Dimethyl-	Trimethyl-	
1	1	Saturated Sb_2O_3	2	0.8	0.001
	2	Saturated Sb_2O_3	1.5	0.8	0.001
	3-5	Controls	< 0.2	< 0.2	< 0.0001
2	1	10 mg Sb/L as PAT[a]	2.1	3.3	0.05
	2	10 mg Sb/L as PAT	2.5	2.5	0.05
	3-5	Controls	< 0.1	< 0.1	< 0.001
3	1	1000 mg Sb/L as PAT	6.9	3.1	0.001
	2	1000 mg Sb/L as PAT	7.1	5.3	0.001
	4-5	Controls	< 0.1	< 0.1	< 0.0001
4	1	10 mg Sb/L as PHHA[b]	< 0.1	< 0.1	< 0.001
	2	10 mg Sb/L as PHHA	< 0.1	< 0.1	< 0.001
	3-5	Controls	< 0.1	< 0.1	< 0.001

[a] – PAT = potassium antimony tartrate
[b] – PHHA = potassium hexahydroxyantimonate

Table 2.4. Continued

Expt.	Culture	Contents	Concentration of Antimony species (ng Sb/mL)		~ % biomethylation
			Dimethyl-	Trimethyl-	
5	1	10 mg Sb/L as PAT	Detected[c]	1.7	0.05
		Labeled Methionine Experiments – See Chapter 4			
6	1-2	100 mg Sb/L as PAT + L-methionine	Detected	20	0.05
	3-4	100 mg Sb/L as PAT	Detected	5	0.01
	5-6	100 mg Sb/L as PAT Nonliving-cell control	< 0.1	< 0.1	< 0.0001
7	1-2	100 mg Sb/L as PAT + L-methionine	Detected	20	0.05
	3-4	100 mg Sb/L as PAT + D-methionine	Detected	6	0.01
		Interaction Experiments – See Chapter 5			
8	1-4	10 mg Sb/L as PAT	Detected	0.5-3.2	0.05
9	1-4	10 mg Sb/L as PAT	Detected	0.6-2.5	0.05
10	1-4	100 mg Sb/L as PAT	Detected	5	0.01
		Also Analyzed Headspace Gases – See Chapter 3			
16	1	1000 mg Sb/L as PAT	Detected	2.5	0.001
	2	1000 mg Sb/L as PAT	Detected	4	0.001
	3	1000 mg Sb/L as PAT	Detected	6	0.001
	4	Control	< 0.1	< 0.1	< 0.00001

[c] – Where dimethylantimony species were detected but not quantified the peak area of dimethylstibine in the HG-GC-AAS chromatogram was approximately the same as the peak area of trimethylstibine.

2.3.2.1 Inorganic Antimony(III) Compounds as a Substrate for Biomethylation

Nonvolatile dimethylantimony and trimethylantimony species were detected (as dimethylstibine and trimethylstibine) in all active *S. brevicaulis* cultures that had been incubated with the inorganic antimony(III) compounds, potassium antimony tartrate or antimony trioxide (Table 2.4: Experiments **1-3**, **5-10**, and **16**). The amounts of dimethylantimony and trimethylantimony species detected were the same for media that was sterilized by filtration and media that was sterilized by autoclave; in other words, the dimethylantimony and trimethylantimony species are not degraded in the autoclave.

When *S. brevicaulis* media samples from cultures that were incubated with inorganic antimony(III) compounds were analyzed by using HG-GC-AAS, two approximately equal size peaks were observed in the HG-GC-AAS chromatograms with retention times corresponding to dimethylstibine and trimethylstibine (Figure 2.3).

Because dimethylantimony compounds are not available as analytical standards, the concentrations tabulated for dimethylantimony species are only an estimate based on the assumption that peak areas in the HG-GC-AAS chromatograms are proportional to the antimony concentration; that is, the detector response is independent of species. This assumption is not likely to be valid because the HG-GC-AAS calibration curves of inorganic antimony(III) and trimethylantimony dichloride have different slopes (Figure 2.4). This may arise from either differences in efficiency of hydride generation, or differences in the atomization efficiency of stibine and trimethylstibine. If it is assumed that this difference in response would arise from the differing atomization of stibine and trimethylstibine then the response of dimethylstibine would probably be expected to fall somewhere between the two. However, the dimethylantimony species,

which derivatizes to dimethylstibine, probably has different hydride-derivatization efficiency from that of trimethylantimony dichloride, and so these results must only be taken as estimates. In fact, it is also assumed that the trimethylantimony species, detected in *S. brevicaulis* media, has a hydride-derivatization efficiency similar to that of trimethylantimony dichloride standard. Ideally, dimethylantimony and trimethylantimony species should be analyzed by using HPLC-ICP-MS, where, in most cases, detector response is independent of species. Nevertheless, the detection of these dimethylantimony and trimethylantimony species in the media indicates that biomethylation of inorganic antimony(III) species is occurring.

No stibines were detected when media samples were run through the HG-GC-AAS system without the addition of sodium borohydride. Therefore, the dimethylstibine and trimethylstibine, detected after performing hydride derivatization, are derived from dimethylantimony and trimethylantimony species, and are not liberated by simply purging the medium.

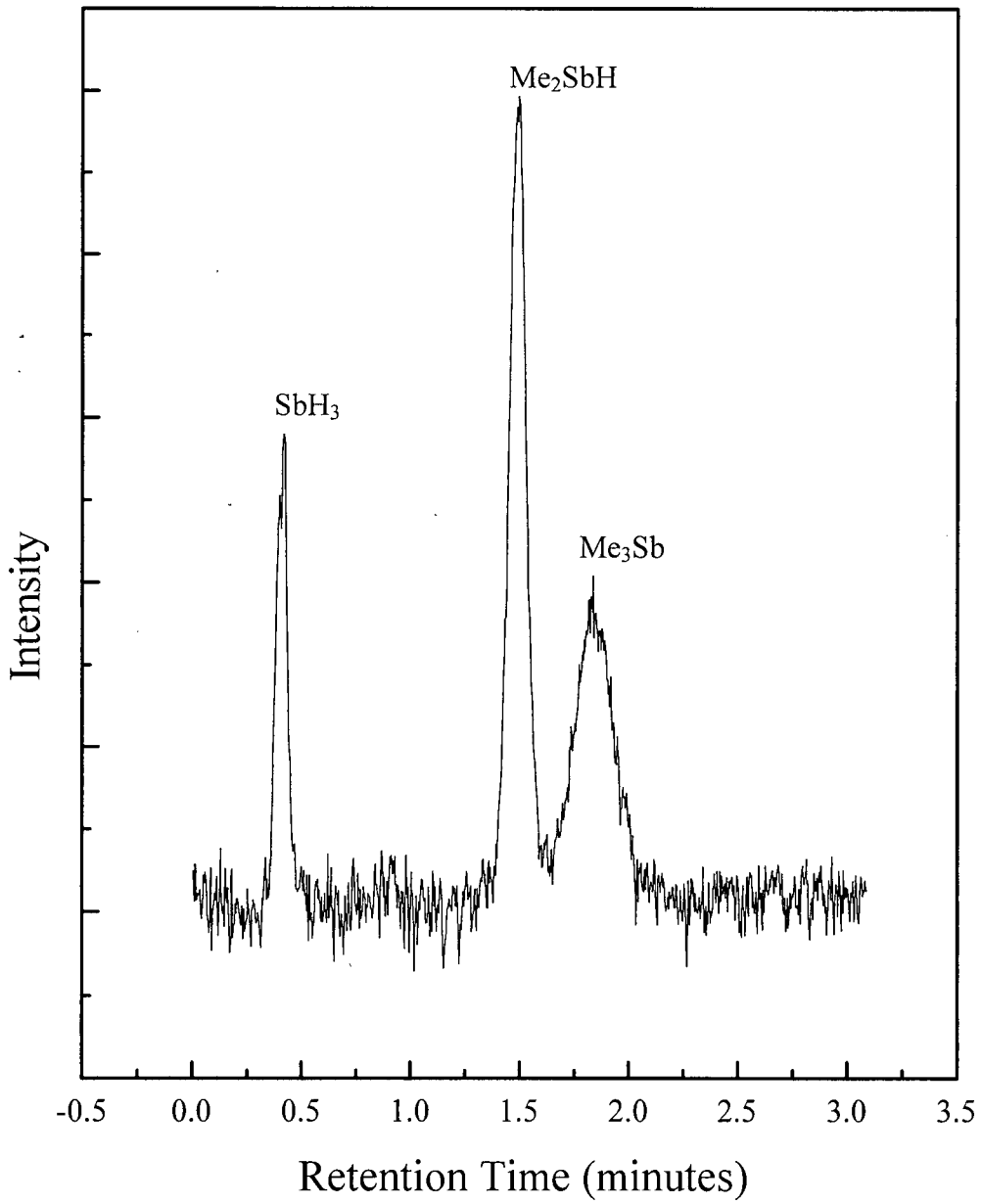

Figure 2.3. HG-GC-AAS chromatogram for medium in which *S. brevicaulis* had been growing with potassium antimony tartrate (10 mg Sb/L) for 1 month.

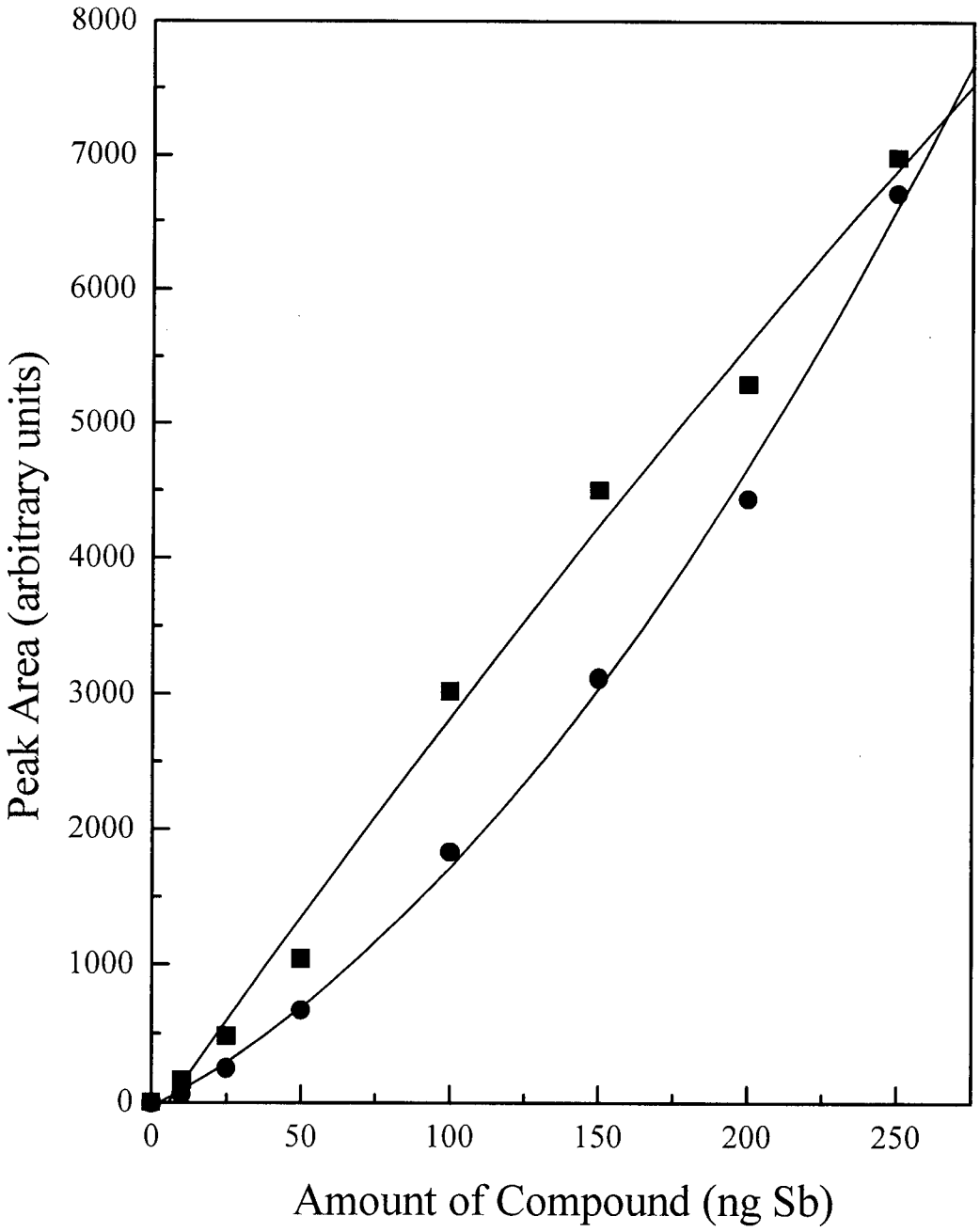

Figure 2.4. HG-GC-AAS calibration curves for potassium antimony tartrate (■) and trimethylantimony dichloride (●).

The final column in Table 2.4 gives the percentage antimony biomethylation, which is very small. In contrast, much larger yields occur for the biomethylation of inorganic arsenic to trimethylarsine, for example when *S. brevicaulis* was incubated with sodium arsenite for 1 month, ~4% of the inorganic arsenic was converted to trimethylarsenic species.

No alkylantimony species were extracted from *S. brevicaulis* biomass by using methanol/water.

In Experiment **1**, cultures containing a saturated solution of antimony trioxide (~4 mg Sb/L), dimethylantimony and trimethylantimony species were detected in the media after 10 days. Higher levels were measured in samples taken at the end of incubation (1 month). The amounts detected were in the low μg Sb/L range.

No alkylantimony species were detected in the controls at any time; generally, the detection limit was 0.1 μg Sb/L as trimethylantimony dichloride, for a typical sample volume of 10 mL, which was used when the concentration of dimethylantimony and trimethylantimony species was low.

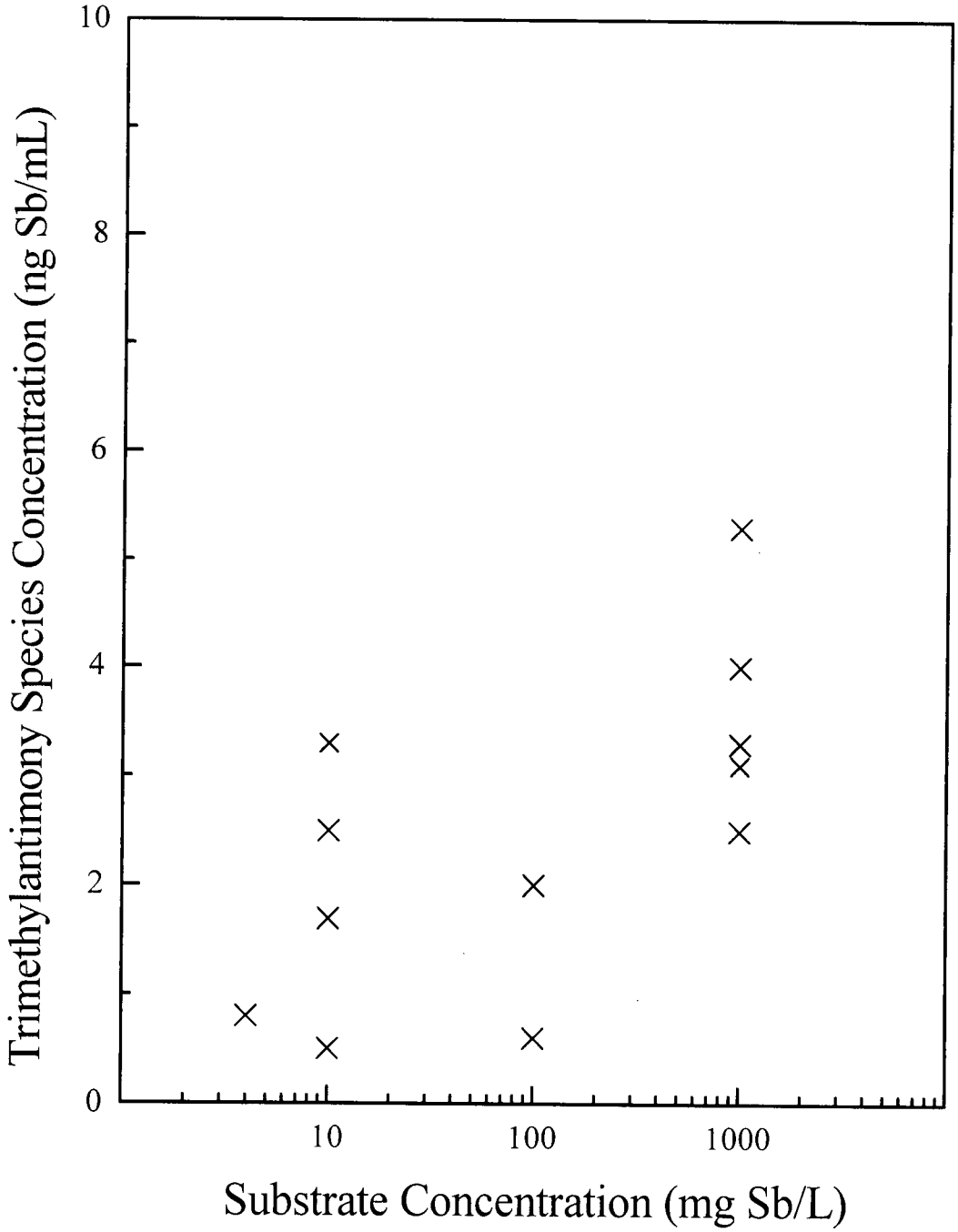

Figure 2.5. Concentration of trimethylantimony species in medium of *S. brevicaulis* after one month of incubation with inorganic antimony(III) species at various concentrations.

Potassium antimony tartrate is more soluble than antimony trioxide, so this enabled experiments to be performed with media that had concentrations of 10, 100, or 1000 mg Sb/L (Table 2.4: Experiments **2**, **3**, **5-10**, and **16**) as potassium antimony tartrate. Dimethylantimony and trimethylantimony species were detected in media samples for all 30 viable cultures. Increasing the concentration of the substrate, potassium antimony tartrate, did not significantly increase the amounts of dimethylantimony and trimethylantimony species produced (Figure 2.5). No organoantimony species were detected in any of the 15 controls.

In Experiment **2**, the contents of media samples were determined every week for 5 weeks and the time-course for the production of dimethylantimony and trimethylantimony species (Figure 2.6), seems to indicate that production is proportional to the amount of biomass as visually observed. The concentrations of the trimethylantimony and dimethylantimony species, increased over time until growth had visibly ceased, after which time the concentrations of the species remained constant.

The addition of sodium arsenite, L-methionine, or D-methionine effected the amounts of dimethylantimony and trimethylantimony species produced, which is discussed in later chapters. Trimethylstibine was detected in headspace gases in Experiment **16**, which is discussed in Chapter 3.

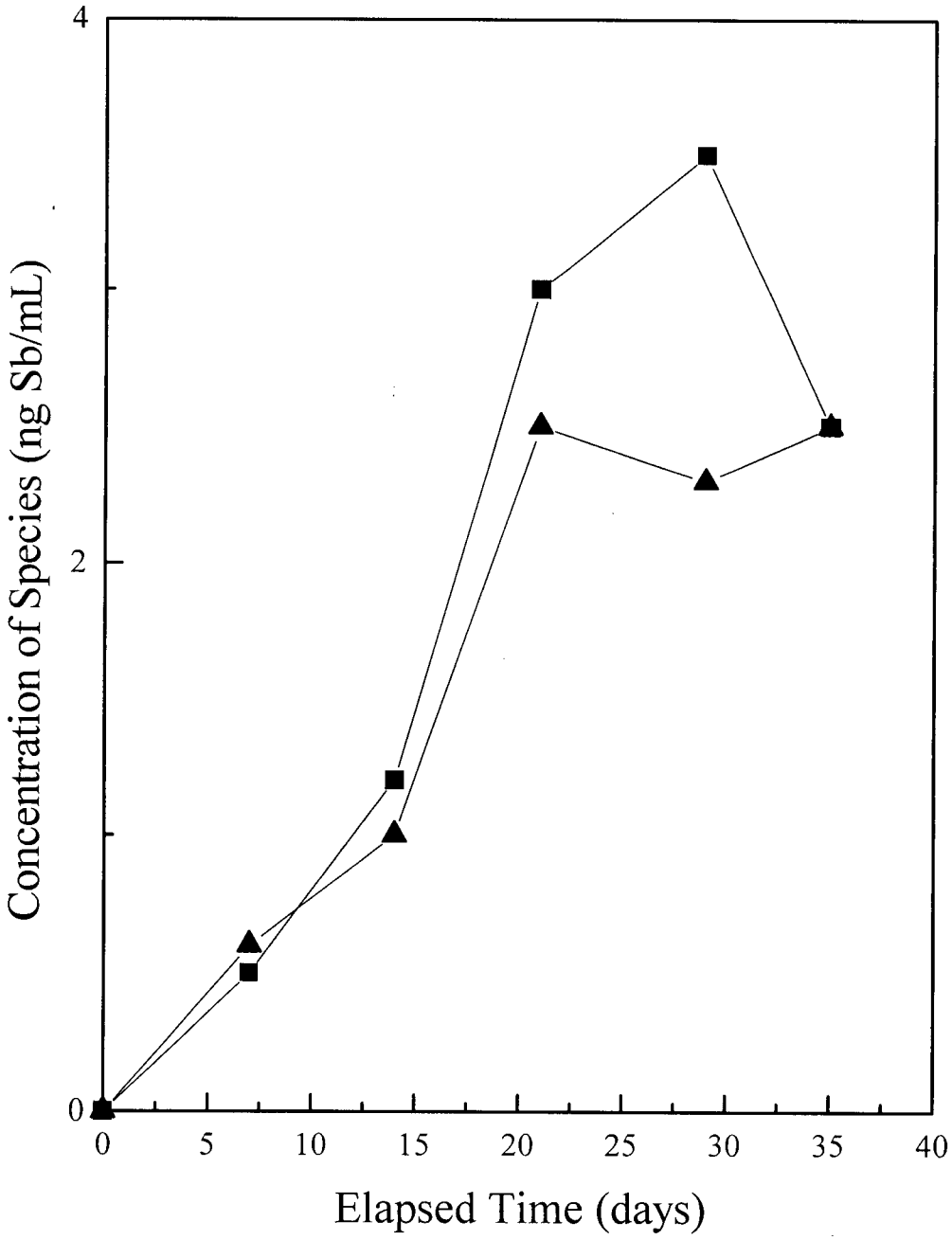

Figure 2.6. Time-course for the production of nonvolatile alkylantimony species by *S. brevicaulis*: Concentrations of dimethylantimony (▲) and trimethylantimony (■) species in samples of medium taken every 7 days during Experiment **2**.

2.3.2.2 Inorganic Antimony(V) Compounds as a Substrate for Biomethylation

When *S. brevicaulis* was incubated with the antimony(V) compound potassium hexahydroxyantimonate (Experiment **4**), no organoantimony species were detected in the media or in cell extracts after one month of growth (Table 2.4). Determination of total antimony in the media by using HG-GC-AAS indicated that the uptake of potassium hexahydroxyantimonate by *S. brevicaulis* is minimal. No inorganic antimony(III) species were detected in the media by using HG-GC-AAS.

2.3.3 Characterization of the Dimethylantimony and Trimethylantimony Species

In Experiments **1-3**, **5-10** and **16**, the chromatograms obtained by using HG-GC-AAS contained two peaks, assigned as dimethylstibine and trimethylstibine that are derivatives of dimethylantimony and trimethylantimony species, respectively. The dimethylantimony and trimethylantimony species are thought to be intermediates on the pathway to trimethylstibine. These assignments are now further discussed.

2.3.3.1 Confirmation of the Identity of the Species

Initially, the two peaks in the HG-GC-AAS chromatograms, attributed to dimethylantimony and trimethylantimony species, were confirmed as arising from species containing antimony by using AAS in two ways. When detection was carried out at 231.2 nm, peaks were seen in the HG-GC-AAS chromatogram with approximately half the peak areas of the peaks detected at the usual wavelength of 217.6 nm. This was also the case for the trimethylantimony dichloride standard, the decrease in signal intensity

being due to the lower extinction coefficient for antimony at 231.2 nm. In addition, no peaks were seen when the monochromator was tuned to 217.0 nm, corresponding to a line in the AA lamp at which antimony atoms do not absorb, indicating that the absorption at 217.6 nm is not arising from broad-band molecular absorption.

Although the retention times of the two stibines detected suggest that they are dimethylstibine and trimethylstibine, the remote possibility existed that they might be ethylstibine and methylethylstibine. Thus, it was necessary to further confirm the identity of the stibines by using GC-MS (ion trap). The details of how this was done are provided in Chapter 4. When stibines, produced by sodium borohydride derivatization of media samples, were injected into the GC-MS the stibine with the retention time of trimethylstibine produced the expected mass spectrum (Figure 2.7). This mass spectrum is a little different from that reported by NIST (Quadrupole MS) for trimethylstibine (Figure 1.2), in that the molecular ion is deprotonated, but this is not unusual and a similar MS was reported by Feldmann *et al.*[6], who used an ion trap MS. Likewise, the peak with the retention time of dimethylstibine produced the expected mass spectrum (Figure 2.7).

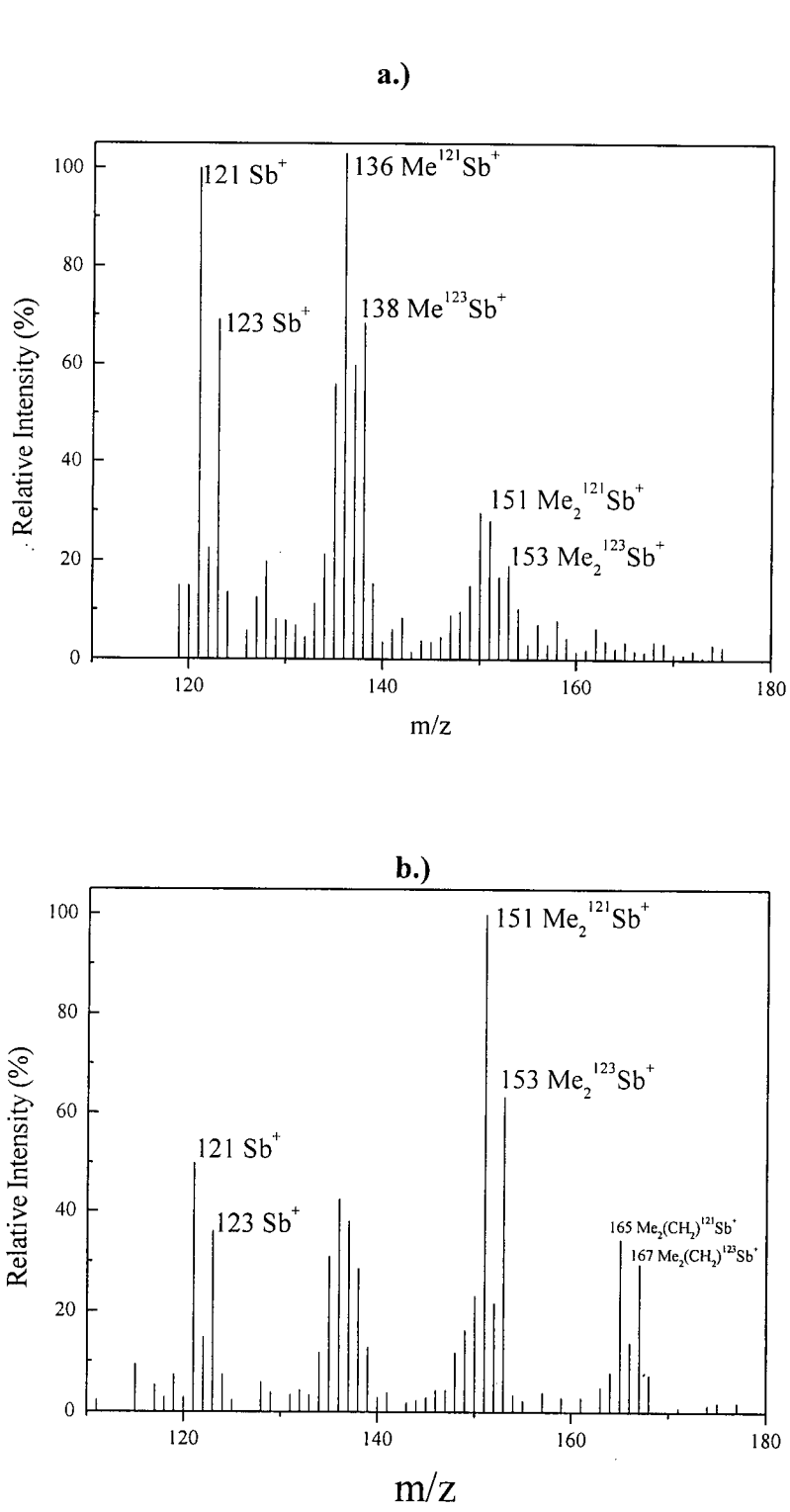

Figure 2.7. GC-MS mass spectra of dimethylstibine (a) and trimethylstibine (b) obtained by hydride derivatization of *S. brevicaulis* medium after 1 month of incubation with potassium antimony tartrate.

2.3.3.2 Evidence that The Dimethylantimony Species is not an Analytical Artifact

Dodd *et al.*[30] demonstrated that demethylation of trimethylantimony species during hydride generation can result in the formation of stibine, methylstibine and dimethylstibine, seen as additional peaks in the chromatogram. This phenomenon is especially prevalent at low pH[31], although it can be minimized by rigorous exclusion of oxygen from the HG-GC-AAS system[10]. In this study, the dimethylstibine peak is unlikely to be an artifact because trimethylantimony dichloride, under the same analysis conditions, shows negligible demethylation, only trimethylstibine is produced. Also, when performing standard additions on the samples the trimethylstibine peak increased in direct proportion to the amount of trimethylantimony dichloride spike added, whereas the dimethylstibine peak remained effectively constant (Figure 2.8).

Furthermore, irrefutable evidence was obtained to show that the dimethylantimony species is not an artifact by separating the dimethylantimony species from the trimethylantimony species and analyzing these species in isolation by using HG-GC-AAS. A sample of medium that was known to contain dimethylantimony and trimethylantimony species was passed through alumina that had only been rinsed with water. The dimethylantimony species was retained but the trimethylantimony species eluted (Eluate 1, Figure 2.9). The dimethylantimony species was recovered by rinsing the alumina with ammonium carbonate (Eluate 2, Figure 2.9). Unfortunately inorganic antimony(V) species behave in a similar manner to the dimethylantimony species so this procedure is not yet suitable for obtaining pure dimethylantimony species for further studies.

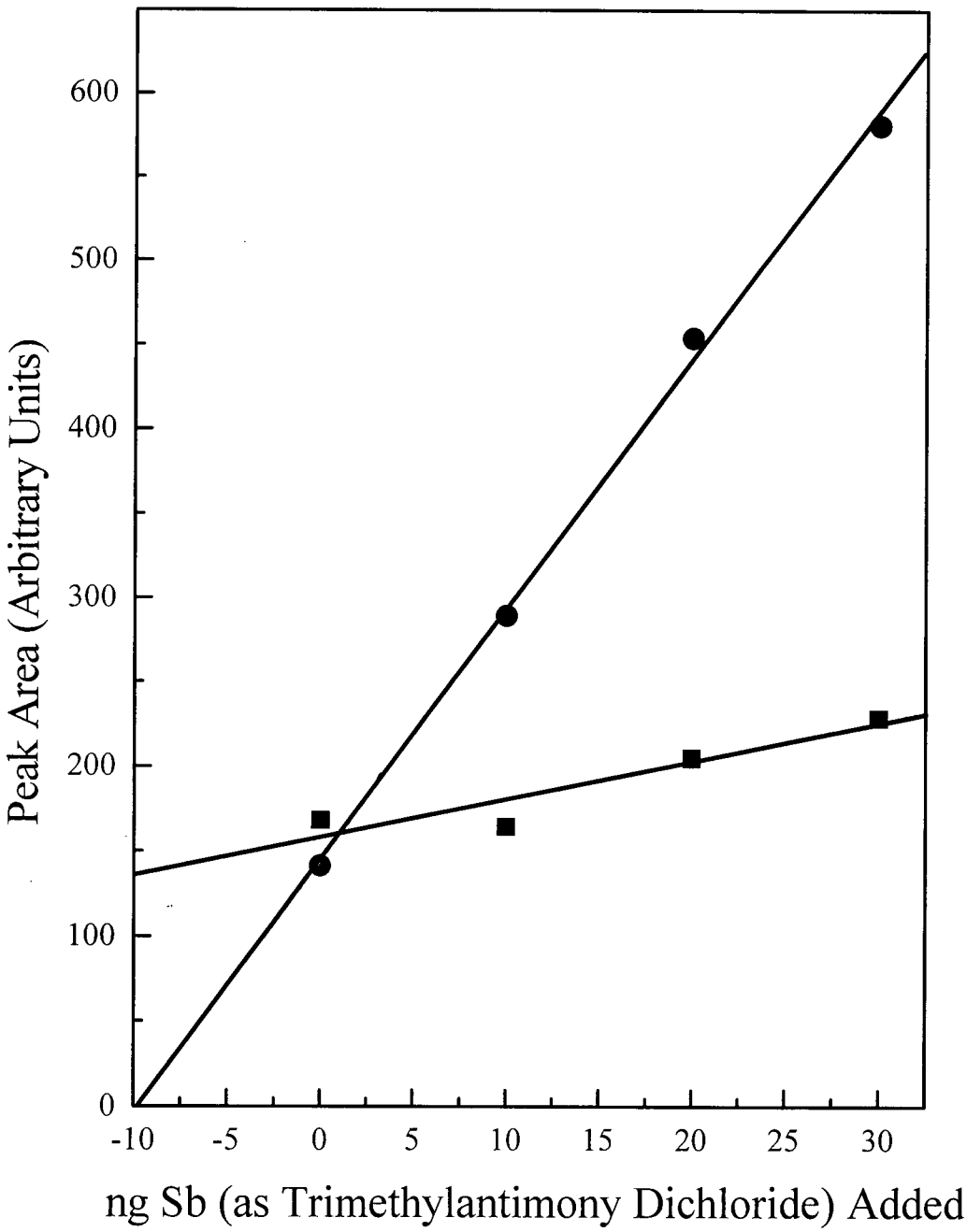

Figure 2.8. Standard additions curve for the addition of trimethylantimony dichloride to samples of media (4 mL taken after 21 days of growth), from the experiment where *S. brevicaulis* was grown in the presence of 10 mg Sb/L as potassium antimony tartrate. (•) Trimethylstibine peak area; (■) dimethylstibine peak area.

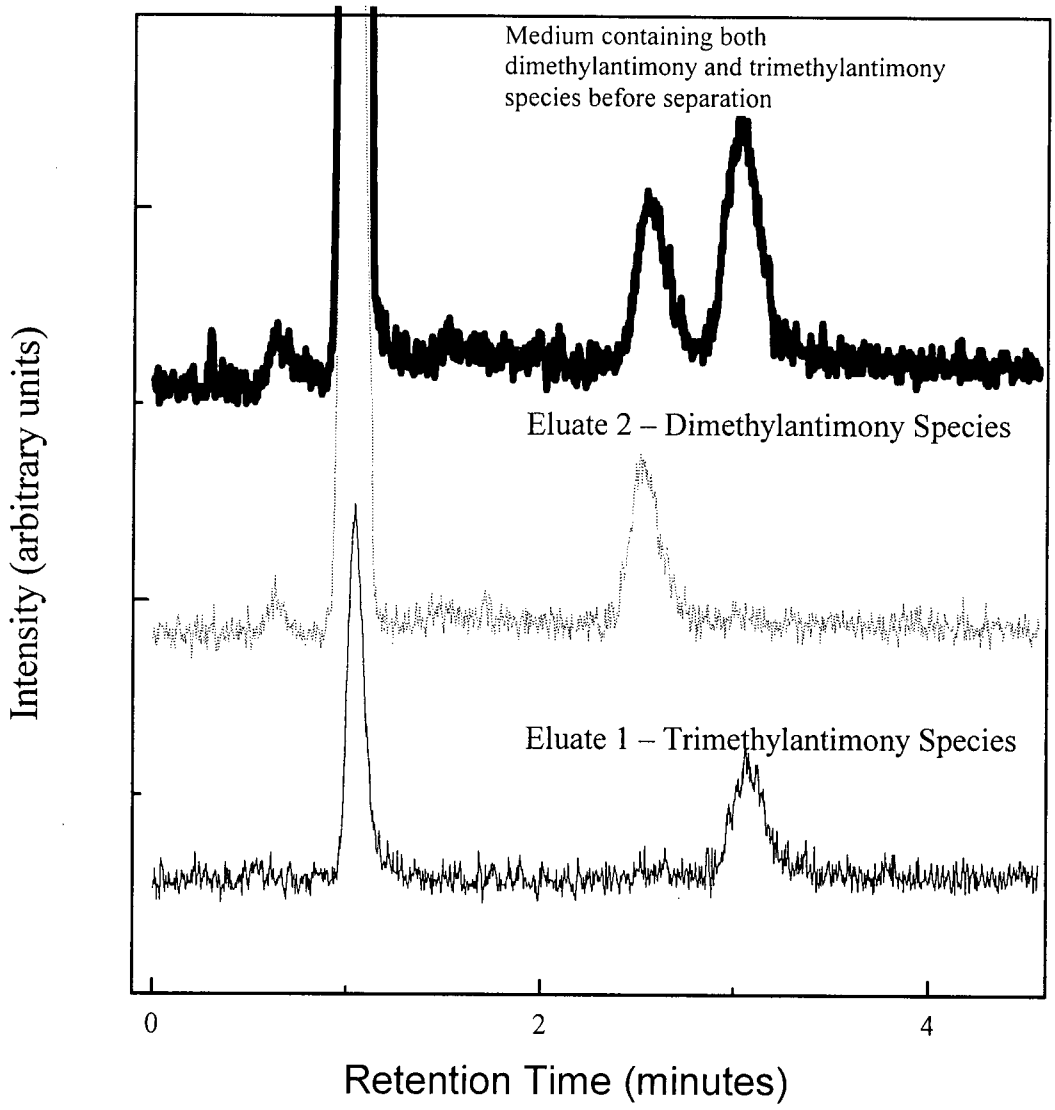

Figure 2.9. HG-GC-AAS chromatograms of dimethylantimony and trimethylantimony species obtained as eluates from basic alumina.

2.3.3.3 Evidence that The Dimethylantimony Species is a True Intermediate and Not a Product of Trimethylstibine Oxidation

Parris and Brinckman[32] studied the oxidation of trimethylstibine and trimethylarsine to better understand the fate of these compounds in the environment. They reported that in unagitated methanol solution, at high concentration, trimethylstibine reacts with air to afford mainly the oxide 'Me_3SbO' accompanied by limited (< 10%) antimony-carbon bond cleavage to afford ill-defined products; they found evidence for the formation of a dimethylantimony species by using ^{1}H-NMR. Extrapolating these results to environmental conditions is difficult but they seem to indicate that at low concentration oxidation of trimethylstibine probably does not result in significant antimony-carbon bond cleavage (the reactions that result in antimony-carbon bond cleavage are less likely at low trimethylstibine concentration). Nevertheless, if the conclusions of Parris and Brinckman are to be believed it is possible that the dimethylantimony species detected in the medium of *S. brevicaulis* cultures could arise from trimethylstibine oxidation[19]. In order to refute this possibility the oxidation products of trimethylstibine were determined. Trimethylstibine was injected into minimal-salts/glucose media in 15 mL septa-capped vials containing a headspace of laboratory air, and the oxidation-products determined, by using HG-GC-AAS. The only significant product of trimethylstibine oxidation is a trimethylantimony species (Figure 2.10).

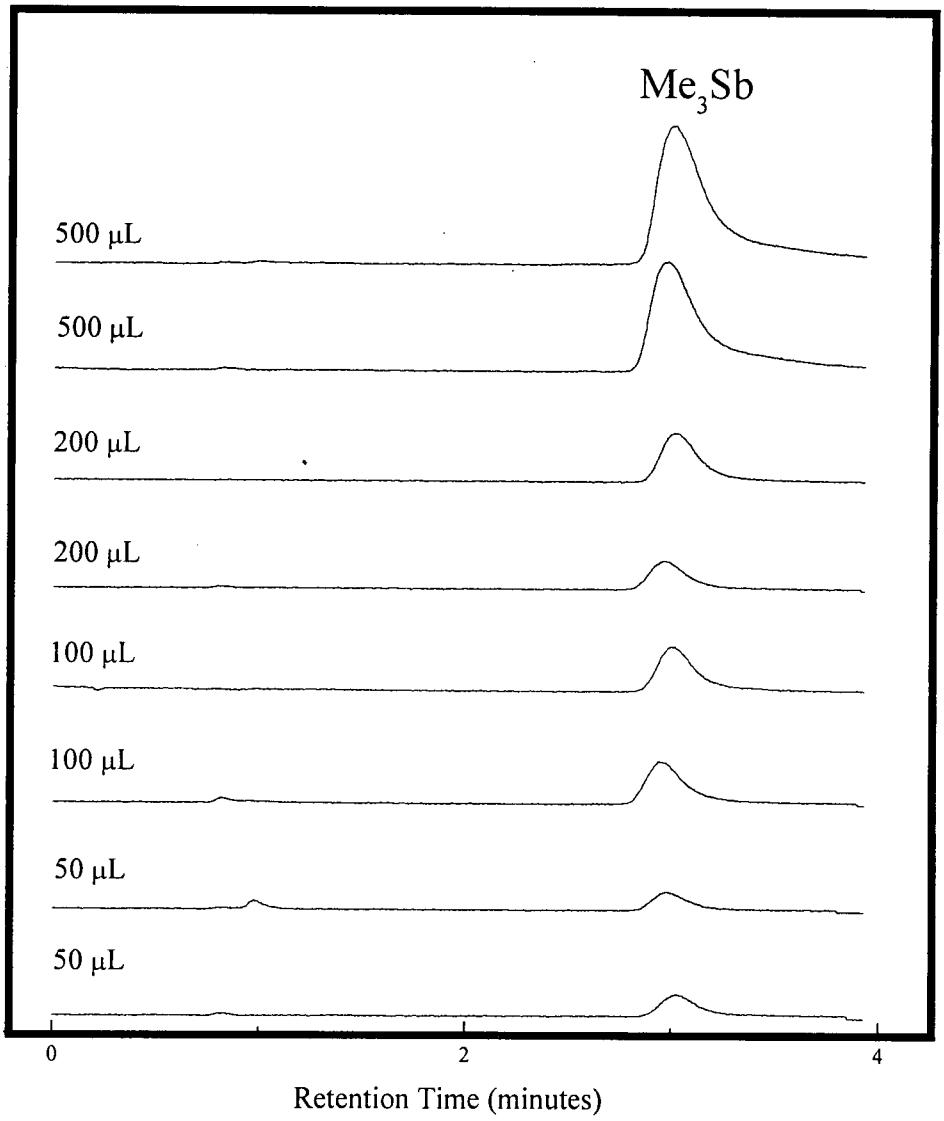

Figure 2.10. HG-GC-AAS chromatograms of media samples containing the products of trimethylstibine oxidation. The volumes given are that of Me_3SbCl_2 (100 mg Sb/L) which was placed in a 15 mL vial and derivatized with sodium borohydride to form trimethylstibine which was collected from the vial headspace. This headspace gas (1 mL) was subsequently injected into a second vial, containing medium, where the oxidation occurs.

2.3.4 Inorganic Antimony Species in Media

The culture containing potassium hexahydroxyantimonate and *S. brevicaulis* (Experiment **4**) contained no detectable antimony(III) species after one month of incubation, determined by using HG-GC-AAS. This is a significant difference from the behavior of arsenate, which is rapidly reduced by *S. brevicaulis* to arsenite[23]. In order to biomethylate inorganic antimony(V), it would first need to be reduced to antimony(III) if the Challenger mechanism applies to antimony. Thus, the fact that antimony(V) is not reduced by cultures of *S. brevicaulis* and is also not biomethylated is consistent with the notion that antimony biomethylation proceeds via the Challenger mechanism.

In Experiment **5**, where *S. brevicaulis* was incubated with potassium antimony tartrate, the inorganic antimony(III) concentration slowly decreased. However, the total antimony concentration remained constant (Figure 2.11), determined by using HG-GC-AAS. This was also observed in nonliving controls. Therefore, some abiotic oxidation of inorganic antimony(III) species to inorganic antimony(V) species can take place. In contrast, as expected, no arsenic(III) oxidation occurred in a culture containing sodium arsenite.

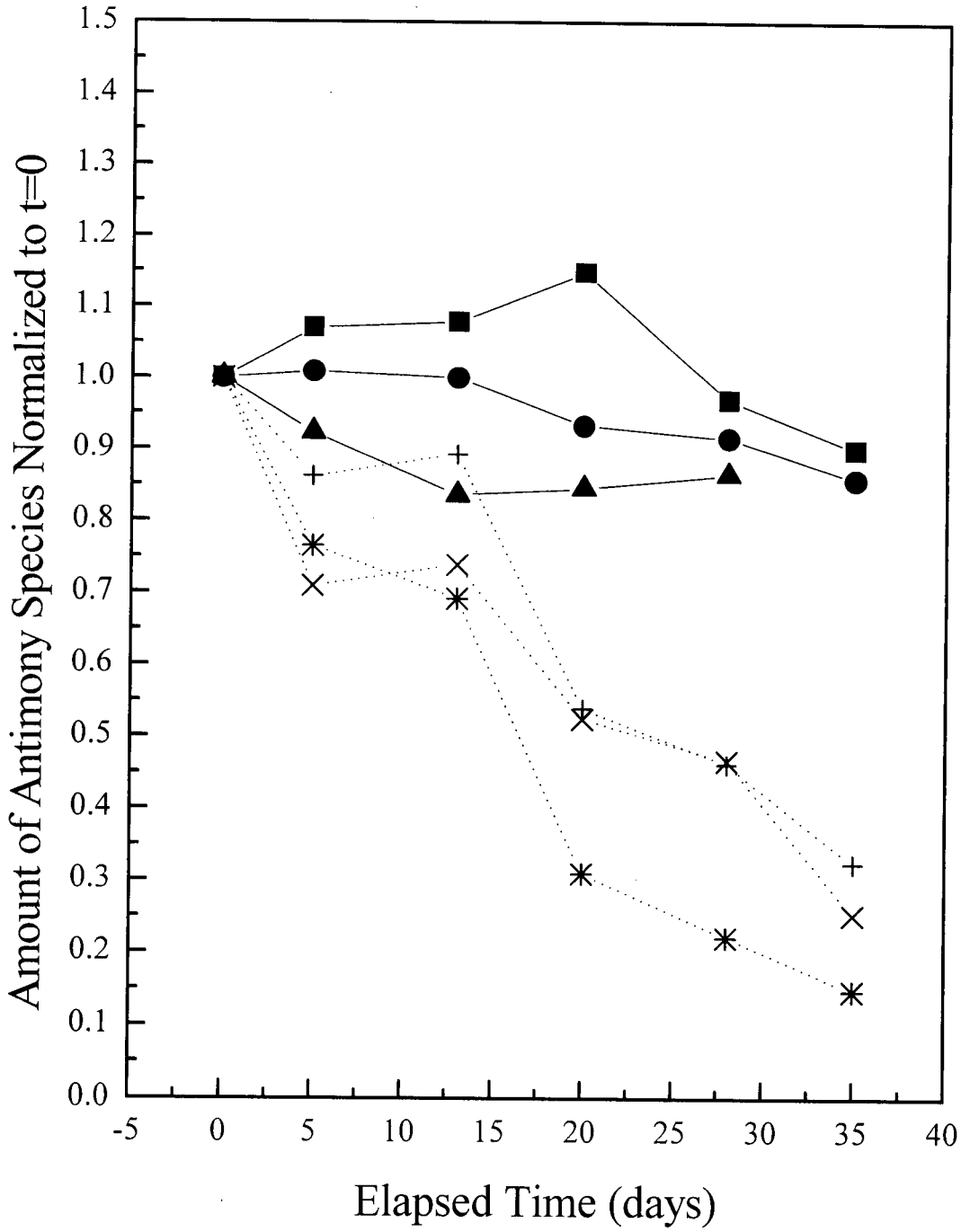

Figure 2.11. Relative (to t = 0) concentrations of antimony(III) and total antimony in cultures of *S. brevicaulis*, from Experiment **5**, over the period of a month: antimony(III), culture 1 (+); antimony(III), culture 2 (X); antimony(III), control (✳); total antimony, culture 1 (■); total antimony, culture 2 (●); total antimony, control (▲);

2.4 SUMMARY

Cultures of *Scopulariopsis brevicaulis* were incubated with potassium antimony(III) tartrate, antimony(III) trioxide, or potassium hexahydroxyantimonate(V) for one month. The contents of the media were determined by using SPE, and HG-GC-AAS. For the first time, nonvolatile dimethylantimony and trimethylantimony species were detected in active cultures of *S. brevicaulis* that had been incubated with inorganic antimony(III) compounds. Alkylantimony species were not detected in media from cultures that were incubated with the antimony(V) compound, potassium hexahydroxyantimonate. Alkylantimony species were not formed in controls (containing: killed cells, medium and antimony compounds; or medium and antimony compounds). The identity of the species was further confirmed by using GC-MS (ion trap). There was no evidence to support the notion that the dimethylantimony species is a product of trimethylstibine oxidation. Furthermore, the possibility that the dimethylantimony species is an analytical artifact was eliminated. Inorganic antimony speciation in active cultures of *S. brevicaulis* was significantly different from inorganic arsenic speciation: Antimony(III) slowly oxidized to antimony(V) and antimony(V) did not undergo any transformation. In contrast, inorganic arsenic(III) species are stable in cultures of *S. brevicaulis* and inorganic arsenic(V) species are rapidly reduced to inorganic arsenic(III) species in cultures of *S. brevicaulis*[23].

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CHAPTER 3

AEROBIC PRODUCTION OF TRIMETHYLSTIBINE BY *SCOPULARIOPSIS BREVICAULIS*

3.1 INTRODUCTION

When cultures of the filamentous fungus *Scopulariopsis brevicaulis* are incubated with inorganic arsenic compounds trimethylarsine is produced[1,2]. There are no major chemical differences between arsenic(III) and antimony(III) that might rule out antimony biomethylation, although antimony(III) is not as nucleophilic as arsenic(III)[3] and the solution chemistries of antimony and arsenic species are different[4] (this could prevent biomethylation by precluding cell uptake). Nevertheless, many have believed that *S. brevicaulis* should be able to biomethylate inorganic antimony species to trimethylstibine. The earliest results, which relied on mass-transport studies, gave indications that *Penicillium notatum* was able to volatilize the antimony(V) compounds potassium antimonate and phenylstibonic acid[5]. However, there was no indication that *S. brevicaulis* could volatilize inorganic antimony compounds[5].

The possibility that microorganisms, especially *S. brevicaulis*, could produce trimethylstibine (or stibines) received renewed attention after Richardson[6] proposed the toxic-gas hypothesis for sudden infant death syndrome (SIDS) (Section 1.5). Initial attempts to detect trimethylstibine in headspace gases of aerobic cultures of *S. brevicaulis* were unsuccessful[7,8]; however, the detection limits in these early studies were greater than 50 ng Sb.

In partially anaerobic systems, such as landfills and sewerage treatment plants, trimethylstibine commonly occurs along with trimethylarsine[9-11]. In these environments, it is likely that trimethylstibine is produced by microorganisms that biomethylate inorganic antimony species, although an abiotic source, such as transmethylation by alkylmetal species, cannot be ruled out. Guyleyuk and coworkers[12] first demonstrated the production of trimethylstibine by microorganisms. They incubated mixed anaerobic cultures, obtained from soils, with potassium antimony tartrate and analyzed headspace gases by using GC-MS. A similar study was performed by Jenkins *et al.*[13] who also detected trimethylstibine in headspace gas samples of anaerobic cultures.

Trimethylstibine was detected, by using GC-MS, in headspace gases of *S. brevicaulis* cultures, under *anaerobic* conditions[14,15], after a period of aerobic incubation with potassium antimony tartrate. Although *S. brevicaulis* is not an anaerobic microorganism, this observation was used as evidence that trimethylstibine is produced in the aerobic phase of growth. It was believed that trimethylstibine was not detected during the aerobic incubation, by using GC methods, because it is rapidly oxidized to nonvolatile species. However, it is also possible that under aerobic conditions trimethylstibine is not produced in sufficient quantities to be detected and under anaerobic conditions nonvolatile trimethylantimony(V) species in the medium, produced during aerobic growth (Chapter 2), are reduced to produce large amounts of trimethylstibine that are readily detected.

As mentioned, some researchers have suggested that trimethylstibine is not detected in aerobic systems because trimethylstibine is rapidly oxidized[8,14,15]. This proposal is based on the conclusions of Parris and Brinckman[16] who performed stability

studies on trimethylstibine, but at high partial pressures. Unfortunately, little is known about the stability of trimethylstibine at environmentally relevant concentrations. Bamford and Newitt[17] studied the gas phase interaction of trimethylstibine with oxygen. Some of the data are shown in Figure 3.1. Their procedure was to allow oxygen into a bulb containing trimethylstibine. In curve (1), the initial departure from linearity signifies *slow* reaction of the gases. Higher pressure of trimethylstibine results in faster reaction, curve (2). Parris and Brinckman[16] used these data to estimate a rate constant for the oxidation. To do this they had to make a number of unjustified assumptions including 'the reaction is homogeneous and second order.' Like many other oxidations, this reaction is probably a chain process, and even though at high pressures trimethylstibine and oxygen can ignite, their interaction at low pressure cannot be predicted because it depends on chain breaking and making reactions, either of which can occur on surfaces.

The fact that landfill gases can be collected in Tedlar bags one day and analyzed successfully for trimethylstibine some days later speaks for the stability of the species[18]. The often-cited conclusions of Parris and Brinckman have little relevance to situations where only trace quantities of trimethylstibine are produced; e.g. from *S. brevicaulis* cultures. If *S. brevicaulis* does produce trimethylstibine then it should be detected under aerobic conditions if sufficiently sensitive analytical methods are used.

The presence of trimethylstibine in headspace gases of aerobic cultures of *S. brevicaulis* was implied by the measurement of antimony in remote nitric acid traps[14,15]. However, antimony transport from cultures could be the result of the formation of other volatile species such as stibine (SbH_3).

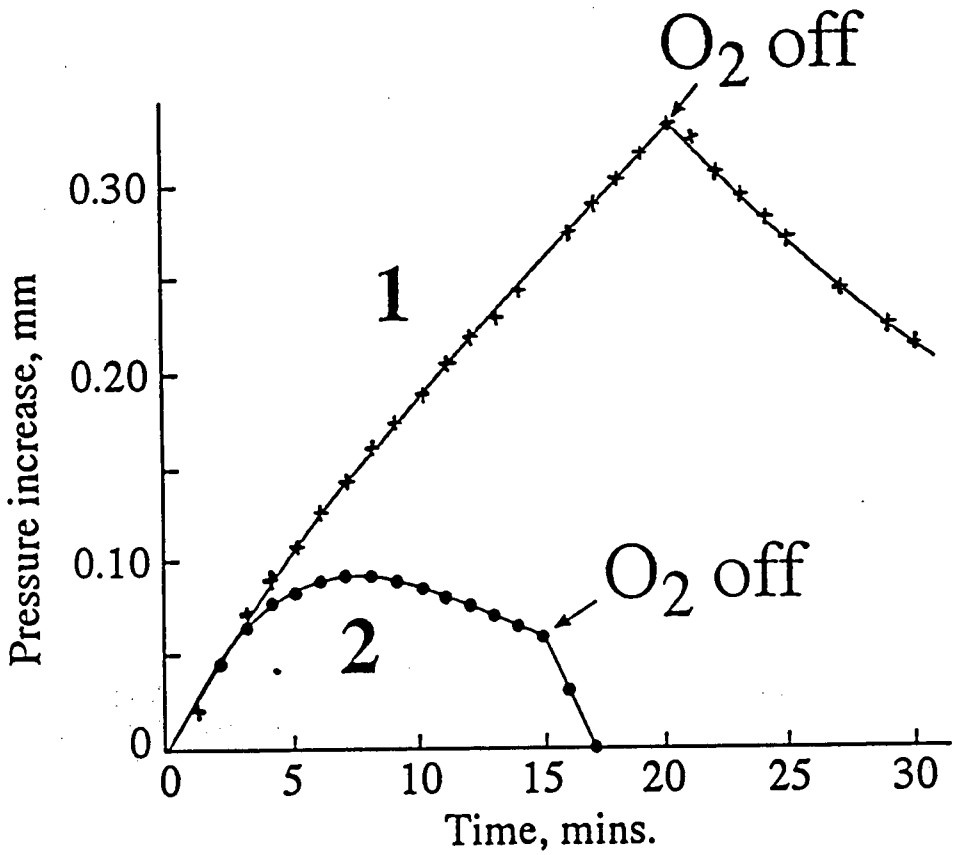

Figure 3.1. Pressure increase versus time for a reaction bulb, which initially contained 0.49 mm (Curve 1) or 0.89 mm (Curve 2) trimethylstibine, and had oxygen bled in at the rate of 0.03 mm/min. (pressure is mm H_2SO_4). From reference 17.

More recently, Craig *et al.*[19] were able to detect trimethylstibine (< 2 – 50 ng Sb) produced by aerobic cultures of *S. brevicaulis* that were incubated with potassium antimony tartrate. Cultures were continuously purged with sterile air and the headspace gases were trapped in Tenax-TA traps at room temperature. Unfortunately, reproducibility was poor; this is most likely because of oxidation of trimethylstibine on the Tenax-TA traps at room temperature over the 5 to 8 days of trapping. The detection limit of their technique was 2 ng Sb.

The aim of the experiments described in this chapter was to assess if aerobic cultures of *S. brevicaulis*, incubated with potassium antimony tartrate, are indeed able to produce trimethylstibine. Trimethylstibine was detected by using one of the most sensitive techniques available, that is GC-ICP-MS (detection limit ~1 pg Sb), and methodology that minimized sample loss by oxidation, that is continuous purging of cultures and trapping at - 78 °C. This work was published in reference 20.

In a preliminary series of experiments, cultures of *S. brevicaulis* were incubated with a variety of antimony compounds and trimethylstibine was only detected sporadically although trimethylarsine was often detected. However, in these experiments, latex tubing was often used for the transfer of gases and it is believed that a significant proportion of trimethylstibine was lost by sorption to the latex. Thus, these preliminary experiments are not described here, but are detailed in reference 21. The apparatus used in the experiments described here was constructed entirely of glass and Teflon.

3.2 EXPERIMENTAL

3.2.1 Reagents

All reagents were of analytical grade or better and obtained from common distributors. Nitric acid (1% w/w) was prepared by dissolving an appropriate volume of concentrated nitric acid (Seàstar) in deionized water. A rhodium internal standard solution (10 ng Rh/mL) was prepared by diluting rhodium trichloride (1 mg Rh/L) in to 1% w/w nitric acid. Standards of antimony (25 ng Sb/mL) and arsenic (25 ng As/mL) were prepared by diluting potassium hexahydroxyantimonate (1 mg Sb/L) and sodium arsenate (1 mg As/L) solutions, in to the rhodium/nitric acid solution.

3.2.2 Details of *Scopulariopsis brevicaulis* Cultures

Medium (400 mL minimal-salts/glucose (Section 2.2.1)) was seeded with 40 mL of *S. brevicaulis* (ATCC #7903) mycelial balls (20-30 balls, ~1 mm diameter). The cultures were maintained in 1 L Erlenmeyer flasks capped with cotton stoppers. After one month of incubation (26 °C on a rotary shaker), the medium was decanted off the cultures and 400 mL of freshly prepared medium added. The potassium antimony tartrate (or trimethylantimony dichloride in the case of Experiment **16**/Culture 3) was added (0.2 μm filter sterilized) (Table 3.1). Each culture was transferred to a 1 L Erlenmeyer flask topped with a ground glass male joint (Pyrex #4980, stopper #9) and capped with a female ground glass joint (40/38) that was fitted with an inlet glass tube (this reached well below the surface of the medium) and a short length of outlet tubing (glass).

The Erlenmeyer flasks were shaken horizontally (~135 rpm, 1.75 inch displacement), maintained at 26 °C, and keep in the dark to minimize possible degradation of trimethylstibine by UV light. At the end of the experiments the average culture dry weight was estimated, by filtering off the biomass, then rinsing the biomass with water (no effort was made to completely remove all medium) and drying at 150 °C. The average dry weight was 2.5 g (range: 2.2 - 2.9 g).

Table 3.1. Experimental Details.

Expt.	Culture	Contents of Culture	Sample time(duration) Day(hours)	Comments
16	1	1000 mg Sb/L as PAT [a]	6(20), 7(21), 10(12), 15(6)	Analyzed medium
	2	1000 mg Sb/L as PAT	6(20), 7(21), 10(12), 15(6)	Analyzed medium
	3	1 mg Sb/L as Me_3SbCl_2	6(20), 7(21), 10(12), 15(6)	
	4	Control - No added Sb	6(20), 7(21), 10(12), 15(6)	
17	1	1000 mg Sb/L as PAT	5(12.5), 7(10), 9(12), 16(11), 18(12)	
	2	1000 mg Sb/L as PAT	5(12.5), 7(10), 9(12), 16(11), 18(12)	

[a]PAT=potassium antimony tartrate

3.2.3 Details of Headspace Gas Sampling

The inlet glass-tube of the Erlenmeyer flask was connected, via a filter (0.2 μm Supor Acrodisc 25, Gelman Sciences), to a cylinder of compressed air. The outlet tubing was connected to another 0.2 μm filter. The inlet filter prevents contamination of the cultures, whereas the outlet filter prevents the introduction of antimony containing aerosols into the traps. Throughout the experiment, there was a constant flow of compressed air through each flask (~10 mL/min). At various stages (Table 3.1) of the experiment a U-shaped glass trap (22 cm by 6 mm o.d.) was connected via PTFE tubing to the outlet filter. The U-tube trap was packed with 10% Supelcoport SP-2100 on Chromosorb (45 - 60 mesh); a small silanized glass-wool plug held the packing material in place. The U-tube trap was cooled in a large dry-ice/acetone filled Dewar flask (- 78 °C). The traps were left in place for 6 hours or more (see Table 3.1 for sampling times). Gas flow rates were measured at the beginning and at the end of sampling and did not significantly change.

The sampling-traps were capped with rubber septa, and stored at - 78 °C, in a dry-ice freezer or in a dry-ice/acetone bath, until analysis. The contents of the sampling traps were determined by using GC-ICP-MS (Section 3.2.5).

3.2.4 Determination of the Efficiency of the Gas Trapping Procedure

In order to check the stability of trimethylstibine in the continuous trapping system, trimethylstibine (~ 3 ng Sb, total) was slowly bubbled (1 hour) through medium containing *S. brevicaulis* and then trapped. The trimethylstibine was generated by sodium

borohydride reduction of trimethylantimony dichloride in an autosampler vial, and a sample taken using a gas syringe. The trimethylstibine was injected into the system via a rubber septum on a T-piece placed in the gas line directly after the cylinder of compressed air. The configuration of trap, flask, and transfer line was exactly the same as in all of the other experiments.

In a second experiment, the T-piece was placed directly before a trap and the same amount of standard injected rapidly. The contents of the traps were determined by using GC-ICP-MS (Section 3.2.5), and the recovery of trimethylstibine was defined as the ratio of trimethylstibine measured in the first trap to that measured in the second trap.

3.2.5 GC-ICP-MS

The GC-ICP-MS system (Figure 3.2), described by Feldmann[22], was used for determining volatile organometallic compounds in the sample-traps.

The 3-way valve was placed in the vent position so that sudden pressure changes during the following processes would not extinguish the plasma. A U-shaped sample-trap, cooled in a Dewar of dry-ice/acetone (- 78 °C), was connected to the system at position 1 via PTFE Swageloks. Helium (70 mL/min) flowed through the sample trap and into a second trap, at position 2. The second trap, at position 2, was cooled in liquid nitrogen. The sample-trap was removed from the dry-ice/acetone bath, and gently warmed with nichrome wire wrapped around the trap and connected to a variac. Thus, the volatile compounds in the sampling trap were cryo-focused onto the second trap.

After the contents of the sampling trap had been cryo-focused onto the second trap, the 3-way valve was switched to the "inject" position. Then the second trap was removed from the liquid nitrogen, the ICP-MS acquisition was started, and heating of the second trap, with nichrome wire, commenced.

The connection units and the transfer line consisted of Teflon. The heated transfer line was connected to a T-piece. This T-piece was substituted for the elbow, which normally connects the spray chamber of the ICP-MS with the torch. In this way, gas samples were mixed with a nebulized internal standard solution (10 ng Rh/mL in 1% nitric acid) before entering the torch of the ICP-MS. The operating parameters for the ICP-MS are given in Table 3.2. The following channels were sampled during acquisitions: m/z = 75 (As^+), 77 ($Ar^{37}Cl^+$), 103 (Rh^+), 121 (Sb^+), 123 (Sb^+). Only the m/z = 121 channel was used for antimony quantification. The m/z = 77 channel was monitored during the acquisition in order to identify artifact peaks in the arsenic chromatogram produced by $Ar^{35}Cl^+$, arising from volatile chlorine species (e.g. CH_3Cl).

3.2.5.1 Identification of Arsines and Stibines

The arsines and stibines detected in the traps were identified by comparing retention times of standard arsines, or stibines, against the retention times of compounds in the sample. Trimethylarsine and trimethylstibine were generated by sodium borohydride reduction of trimethylarsine oxide and trimethylantimony dichloride, respectively.

Table 3.2. Operating parameters for ICP-MS (VG Plasmaquad PQ2 Turbo)

Forward radio-frequency power	1350 W
Reflected Power	<10 W
Coolant gas flow rate	13.8 L/min
Auxiliary gas flow rate	0.65 L/min
Nebulizer	De Galan
Nebulizer gas flow rate	1.00 L/min
Spray Chamber	Scott, water cooled 4-6 °C
Analysis mode	time resolved, 1 s time slice
Expansion chamber pressure	2 mbar
Intermediate chamber	$<1 \times 10^{-4}$ mbar
Analyzer chamber	2×10^{-6} mbar

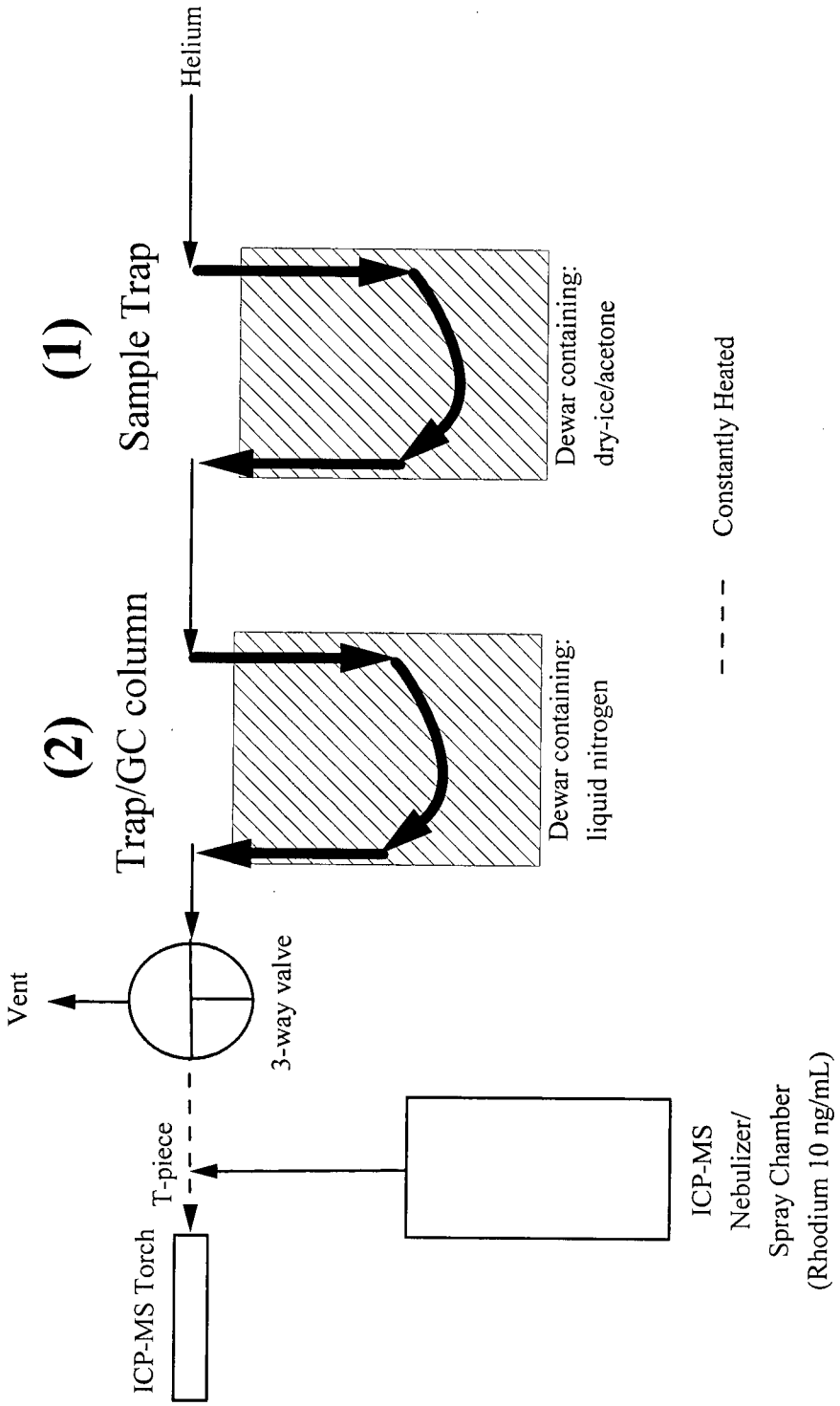

Figure 3.2. GC-ICP-MS apparatus.

3.2.5.2 Semi-quantification of Organometallic Gases

A one-point calibration/internal standard method, previously described by Feldmann[22], was used for semi-quantification of the organometallic gases.

A relative sensitivity factor (RSF_E) for each element (E = As or Sb) was determined. A solution of nitric acid (1% w/w), containing arsenic (25 ng As/mL), antimony (25 ng Sb/mL) and rhodium (10 ng Rh/mL), was continuously nebulized into the plasma. After 5 minutes had elapsed (rinsing), the drain solution from the nebulizer was collected in a container (previously weighed) and simultaneously signal acquisition was started. The drain solution was collected and signals acquired for 10 minutes. The weight of drain solution was determined and used to determine the volume. Similarly, the weight of standard solution taken up over the 15 minute period was determined and converted into a volume. The uptake (U) and drain (D) rates (mL/min) for the nebulizer were then calculated. The concentration of each element in the drain solution ($[E]_D$, ng E/mL) was determined and so the mass transfer rate of each element to the plasma (M_E, ng E/min) could be calculated as follows:

$$M_E = (25 \times U) - ([E]_D \times D) \qquad M_{Rh} = (10 \times U) - ([Rh]_D \times D)$$

The average signal intensities, over the 10 minute period, for each element (I_E, cps) were used to determine the RSF for each element as follows (the intensity of the blank was insignificant in this case):

$$RSF_E = \frac{I_E}{I_{Rh}} \times \frac{M_{Rh}}{M_E}$$

After the RSFs had been determined, the gas samples were analyzed while the internal standard of rhodium (10 ng Rh/mL) was continuously nebulized into the plasma. The peak areas (A_E, counts) for the gaseous species were then measured using Origin Microcal's integration function. For each GC-ICP-MS acquisition the average signal for rhodium was determined (I_{Rh}, cps). The amount of metalloid (m_E, ng) for each GC peak could then be quantified by applying the following formula:

$$m_E = \frac{A_E}{I_{Rh}} \times \frac{M_{Rh}}{RSF_E} \times \frac{1}{60}$$

3.3 RESULTS

3.3.1 Efficiency of the Gas Trapping Procedure

In the experiments designed to determine the efficiency of the sampling procedure approximately 80% of the antimony added as trimethylstibine was recovered. Thus, the trapping appears efficient and loss of trimethylstibine is minimal. The concentration of trimethylstibine employed in this recovery experiment was much greater than that seen in any of the sampling experiments, so the rate of oxidation should have been significantly higher in this experiment. It is presumed that once trimethylstibine is condensed in the Supelcoport trap (- 78 °C) it is not susceptible to oxidation. Hence, in a continuous trapping system where trimethylstibine is formed and flushed from the Erlenmeyer in less than an hour, the duration of the recovery experiment, the trimethylstibine should be readily detected. Although oxidation may occur it is doubtful that it would remove a significant amount of trimethylstibine.

3.3.2 Production of Trimethylstibine by Cultures of Scopulariopsis brevicaulis

Trimethylstibine and trimethylarsine were identified in all headspace gas samples taken during Experiments **16** and **17** in the following ways:

1. The retention times of the volatile antimony and arsenic compounds in the samples matched the retention times of the standard trimethylstibine, and trimethylarsine, produced by sodium borohydride reduction of trimethylantimony dichloride and trimethylarsine oxide, respectively.
2. There was no disturbances in the ICP-MS plasma at these retention times as determined by monitoring the rhodium internal standard (Figure 3.4).
3. There was no response for the m/z = 77 channel ($Ar^{37}Cl^{+}$) (Figure 3.4).
4. The average ratio of peak areas for m/z = 121 to m/z = 123 was determined to be 1.3 (c.f. the expected natural abundance ratio for $^{121}Sb/^{123}Sb$ of 1.34).

Results are summarized in Table 3.3. All samples, taken over the two weeks, in Experiment **16** gave a significant peak (average S/N ~1000) in the GC-ICP-MS chromatogram. This preliminary data was not quantified. The average response for trimethylstibine in the culture containing trimethylantimony dichloride was about six times more than that for the cultures containing potassium antimony tartrate.

Table 3.3. Summary of results from the analysis of headspace gases obtained from cultures of *S. brevicaulis* that were incubated with antimony compounds.

Expt.	Culture	Contents of Culture	Results	Comments
16	**1**	1000 mg Sb/L as PAT	Me_3Sb detected	Medium: 2.5 ng Sb/mL as trimethylantimony species
	2	1000 mg Sb/L as PAT	Me_3Sb detected	Medium: 4 ng Sb/mL as trimethylantimony species
	3	1 mg Sb/L as Me_3SbCl_2	Me_3Sb detected	
	4	Control - No added Sb	TRACE of Me_3Sb detected	
17	**1**	1000 mg Sb/L as PAT	10 ng Sb as Me_3Sb + 20 ng As as Me_3As	
	2	1000 mg Sb/L as PAT	10 ng Sb as Me_3Sb + 20 ng As as Me_3As	
--	--	*System blank*	No Me_3Sb detected No Me_3As detected	

In Experiment **17**, the two cultures containing potassium antimony tartrate produced trimethylstibine that was detected in all headspace gas samples (Table 3.3). The GC-ICP-MS peak areas were similar to those seen in Experiment **16**.

In the control culture (potassium antimony tartrate not added), only traces of trimethylstibine were detected. No volatile antimony or arsenic compounds were detected in the system blank; this blank sample was obtained by passing compressed air through the tubing and filters and then through the cold (- 78 °C) U-tube trap (the Erlenmeyer flask of culture was omitted). The absolute detection limit for the GC-ICP-MS was ~1 pg Sb.

A typical single ion chromatogram for antimony (m/z = 121), for a headspace gas sample, from an active culture grown in the presence of potassium antimony tartrate, is shown in Figure 3.3. Also shown is the antimony chromatogram for a gas sample taken at the same time from the control culture that did not contain potassium antimony tartrate.

In all these experiments, significant amounts of trimethylarsine were detected, even though no inorganic arsenic compounds were added to the medium. Typical single ion chromatograms, for a single trap, for arsenic and antimony, are shown in Figure 3.4. Although similar amounts of trimethylstibine and trimethylarsine were detected, it is important to realize that no arsenic was added to these cultures and the concentration of antimony in the cultures was 1000 mg Sb/L. The arsenic that is volatilized must arise from impurities in the medium (the concentration of arsenic in the medium was < 2 ng As/mL). When expressed as a percentage of inorganic metalloid volatilized it is apparent that arsenic (> 3% volatilized) is much more readily volatilized than antimony (0.0000025% volatilized), or, to state it another way, arsenic volatilization by *S. brevicaulis* is at least one million times more efficient than antimony volatilization in this case.

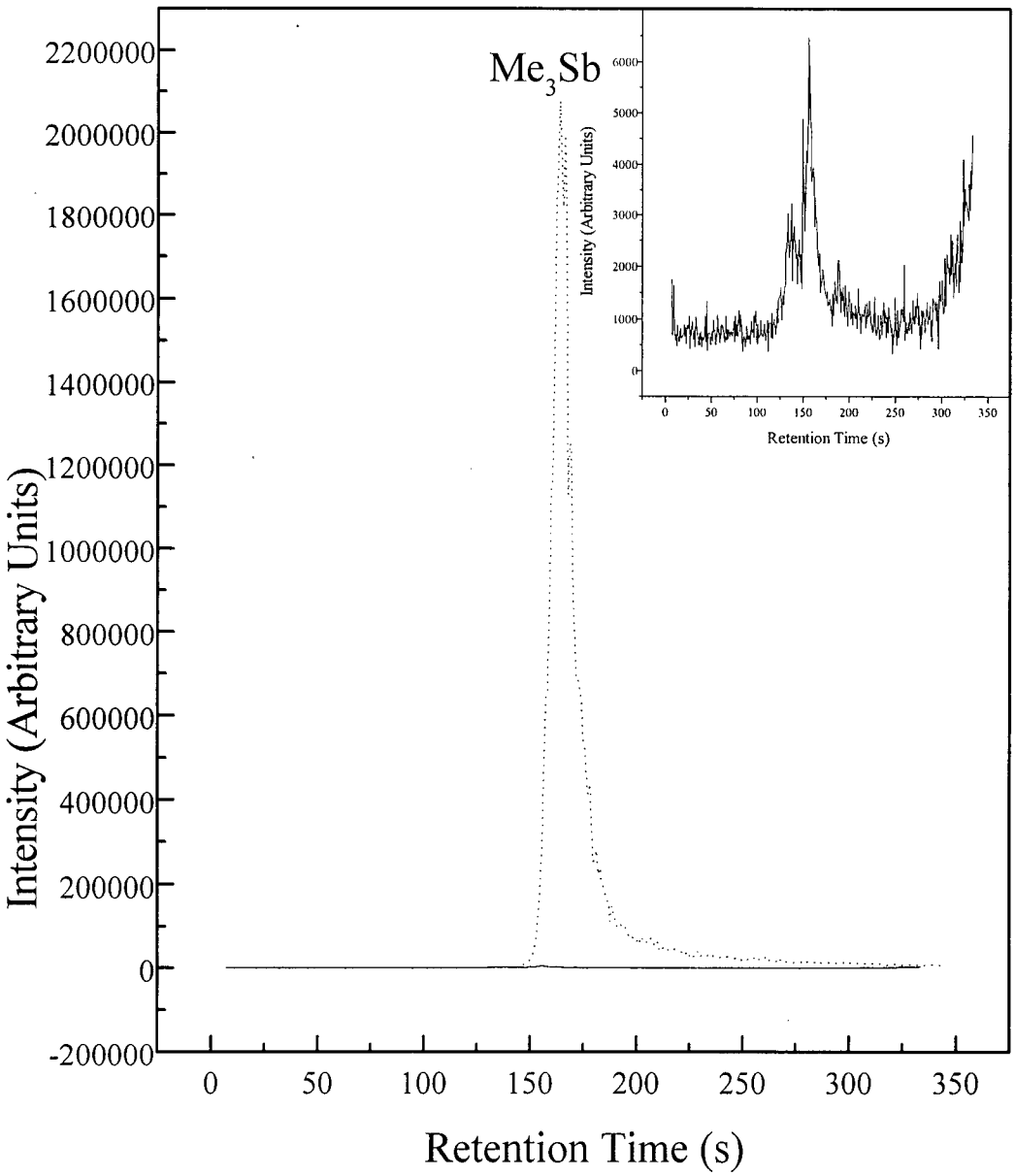

Figure 3.3. GC-ICP-MS single ion chromatogram for antimony (m/z = 121) for culture headspace samples taken six days into Experiment **17**.

..... Sample taken from Culture 1, containing potassium antimony tartrate.

——— Control sample taken from Culture 3, which did not have potassium antimony tartrate added. Inset: zoom plot of control sample.

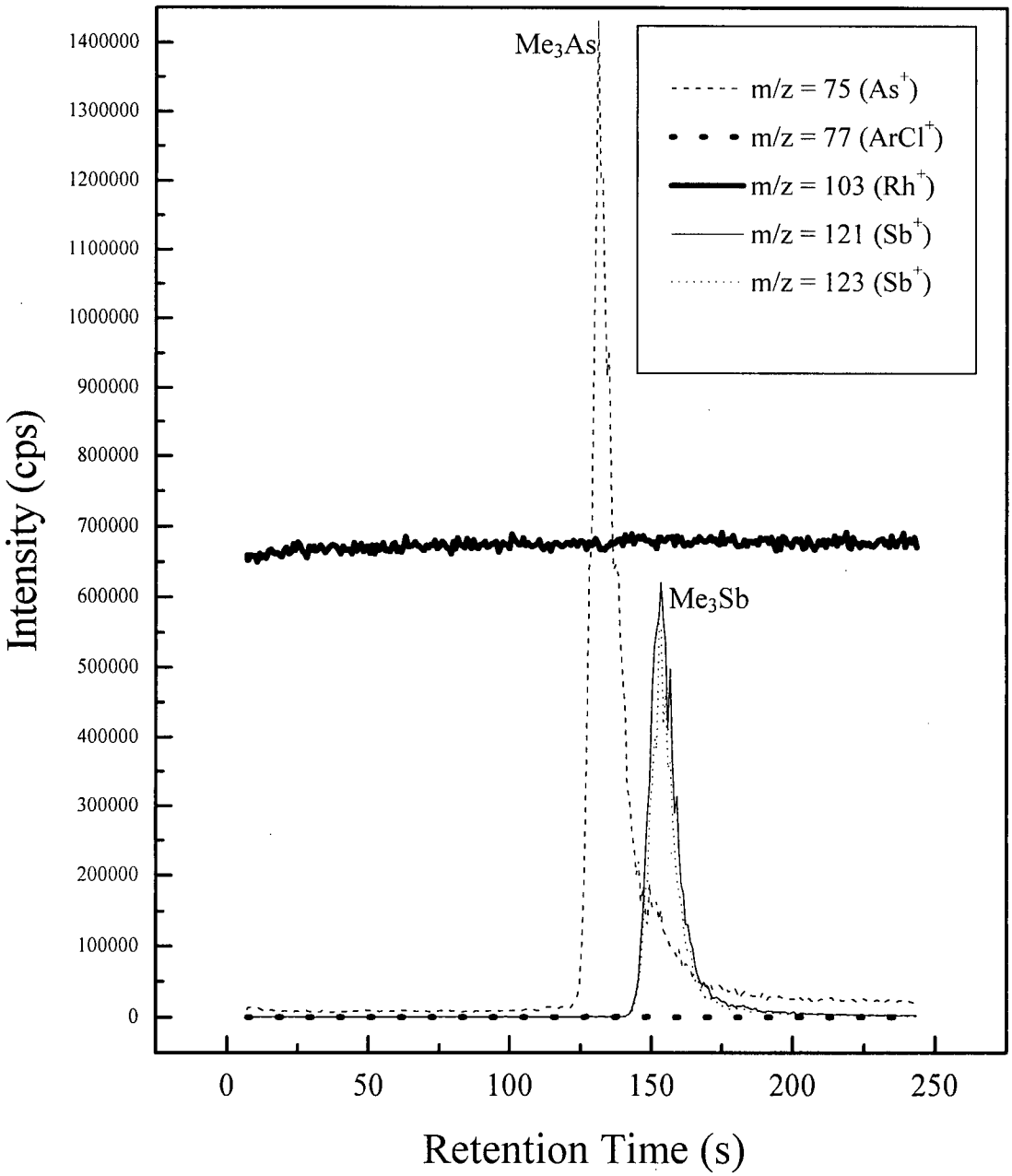

Figure 3.4. GC-ICP-MS single ion chromatograms from a headspace sample taken from Culture 1 of Experiment **17** after 5 days.

Table 3.4. Sampling times, amounts of trimethylarsine and trimethylstibine trapped, and amounts trapped per hour of sampling, for the cultures in Experiment **17** containing 1000 mg Sb/L.

Elapsed Time (days)	**Sampling Time (Hours)**	**Amount of Trimethyl-arsine In Trap (pg As)**	**Amount of Trimethyl-stibine In Trap (pg Sb)**	**Trimethyl-arsine (pg As/hour)**	**Trimethyl-stibine (pg Sb/hour)**
Culture 1					
5	12.5	925	525	74	42
7	10	570	130	57	13
9	12	528	180	44	15
16	11	352	176	32	16
18	12	360	60	30	5
Culture 2					
5	12.5	750	538	60	43
7	10	480	260	48	26
9	12	564	204	47	17
16	11	660	143	60	13
18	12	720	156	60	13

In Experiment **17**, the rates of production of trimethylstibine and trimethylarsine were determined (Table 3.4) and by extrapolating this information over the entire experiment a time-course for cumulative trimethylstibine produced was constructed (Figure 3.5). It was assumed that the rate of trimethylstibine production between taking samples is the same as that during sampling. The period of maximum trimethylstibine production was near the beginning of the experiment. This is probably because the use of a large amount of biomass rapidly depleted the medium, and so the rate of growth

significantly decreased after a few days. For both experimental replicates approximately 10 ng Sb as trimethylstibine was produced over the course of the entire experiment (18 days). The actual amount produced may be higher than this if sampling is inefficient or if trimethylstibine is oxidized. The oxidation of trimethylstibine is unlikely to be significant at these low concentrations; thus, the amounts of trimethylstibine produced are unlikely to be much greater than that estimated.

3.3.3 Comparison of Amounts of Trimethylstibine and Nonvolatile Alkylantimony Species Produced

The average concentration of trimethylantimony species found in the medium of *S. brevicaulis* cultures that were incubated with potassium antimony tartrate or antimony trioxide for one month is 4 ng Sb/mL (Chapter 2). Thus, for 400 mL of medium the total amount of nonvolatile trimethylantimony species produced over one month is approximately 200 ng Sb. This is significantly more than the 10 ng Sb as volatile trimethylstibine that was produced over 18 days in these experiments. Therefore, the predominant products of antimony biomethylation by *S. brevicaulis* are nonvolatile dimethylantimony and trimethylantimony species (~ 95%). The final reduction product in the biomethylation sequence, trimethylstibine, is only a minor product (~ 5%). This is not unusual; in the case of arsenic, when the concentration of substrate sodium arsenite is $\leq$ 1 mg As/L the predominant product is nonvolatile trimethylarsine oxide that is found in the medium[23].

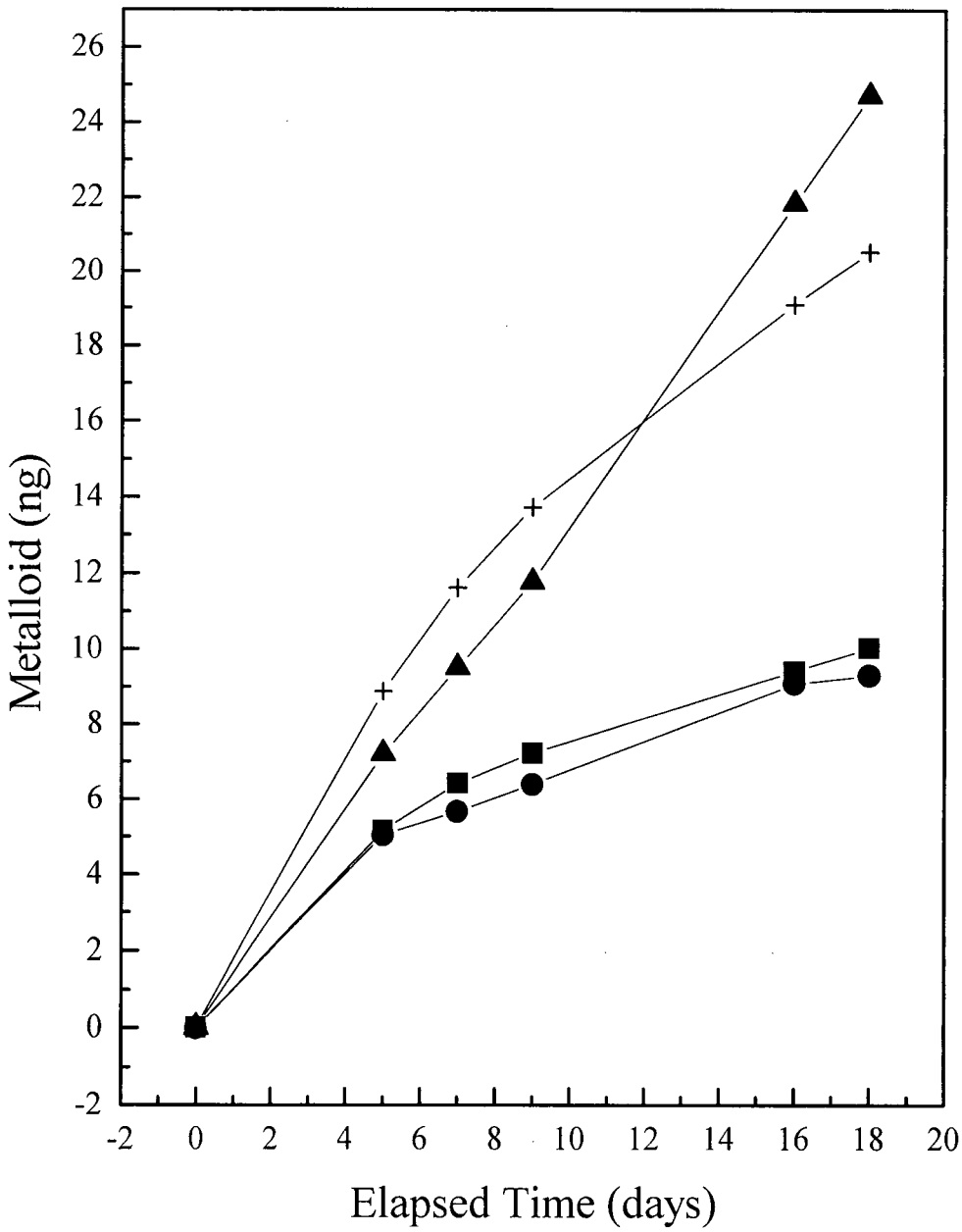

Figure 3.5. Time-course for the production of trimethylstibine and trimethylarsine from two cultures of *S. brevicaulis* containing 1000 mg Sb/L as potassium antimony tartrate (Experiment **17**). The estimated total amount of volatile metalloid produced (between t=0 and the time of sampling) is plotted against time.

+ Trimethylarsine culture 1
▲ Trimethylarsine culture 2
● Trimethylstibine culture 1
■ Trimethylstibine culture 2.

3.3.4 Comparison with Other Results

Previous researchers had been unable to reproducibly detect trimethylstibine in the headspace of antimony rich *S. brevicaulis* cultures[7,8]. It is now evident that in these studies, the detection limits (> 50 ng Sb) for the methodologies used were much higher than what we have found to be required (10 ng Sb, if sampling over 18 days).

Jenkins *et al.*[14,15] described a study where "trimethylstibine" was detected by trapping in remote nitric acid traps. The levels produced ranged from 260 to 4107 ng Sb over ~12 days, depending on the substrate. This quantity is significantly higher than the 10 ng Sb reported here. Differences in fungal strain and medium make comparisons of results difficult (i.e. Jenkins *et al.*[14,15] used *S. brevicaulis* IMI 17297 in malt extract broth; in this research *S. brevicaulis* ATCC 7903 in minimal-salts/glucose medium was used). However, the surprisingly high levels of volatile antimony measured, which also markedly differ from other results that this group obtained[19], may be the result of the production of stibines other than trimethylstibine (e.g. SbH_3).

During the course of the present studies, Craig *et al.*[19] performed similar experiments to the ones detailed here. That is, *Scopulariopsis brevicaulis* (IMI 17297) was incubated in minimal-salts/glucose medium (100 mL) that contained potassium antimony tartrate (20-120 mg Sb/L) and the cultures were continuously purged with sterile air, and over 5, or 8, days of incubation volatile species were trapped on Tenax TA. After 8 days of incubation at 28 °C, the dry-weights of the cultures were between 0.9 and 1.2 g. Analysis of gaseous species, trapped on Tenax TA at room temperature, was performed by using GC-AAS, or GC-MS. For the 5 day incubation, trimethylstibine

(25-50 ng) was detected in two of the three experiments performed. GC-MS analysis was used to confirm the identity of trimethylstibine isolated from 5-day culture headspace gases. Details on how quantification was performed were not supplied. For the 8 day incubation, trimethylstibine (5 ng) was detected in only one of the four experiments performed. The large variation in the amounts of trimethylstibine trapped was attributed to the low stability of trimethylstibine on Tenax-TA at room temperature. However, these results are reassuring since partial stability of trimethylstibine at room temperature infers that at - 78 °C the species would be stable. Furthermore, the amounts of trimethylstibine they detected are not significantly different from the amounts detected in this research.

3.4 CONCLUSIONS AND FUTURE WORK

Trimethylstibine (10 ng Sb) was detected in aerobic cultures of *S. brevicaulis* that were incubated with potassium antimony tartrate. Similar quantities of trimethylarsine were detected even though no arsenic was added to the medium. It is possible that the quantities of trimethylstibine are underestimated if trimethylstibine is lost by oxidation or sorption, but the recovery studies, and other results, suggest that this is unlikely. Nevertheless, it should be possible to verify this conclusion. One reliable method of doing this would be to spike the continuous flow of compressed air flowing through the cultures with a continuous flow of trimethylstibine. By varying the spike flow a standard additions curve could be calculated. This approach, although difficult, would have the advantages of accounting for losses due to oxidation, sorption and inefficient trapping. It would also eliminate errors that may arise from the use of aqueous solutions to calibrate gaseous samples.

One of the reasons for research into the volatilization of antimony by *S. brevicaulis* has been the hypothesis associating antimony volatilization with sudden infant death syndrome[6]. The present results, subsequently confirmed by other researchers[19], show that trimethylstibine is produced by aerobic cultures of *S. brevicaulis* but detection of the quantities produced requires the most sensitive analytical methodology available. In the reported experiments, it was necessary to use large amounts of biomass and substrate to obtain detectable amounts of trimethylstibine. The finding of trimethylstibine in these ideal experiments offers little support for the notion that enough trimethylstibine could be produced in a cot environment to be of harm to a child. This same conclusion was also reached by the Limerick committee[24], after performing much more extensive research.

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CHAPTER 4

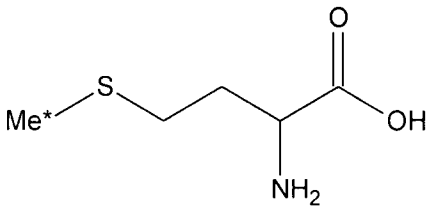
THE BIOMETHYLATION OF ANTIMONY AND ARSENIC BY *SCOPULARIOPSIS BREVICAULIS*: D-METHIONINE AND L-METHIONINE AS METHYL DONORS

4.1 INTRODUCTION

The mechanism of arsenic biomethylation, first proposed by Challenger[1], involves a series of oxidative-methylation and reduction steps (Figure 1.1). At the heart of this mechanism is the oxidative addition of a methyl carbocation to nucleophilic arsenic(III) species; the subsequent reduction of the arsenic(V) product allows the cycle to continue. Antimony and arsenic have similar redox properties. Antimony(III) species are not as nucleophilic as arsenic(III) species[2], but there are no significant chemical reasons why antimony should not be biomethylated in the same way as arsenic. In this chapter, one aspect of the arsenic and antimony biomethylation mechanism is examined, that is the source of the methyl group; this research was done primarily to see if arsenic and antimony are biomethylated in a similar manner.

To support his proposed mechanism, and to establish the source of the methyl group in arsenic biomethylation, Challenger[1] isolated trimethylarsine from cultures of *S. brevicaulis*, incubated with $^{14}CH_3$-D,L-methionine (Figure 4.1) and sodium arsenite. The trimethylarsine contained significant levels of ^{14}C, thus Challenger demonstrated that the carbon in the methyl groups, and probably the methyl groups, in arsenic biomethylation come from methionine. Subsequently, Cullen *et al.*[3] demonstrated that

the methyl group of L-methionine is transferred *intact* (as opposed to transfer of, for example, CH_2), by addition of CD_3-L-methionine to a culture and trapping the evolved trimethylarsine which was then introduced directly into a mass spectrometer. The mass spectrum showed that the trimethylarsine contained significant amounts of CD_3. These authors suggested that the biological methyl donor is S-adenosylmethionine (SAM). Most recently, Cullen *et al.*[4] performed experiments with cultures of a yeast, *Cryptococcus humicolus* (which was then classified as *Apiotrichum humicola*, but when first isolated it was classified as *Candida humicola*), containing CD_3-L-methionine and sodium arsenite. After incubation, analysis of the culture medium by using HG-GC-MS demonstrated that CD_3 was present in nonvolatile dimethylarsenic(V) and trimethylarsenic(V) species. These nonvolatile species should be found in the medium according to Challenger's mechanism for arsenic biomethylation. Since L-methionine is the precursor for S-adenosylmethionine, these experiments imply that SAM is the source of the methyl groups. Nonvolatile alkylarsenic species were detected by Cullen *et al.* in medium of *S. brevicaulis* cultures, however experiments to determine the methyl source were not carried out.



*Me-L-methionine

*Me = CD_3 (Cullen *et al.*[3]), $^{14}CH_3$ (Challenger[1]) or $^{13}CD_3$ (this work).

CD_3-D-methionine (this work)

Figure 4.1. Substrates used to probe the methyl source in biomethylation.

For both antimony and arsenic, biomethylation may either be enzymatic, or non-enzymatic. Any difference in the ability of D-methionine to act as methyl donor, compared with L-methionine, would arise if the biomethylation process were stereospecific, which is most likely if an enzyme is involved. Mammalian methyltransferase enzymes, that perform arsenic biomethylation, have been isolated[5].

In this chapter, experiments are described that were performed to determine the incorporation of labeled methyl groups from $^{13}CD_3$-L-methionine and CD_3-D-methionine into alkylantimony and alkylarsenic species during biomethylation by *S. brevicaulis*. HG-GC-MS was used to determine if labeled groups were present in trimethylantimony and trimethylarsenic species produced over one month of incubation of *S. brevicaulis* with inorganic arsenic(III) or antimony(III) compounds, and D-methionine or L-methionine. Labeling with $^{13}CD_3$ or CD_3 was chosen, rather than ^{14}C labeling, both for convenience and to distinguish intact methyl transfer from a possible transfer of CH_2. From the MS perspective, $^{13}CD_3$ is a better label than CD_3 because extra peaks that arise in the MS due to the presence of a label are most easily distinguished from interfering fragments the greater the mass increase on label incorporation; this is especially true for antimony because of its unique isotope distribution. Unfortunately, this label was only available for L-methionine.

4.2 EXPERIMENTAL

4.2.1 Reagents

All reagents were of analytical grade or better and obtained from common distributors, except where noted. $^{13}CD_3$-L-methionine (methyl: ^{13}C 90 atom %; D 98 atom %) was obtained from BOC Prochem (Deer Park Rd., London, UK). CD_3-D-methionine (90% CD_3) was kindly synthesized by Changqing Wang by using a literature method[6]. Stock solutions of sodium arsenite (1000 mg As/L) and potassium antimony tartrate (5000 mg Sb/L) were freshly prepared by dissolving the appropriate amount of solid in deionized water.

4.2.2 Details of *S. brevicaulis* Cultures

Submerged cultures of *S. brevicaulis* (ATCC #7903) mycelial balls were prepared by adding 100 mL of a seed culture to 300 mL of minimal-salts/glucose medium (Section 2.2.1) in 1 L Erlenmeyer flasks.

For cultures 5 and 6 of Experiment **6** (nonliving controls): cultures of *S. brevicaulis*, which had been incubated in 400 mL of medium for a month, were autoclaved, the old medium decanted off, and 300 mL of fresh medium added.

Appropriate amounts of stock solutions of potassium antimony tartrate and sodium arsenite were added to the cultures, via 0.2 μm syringe filters, to give the concentrations specified in Table 4.1. For each culture the appropriate methionine compound (0.1 g), as detailed in Table 4.1, was dissolved in 10 mL of water and added

via a syringe filter (0.2 μm). The Erlenmeyer flasks were shaken horizontally (~135 rpm, 1.75 inch displacement) and maintained at 26 °C for 1 month. After incubation, the cultures were autoclaved (20 minutes, 121 °C, 19 psi) and analyzed as detailed in Section 4.2.3.

Table 4.1. Contents of cultures.

Expt.	Cultures	Potassium Antimony Tartrate	Sodium Arsenite	Methionine
6	**1 – 2**	100 mg Sb/L	Not added	0.1 g $^{13}CD_3$-L-methionine
6	**3 – 4**	100 mg Sb/L	Not added	Not added
6	**5 – 6**[a]	100 mg Sb/L	Not added	0.1 g CH_3-L-methionine
7	**1 – 2**	100 mg Sb/L	Not added	0.1 g $^{13}CD_3$-L-methionine
7	**3 – 4**	100 mg Sb/L	Not added	0.1 g CD_3-D-methionine
7	**5 – 6**	Not added	10 mg As/L	0.1 g CD_3-D-methionine
7	**7 – 8**	Not added	10 mg As/L	0.1 g $^{13}CD_3$-L-methionine

[a] – Cultures 5 and 6 in experiment 6 were nonliving controls that contained: autoclaved *S. brevicaulis* biomass, medium, potassium antimony tartrate and methionine.

4.2.3 Details of Analysis

HG-GC-MS was used to analyze medium, after inorganic antimony(III) and arsenic(III) species were removed by using SPE (Section 2.2.5.1). Two approaches were used to perform hydride derivatization in preparation for GC-MS. When the concentration of species was known to be high (as was the case for alkylarsenic species) the sample was derivatized in septa-capped vials and the vial headspace was injected into the GC-MS. When a sample contained low concentrations of species (as was the case for antimony) the semi-continuous flow HG-GC-AAS system (Section 2.2.5.2) was used to generate a large quantity of hydrides that were obtained as fractions from the GC column.

4.2.3.1 HG-GC Fraction Collection

HG-GC was performed as described in Section 2.2.5.2, but the AAS was disconnected from the system. The gaseous effluent that eluted from the GC column was collected as fractions in evacuated, septa capped (PTFE faced silicon, 16 mm, Supelco), 15 mL vials. The flow rate from the column was 40 mL/min, so one fraction was taken every 20 seconds. To obtain sufficient levels of trimethylstibine and dimethylstibine in the collected fractions generally 10 to 20 mL of culture medium needed to be used in a HG-GC run. One run was also performed where the effluent from the GC column, of the HG-GC system, was analyzed by using an atomic absorption spectrometer as the detector (HG-GC-AAS). This was done to measure the retention times of dimethylstibine and trimethylstibine, to determine in which fractions these compounds would be found.

4.2.3.2 GC-MS (ion trap)

The operating conditions of the GC-MS are given in Table 4.2. The injection volume was 1 mL, taken from the vial using a gas tight syringe (Precision Sampling Corp).

Table 4.2. Operating parameters for the GC-MS (ion trap).

MS (ion trap): Saturn 4D, Varian Ltd.	
Mass range	70-200 m/z
Scan time	0.4 s
Segment Length	5 min
Peak threshold	0 counts
Mass defect	0 mm / 10
Background	150 m/z
Ion mode	Electron Impact
Manifold temperature	260 °C
GC: Star 3400Cx / 1078 injector, Varian Ltd.	
Injector temperature	200 °C
Column temperature program	40 °C, 15 °C/min, 150 °C
Transfer line temperature	200 °C
Capillary column	PTE^{TM}-5, 30 m * 0.32 mm, 0.25 μm, Supelco 2-4143

4.2.4 Details of Quantification

4.2.4.1 Quantification of Labeled Methyl Groups in Trimethylantimony Species

The fragmentation patterns of stibines containing $^{13}CD_3$ and CD_3 groups are unknown but they are unlikely to be much different from that of the unlabeled stibines (this is the case for arsines[3]), so for the purposes of quantification the fragmentation patterns are assumed to be the same.

Ideally the calculation of how much labeled methyl group has been incorporated into trimethylstibine would be based on all three methyl containing fragment ions; i.e. $MeSb^+$, Me_2Sb^+, and Me_3Sb^+. But because the parent ions are not abundant in the mass spectrum and the $MeSb^+$ region of the mass spectrum is complex, quantification was done only on the Me_2Sb^+ (m/z = 151, 153) fragment ions assuming that the probability of finding a labeled methyl group in a fragment ion is the same for all fragments.

The fraction of $^{13}CD_3$ present in $Me_3{}^{121}Sb$ ($f_{(^{13}CD_3\text{-}^{121}Sb)}$), and in $Me_3{}^{123}Sb$ ($f_{(^{13}CD_3\text{-}^{123}Sb)}$) can be calculated by using the following formula:

$$f_{(^{13}CD_3\text{-}^{121}Sb)} = \frac{A_{155} + 2 \times A_{159}}{2 \times (A_{151} + A_{155} + A_{159})} \qquad f_{(^{13}CD_3\text{-}^{123}Sb)} = \frac{A_{157} + 2 \times A_{161}}{2 \times (A_{153} + A_{157} + A_{161})}$$

Where A_{151}, A_{153}, A_{155}, A_{157}, A_{159} and A_{161} are the peak areas, at the trimethylstibine retention time, of the ion chromatograms for m/z = 151, 153, 155, 157, 159 and 161, respectively. The fraction of CD_3 present in $Me_3{}^{121}Sb$ ($f_{(CD_3\text{-}^{121}Sb)}$) and $Me_3{}^{123}Sb$ ($f_{(CD_3\text{-}^{123}Sb)}$) was calculated in an analogous fashion, that is:

$$f_{(CD_3\text{-}^{121}Sb)} = \frac{A_{154} + 2 \times A_{157}}{2 \times (A_{150} + A_{154} + A_{157})} \qquad f_{(CD_3\text{-}^{123}Sb)} = \frac{A_{156} + 2 \times A_{159}}{2 \times (A_{152} + A_{156} + A_{159})}$$

In using these procedures, it is also assumed that the amounts of other interfering fragment ions are not significant and that the instrument response for labeled stibines is the same as that for unlabeled stibines. The final assumption made is that the efficiency of hydride derivatization is the same for both labeled and unlabeled trimethylantimony species.

4.2.4.2 Quantification of Labeled Methyl Groups in Trimethylarsenic Species

Other researchers have reported that the fragmentation patterns of $(CD_3)_3As$, $CD_3(CH_3)_2As$ and $(CH_3)_3As$ in a quadrupole mass spectrometer are essentially the same[3]. In this work, it is reasonable to assume that the fragmentation patterns of both labeled and unlabeled trimethylarsine are the same. Quantification of the percentage incorporation of labeled methyl groups into trimethylarsenic species is best done based on the parent ion ($(CH_3)_3As^+$; m/z = 120), which, in the ion trap mass spectrometer, is almost completely deprotonated ($(CH_3)_2AsCH_2^+$; m/z = 119). Quantification of the fraction of labeled methyl groups in the dimethylarsenic fragment ion is not possible because the fragmentation pattern in this region of the MS is complicated. Therefore, the percentage incorporations were calculated as follows:

$$f(^{13}CD_3\text{-}^{75}As) = \frac{A_{122} + A_{123} + 2 \times (A_{126} + A_{127}) + (3 \times A_{130})}{3 \times (A_{119} + A_{122} + A_{123} + A_{126} + A_{127} + A_{130})}$$

$$f(CD_3\text{-}^{75}As) = \frac{A_{121} + A_{122} + 2 \times (A_{124} + A_{125}) + (3 \times A_{127})}{3 \times (A_{119} + A_{121} + A_{122} + A_{124} + A_{125} + A_{127})}$$

Again, as for antimony, it is assumed that:

1. There are no interfering fragment ions in the mass spectrum.
2. The instrument response for labeled arsines is the same as the response for unlabeled arsines.
3. The efficiency of hydride derivatization is the same for both labeled and unlabeled trimethylarsenic species.

4.3 RESULTS

4.3.1 Preliminary Remarks

Cultures 5 and 6, in Experiment **6**, consisted of autoclaved *S. brevicaulis*, media, potassium antimony tartrate and CH_3-L-methionine. No alkylantimony species were detected in these nonliving controls after 1 month of incubation. This indicates that biomethylation of antimony, with L-methionine as the methyl source, is not a passive process.

For all the active cultures of *S. brevicaulis* that contained potassium antimony tartrate (Cultures: **6/1** – **6/4** and **7/1** – **7/4**), dimethylantimony and trimethylantimony species were detected in media samples after 1 month of growth. For the cultures that contained L-methionine, the concentrations of trimethylantimony species in the media were approximately 20 ng Sb/mL (Cultures: **6/1** and **6/2**) and 6 ng Sb/mL (Cultures: **7/1** and **7/2**). For the cultures that contained no added D-methionine or L-methionine (Cultures: **6/3** and **6/4**), the concentrations of trimethylantimony species in the media were approximately 5 ng Sb/mL. For the cultures that contained D-methionine (Cultures: **7/3** and **7/4**), the concentrations of trimethylantimony species in the media were approximately 2 ng Sb/mL.

After one month of incubation of *S. brevicaulis* with sodium arsenite and D-methionine, cultures **7/5** and **7/6** contained approximately 30 ng As/mL as trimethylarsenic species. After one month of incubation of *S. brevicaulis* with sodium arsenite and L-methionine, cultures **7/7** and **7/8** contained approximately 130 ng As/mL as trimethylarsenic species. Methylarsenic and dimethylarsenic species were not detected.

The assignment of peaks in the mass spectra presented here are only tentative assignments based on the literature fragmentation pathways, and common sense, rather than experimental determination by using MS/MS.

4.3.2 Incorporation of Labeled Methyl Groups of Methionine into Trimethylantimony Species

4.3.2.1 Incorporation of $^{13}CD_3$-L-Methionine Methyl Groups into Trimethylantimony Species

Hydride derivatization of medium, from cultures **6/3** and **6/4**, produced dimethylstibine and trimethylstibine. The trimethylstibine, obtained as a gaseous fraction, was injected into the GC-MS (ion trap) and a typical mass spectrum is illustrated in Figure 4.2a. A typical mass spectrum, for the trimethylstibine originating from a culture that contained $^{13}CD_3$-L-methionine has a number of extra peaks (Figure 4.2b). The extra peaks are consistent with a sample of trimethylstibine containing intact $^{13}CD_3$ methyl groups. This is most apparent for $^{13}CD_3CH_3Sb^+$ (m/z = 155, 157) and $^{13}CD_3CH_3(CH_2)Sb^+$ (m/z = 169, 171) and $(^{13}CD_3)_2(CH_2)Sb^+$ (m/z = 173, 175). These peaks are absent in the mass spectrum of the control trimethylstibine, obtained by hydride derivatization of medium from a culture that contained no labeled methionine.

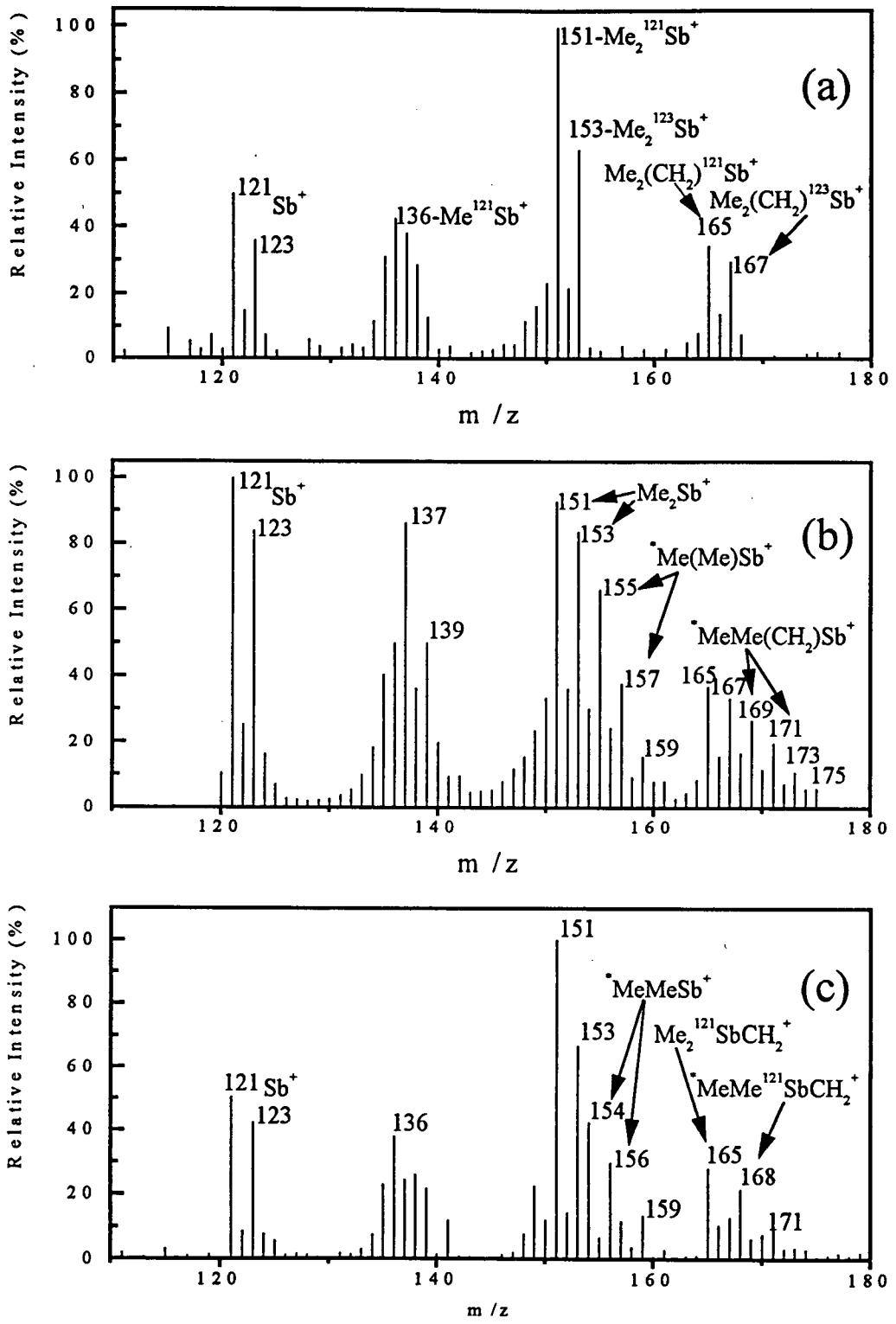


Figure 4.2. The GC-MS (ion trap) mass spectra of trimethylstibine, obtained by hydride derivatization of medium from: **(a)** a culture that did not contain added methionine; **(b)** a culture that contained $^{13}CD_3$-L-methionine ($^{*}Me = {}^{13}CD_3$); **(c)** a culture that contained CD_3-D-methionine ($^{*}Me = CD_3$).

The procedures described in Section 4.2.4.1 were applied to the mass spectra to calculate the percentage of labeled methyl group in the trimethylantimony species (Table 4.3) and it can be seen that there is significant variability, in the percentage of $^{13}CD_3$ incorporated into trimethylstibine, for both analytical replicates and culture replicates. The isotope ratios (Table 4.3) represent the ratios of peak areas for the single ion chromatograms. The natural isotope ratio for antimony is 1.34. The significant variation from the natural isotope ratio, especially for Me_2Sb^+ (151/153), $Me^{13}CD_3Sb^+$ (155/157) and $(^{13}CD_3)_2Sb^+$ (159/161) arises from instrumental error and from the presence of interfering fragments in the mass spectrum. There is no evidence in the total ion chromatograms of co-eluting species. The interfering fragments are most likely due to the multiple fragmentation pathways of trimethylstibine. Thus, the assumptions in calculating the percent of labeled methyl group in alkylantimony species are not substantiated. So the results in Table 4.3 should only be used as an estimate of $^{13}CD_3$ incorporation. On average approximately 47% $^{13}CD_3$, from $^{13}CD_3$-L-methionine, was present in trimethylantimony species.

Table 4.3. Estimate of Percent $^{13}CD_3$ present in the trimethylantimony compound found in media samples of *S. brevicaulis* cultures. See text for discussion of the assumptions made in these calculations.

Contents of Culture	Percent $^{13}CD_3$ in Trimethylantimony			Ion Ratios			
	121	**123**	**Average**	**121/123**	**151/153**	**155/157**	**159/161**
6/1. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	35	28	31	1.32	1.02	1.52	1.81
+ 100 mg Sb/L as potassium antimony tartrate	37	33	35	1.24	1.17	1.32	1.63
	41	33	37	1.17	1.00	1.41	1.95
	27	20	23	1.16	1.11	1.64	2.22
	34	28	31	1.19	0.93	1.44	1.40
	26	20	23	1.16	1.20	1.77	2.10
	38	27	33	1.19	0.89	1.44	2.60
6/2. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	55	49	52	1.12	0.91	1.39	1.46
+ 100 mg Sb/L as potassium antimony tartrate	50	42	46	1.06	1.25	1.57	2.30
	51	48	49	1.24	1.03	1.36	1.33
7/1. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g) + 100 mg Sb/L as potassium antimony tartrate	57	53	55	1.08	1.45	1.25	1.82
7/2. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g) + 100 mg Sb/L as potassium antimony tartrate	52	55	53	1.10	1.09	1.57	0.92
6/3. Control: *S. brevicaulis* + 100 mg Sb/L as potassium antimony tartrate	< 1	< 1	< 1	1.19	1.71	n/a	n/a
6/4. Control: *S. brevicaulis*	< 1	< 1	< 1	1.25	1.50	n/a	n/a
+ 100 mg Sb/L as potassium antimony tartrate	3	5	4	1.19	1.70	n/a	n/a
Standard of Me_3Sb from HG of Me_3SbCl_2	< 1	< 1	< 1	1.12	1.54	n/a	n/a

4.3.2.2 Incorporation of CD_3-D-Methionine Methyl Groups into Trimethylantimony Species

The mass spectrum of trimethylstibine, obtained by hydride derivatization of media from cultures that had been incubated with potassium antimony tartrate and CD_3-D-methionine, contains a number of extra peaks (Figure 4.2c) when compared with a trimethylstibine control, generated from a culture of *S. brevicaulis* that had been incubated with antimony in the absence of labeled methionine (Figure 4.2a). These peaks are most apparent at m/z = 154 ($CD_3CH_3{}^{121}Sb^+$), 156 ($CD_3CH_3{}^{123}Sb^+$), 157 ($(CD_3)_2{}^{121}Sb$) and 159 ($(CD_3)_2{}^{123}Sb$). These extra peaks are consistent with the sample containing intact CD_3 groups.

The quantities of CD_3 incorporated were calculated and are tabulated in Table 4.4. Again, the ion ratios significantly differed from the expected value of 1.34 indicating that the assumptions made in calculating the fraction of CD_3 are not substantiated, and the results should only be used as an estimate. However, the analytical and culture replicates did agree quite well. On average approximately 20% CD_3, from CD_3-D-methionine, was present in trimethylantimony species. That is, the incorporation of methyl groups from D-methionine is about half the incorporation from L-methionine, so there is some stereospecificity that suggests that biomethylation is enzymatic.

Table 4.4. Estimate of Percent CD_3 present in the trimethylantimony species found in media samples of *S. brevicaulis* cultures. See text for discussion of the assumptions made in these calculations.

Contents of Culture	Percent CD_3 in Trimethylantimony			Ion Ratios			
	121	123	Average[b]	121/123	151/153	154/156	157/159
7/3. *S. brevicaulis*+ CD_3-D-methionine(0.1 g)	14	22	18	1.24	2.35	1.46	0.7
+ potassium antimony tartrate (100 mg Sb/L)	21	23	22	1.38	1.54	1.36	1.46
	22	21	21	1.20	1.70	1.49	2.16
7/4. *S. brevicaulis*+ CD_3-D-methionine(0.1 g)	18	17	18	1.11	1.87	2.66	1.39
+ potassium antimony tartrate (100 mg Sb/L)	20	23	22	1.06	1.47	1.38	1.07

4.3.3 Incorporation of Labeled Methyl Groups of $^{13}CD_3$-L-methionine into Dimethylantimony Species

The cultures of *S. brevicaulis* also produced dimethylantimony species, which on hydride generation derivatizes to dimethylstibine. The mass spectrum of dimethylstibine originating from a culture of *S. brevicaulis*, which was grown in the absence of $^{13}CD_3$-L-methionine is shown in Figure 4.3a. The fragmentation pattern for dimethylstibine using ion trap MS is quite complicated. When *S. brevicaulis* was grown in the presence of $^{13}CD_3$-L-methionine the labeled methyl group was significantly incorporated into the dimethylantimony species as can be seen from the mass spectrum (Figure 4.3b), which contains a number of extra peaks for instance at m/z = 140 ($^{13}CD_3{}^{121}Sb^+$) and m/z = 142 ($^{13}CD_3{}^{123}Sb^+$). The fraction of $^{13}CD_3$ incorporated into the dimethylantimony species was determined and is reported in Table 4.5. As was found for the trimethylantimony species, the isotope ratios significantly differed from the expected value of 1.34. The average amount of $^{13}CD_3$ incorporated into dimethylantimony species was approximately 38%. It is significant that for culture **6/1** the incorporations of $^{13}CD_3$ into dimethylantimony and trimethylantimony species were 33% and 30%, respectively and that for culture **6/2** the incorporations of $^{13}CD_3$ into dimethylantimony and trimethylantimony species were 43% and 49%, respectively. That is essentially the same amount of label is incorporated into both dimethylantimony and trimethylantimony species. This is expected if antimony biomethylation proceeds by the Challenger mechanism. The incorporation of CD_3 from CD_3-D-methionine into dimethylantimony species was not measured, but it is most likely that the amounts of CD_3 incorporated into dimethylantimony and trimethylantimony species are the same.

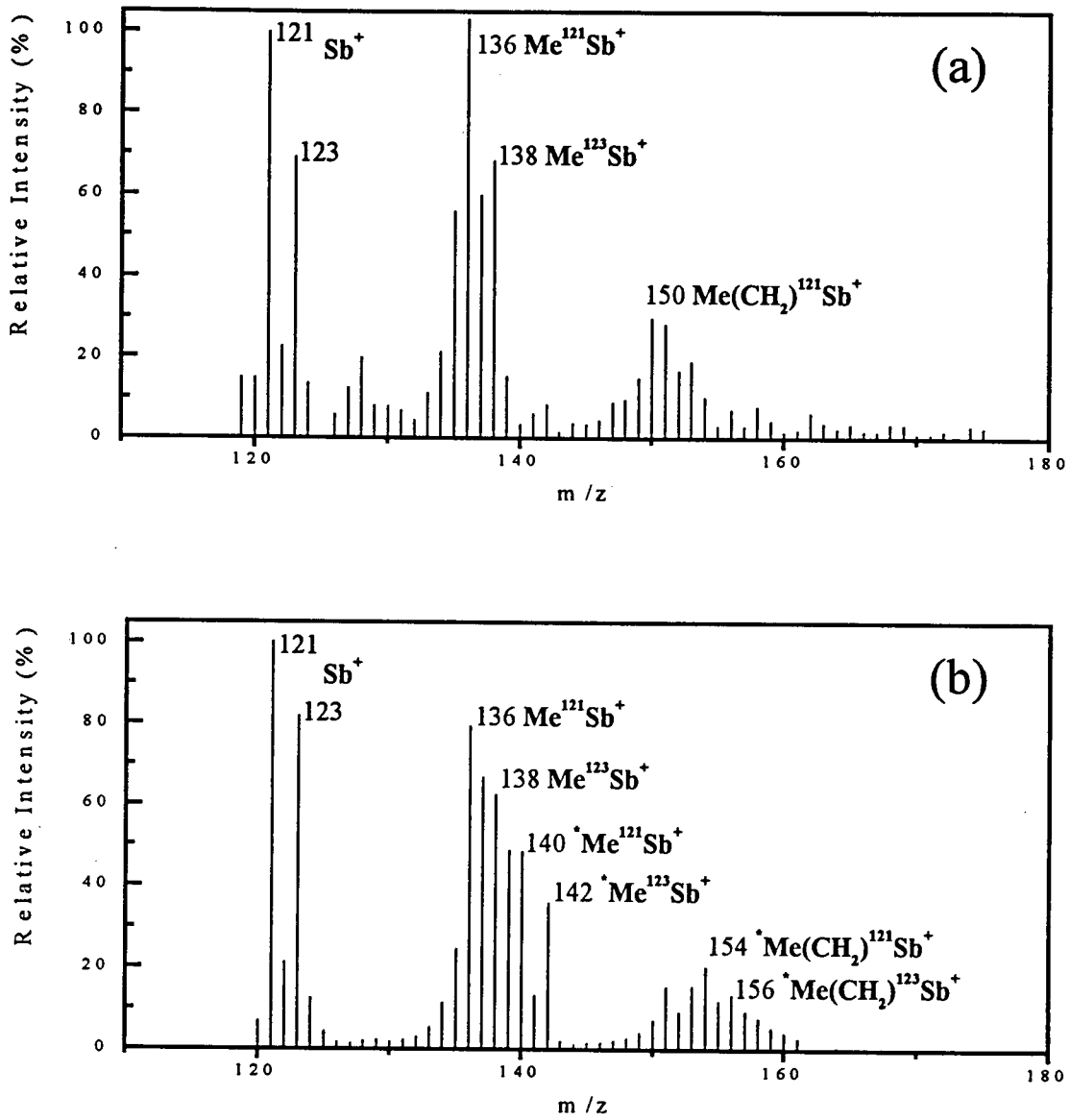


Figure 4.3. The GC-MS (ion trap) mass spectra of dimethylstibine, obtained by hydride derivatization of medium from: (a) a culture that did not contain added methionine; (b) a culture that contained $^{13}CD_3$-L-methionine. $^{*}Me = {}^{13}CD_3$

Table 4.5. Estimate of Percent $^{13}CD_3$ present in the dimethylantimony species found in media samples of *S. brevicaulis* cultures. See text for discussion of the assumptions made in these calculations.

Contents of Culture	Percent $^{13}CD_3$ in Dimethylantimony			Ion Ratios			
	121	**123**	**Average**	**121/123**	**151/153**	**155/157**	**159/161**
6/1. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	38	29	33	1.29	1.01	1.29	2.32
+ 100 mg Sb/L as potassium antimony tartrate	39	28	33	1.25	0.92	1.27	2.45
6/2. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	46	40	43	1.26	1.21	1.08	2.08
+ 100 mg Sb/L as potassium antimony tartrate							
6/3. Control: *S. brevicaulis*	< 1	< 1	< 1	1.31	1.16	n/a	n/a
+ 100 mg Sb/L as potassium antimony tartrate							

4.3.4 Incorporation of Labeled Methyl Groups of $^{13}CD_3$-L-methionine and CD_3-D-methionine, into Trimethylarsenic Species

Hydride derivatization of medium from cultures 5 - 8 of Experiment 7 produced trimethylarsine. The trimethylarsine was collected in septa-capped vials and then injected into the GC-MS (ion trap). A typical mass spectrum of trimethylarsine that contains no labeled-methyl groups is shown in Figure 4.4a. When trimethylarsine, obtained by hydride derivatization of medium from a culture that contained $^{13}CD_3$-L-methionine, is injected into the GC-MS, a number of extra peaks are evident in the mass spectrum (Figure 4.4b) at, for instance, m/z = 123 ($^{13}CD_3CH_3AsCH_2^+$), m/z = 127 (($^{13}CD_3)_2AsCH_2^+$) and m/z = 130 (($^{13}CD_3)_2As^{13}CD_2^+$). A number of peaks also exist in the mass range of m/z = 105 – 113, in no definitive pattern, because trimethylarsine has quite a complex fragmentation pattern in this region, but it is clear, when the MS in this mass range for a labeled culture is compared with unlabeled trimethylarsine, that there has been incorporation of the label. The trimethylarsine mass spectra, for cultures that had been incubated with CD_3-D-methionine (Figure 4.4c), also contained a number of extra peaks, for instance at m/z = 122 ($CD_3CH_3AsCH_2^+$) and at m/z = 125 (($CD_3)_2AsCH_2^+$). The presence of these peaks is consistent with the incorporation of the appropriate intact labeled-methyl groups. The procedures described in Section 4.2.4.2 were applied to the mass spectra to calculate the percentage of labeled methyl group in the trimethylarsenic species (Table 4.4).

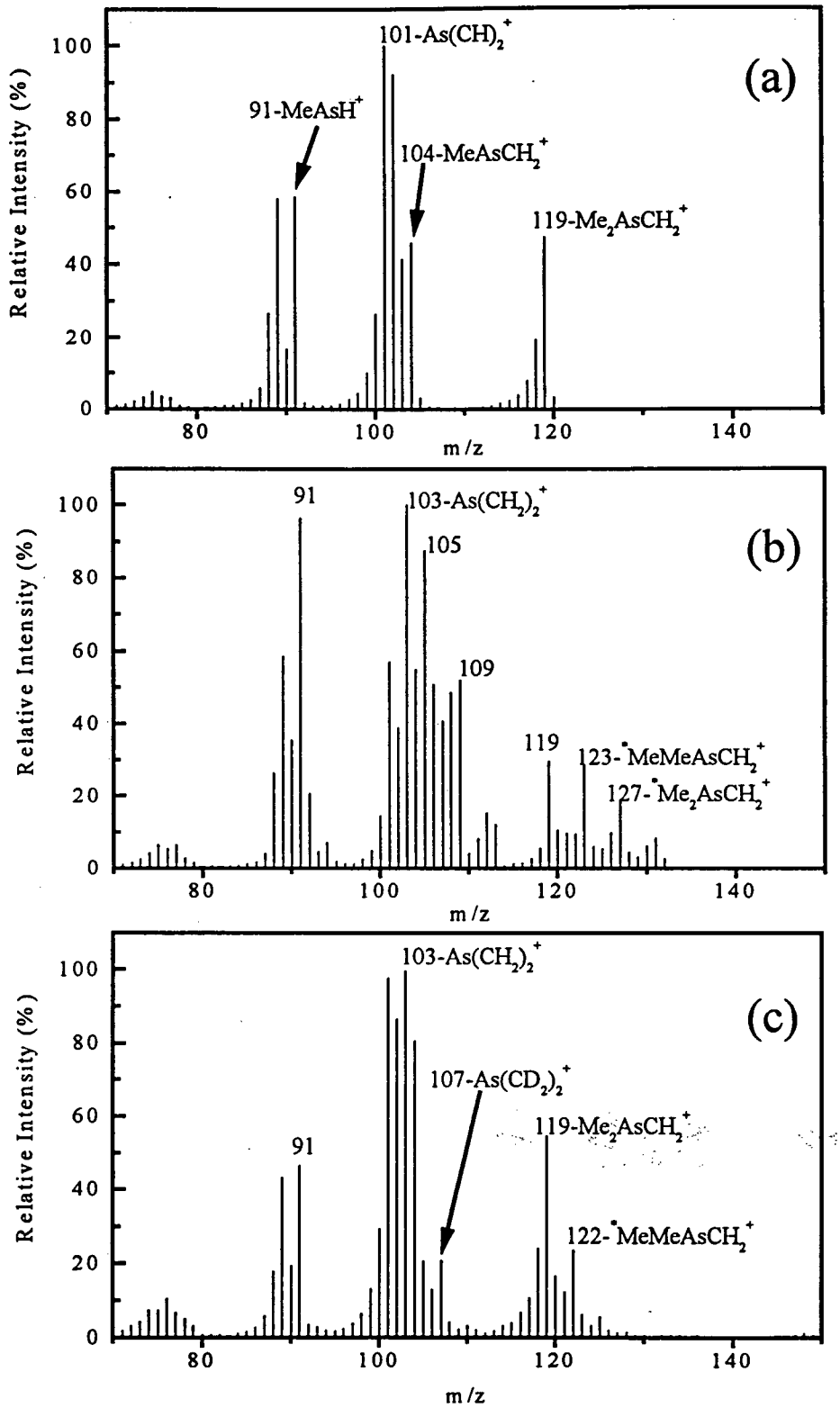


Figure 4.4. GC-MS mass spectra of trimethylarsine, obtained by hydride derivatization of medium from: **(a)** culture that did not contain added methionine; **(b)** culture that contained $^{13}CD_3$-L-methionine ($^*Me = {}^{13}CD_3$); **(c)** culture that contained CD_3-D-methionine ($^*Me = CD_3$).

The same assumptions that were made in quantifying label incorporation into trimethylstibine, were made for trimethylarsine; but isotope ratios cannot be monitored as a check, because arsenic has only one naturally occurring isotope. However, repeatability between culture replicates and analytical replicates was acceptable. The average amount of $^{13}CD_3$, from $^{13}CD_3$-L-methionine, in trimethylarsenic species was 47%. The average amount of CD_3, from CD_3-D-methionine, in trimethylarsenic species was 16%. That is, there was some stereospecificity suggesting that arsenic biomethylation is enzymatic.

Table 4.6. Estimate of Percent CD_3 and $^{13}CD_3$ present in trimethylarsenic species found in media samples of *S. brevicaulis* cultures from Experiment 7. See text for discussion of the assumptions made in these calculations.

Contents of Culture	**Percent *Me[a] in Trimethylarsenic**
7/5. *S. brevicaulis* + CD_3-D-methionine (0.1 g)	11
+ sodium arsenite (10 mg As/L)	15
7/6. *S. brevicaulis* + CD_3-D-methionine (0.1 g)	21
+ sodium arsenite (10 mg As/L)	20
	17
7/7. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	36
+ sodium arsenite (10 mg As/L)	43
7/8. *S. brevicaulis* + $^{13}CD_3$-L-methionine (0.1 g)	54
+ sodium arsenite (10 mg As/L)	53

[a] - *Me = $^{13}CD_3$ or CD_3

4.4 DISCUSSION

Challenger[1] found that cultures of *S. brevicaulis*, grown in the presence of sodium arsenite and $^{14}CH_3$-D, L-methionine, produced trimethylarsine containing 28.3% of ^{14}C labeled methyl group. Cullen *et al.*[3] incubated *S. brevicaulis* with sodium arsenite and CD_3 labeled methionine. The trimethylarsine produced was collected and injected into an MS, and it was demonstrated that the methyl group is transferred *intact*. Cultures of *S. brevicaulis*, grown in the presence of 1 mg As/L as sodium arsenite, also produce mainly a nonvolatile trimethylarsenic species found in the medium[7]. However, the methyl donor for this nonvolatile species was not determined although it was shown that cultures of *Cryptococcus humicolus* (*Candida humicola*), grown in the presence of sodium arsenite and CD_3-L-methionine, produced nonvolatile alkylarsenic species containing 20% CD_3[4]. Unfortunately, the results obtained by these previous researchers[1, 3, 4] cannot be directly compared with the results presented in this chapter, because the experimental methods they used (e.g. cultures and species measured) are vastly different.

When cultures of *S. brevicaulis* were incubated with either sodium arsenite or potassium antimony tartrate under identical conditions (medium, fungal strain, temperature, aeration, time, methionine concentration) approximately equivalent amounts of labeled methyl groups from D-methionine and L-methionine were incorporated into nonvolatile trimethylantimony and trimethylarsenic species; a summary of the results is presented in Table 4.7. Thus, within experimental error, antimony behaves in the same way as arsenic, with respect to the methyl source for biomethylation.

Table 4.7. Summary of results.

Experiment	**% Incorporated**
Antimony + $^{13}CD_3$-L-methionine	47
Arsenic + $^{13}CD_3$-L-methionine	47
Antimony + CD_3-D-methionine	20
Arsenic + CD_3-D-methionine	16

When CD_3-D-methionine was used as a methyl source, for both potassium antimony tartrate (20% CD_3 in trimethylantimony species) and sodium arsenite (16% CD_3 in trimethylarsenic species), the amount of label incorporated decreased. This is expected if biomethylation is an enzymatic process, the stereochemistry of D-methionine would exclude it from the enzyme, but some incorporation may occur if there is racemization of the D-methionine. That is, this work supports the notion that both antimony and arsenic biomethylation occurs via an enzyme-mediated process.

4.5 REFERENCES

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CHAPTER 5

ARSENIC AND ANTIMONY BIOMETHYLATION BY *SCOPULARIOPSIS BREVICAULIS*: INTERACTION OF ARSENIC AND ANTIMONY COMPOUNDS

5.1 INTRODUCTION

The biomethylation of arsenic in the environment, and in humans, is well established[1], and this is significant because the chemical properties and toxicities of alkylarsenic species are different from those of inorganic arsenic species. Alkylantimony species have also been detected in the environment[2-7], and the biomethylation of antimony by some microorganisms, such as *S. brevicaulis* (Chapters 2-4), has been demonstrated in laboratory cultures[8-16]. However, antimony has not been studied to the same extent as arsenic and little is known about the biological properties of organoantimony species.

In almost all previous studies of arsenic and antimony biomethylation, each metalloid was studied in isolation, even though the two elements are usually associated in the environment. In addition, arsenic and antimony are both in Group 15 of the periodic table and so have similar chemistries, therefore it is possible that biological processes involving one of these elements are likely to be affected by the presence of the other. The results from Chapters 2-4 suggest that antimony may be biomethylated by the same putative methyltransferase enzyme that biomethylates arsenic, probably fortuitously. Thus, the simplest interactions that might be expected are direct competition, where for

example, the metalloids might compete for a binding site, a process that would be controlled by the concentration of each metalloid and the strength of binding.

A few studies of antimony and arsenic interactions in mammalian systems have been reported. Bailly *et al.*[17,18] reported that arsenic biomethylation, in rat liver cytosol, is completely inhibited at antimony concentrations of 10^{-5} M (as $SbCl_3$) but few details were given. The results of sister chromatid exchange tests (for genotoxicity) revealed that the combined effects of arsenic and antimony compounds on human lymphocytes are less than predicted from addition of the individual genotoxicities of arsenic and antimony compounds[19]. By using the micronucleus tests with V79 cells it was found that chromosome mutagenicity induced by arsenic(III) was significantly suppressed by antimony(III)[20]. In both of these genotoxicity studies the sub-additive interaction of arsenic and antimony was not attributable to inhibition of cell uptake. Another study reported that human tumor cells that were resistant to arsenite were also resistant to potassium antimony tartrate and vice versa[21]. This cross resistance could be partially attributed to reduced uptake of arsenic and antimony.

Thus, it seems that predicting the effects of arsenic contamination may be significantly complicated by the presence of antimony. In light of this situation Gebel[22], in a letter to Science, called for the measurement of antimony co-contamination in arsenic contaminated waters, and also for "the inclusion of antimony as a putative confounding variable in the toxicity of arsenic in future investigations".

Research on arsenic biomethylation in mammals is currently very topical in order to establish if methylation increases or decreases the carcinogenicity of inorganic arsenic

species, but usually co-exposure to other contaminants is not considered[23]. A significant increase or decrease in arsenic biomethylation could be the result of co-exposure to antimony.

Arsenic biomethylation by the filamentous fungus *Scopulariopsis brevicaulis* has been studied in detail and this fungus has served as a useful model in the study of other systems[24,25]. Antimony biomethylation by *S. brevicaulis* has been discussed previously in this thesis. Some studies on antimony and arsenic biomethylation by *S. brevicaulis* are now described in this chapter. These studies were performed to see if the yields of biomethylation products from one metalloid would be increased or decreased in the presence of the other. In this study, the focus was on the production of nonvolatile biomethylation products that are found in the medium, i.e. the trimethylarsenic and trimethylantimony species that produce trimethylarsine and trimethylstibine, respectively, on hydride derivatization. These are the principle products (>90%) of biomethylation under the conditions of this study. For antimony, the yields of volatile trimethylstibine (10 ng Sb (Chapter 3)) are always much lower than the yields of the trimethylantimony species in the media (300-3000 ng Sb [12,14]). For arsenic, the yields of trimethylarsine are much lower (<1 %) than that of trimethylarsenic species in media when the arsenic substrate concentration is $\leq$1 mg As/L[25].

The average concentration of arsenic in the earths crust (1.8 mg As/kg) is an order of magnitude greater than that of antimony (0.2 mg Sb/kg). However, in local environments the ratio of arsenic to antimony can vary tremendously. For example, the Rapahannock (Fredricksburg, VA) and the Roanoke (Weldon, NC) Rivers, in the USA, have arsenic to antimony ratios of 0.1:1, whereas the Sri Nakarin River (Si Sawat,

Thailand) the ratio is approximately 100:1[26]. These variations reflect differing geochemistry in each river and anthropogenic influences. The ratios of arsenic to antimony employed in this study ranged from 100:1 to 1:1 (for studying antimony biomethylation) and from 2:1 to 0.1:1 (for studying arsenic biomethylation). Thus, the present investigation is environmentally relevant.

5.2 MATERIALS AND METHODS

Submerged cultures of *Scopulariopsis brevicaulis* (ATCC #7903) mycelial balls were prepared by adding 40 mL of a seed culture to 400 mL of minimal-salts/glucose medium (Section 2.2.1) in 1 L Erlenmeyer flasks. Appropriate amounts of solutions of sodium arsenite, sodium arsenate, potassium tartrate, potassium antimony tartrate and potassium hexahydroxyantimonate were added to cultures via 0.2 μm syringe filters to give mixtures of arsenic and antimony compounds, as summarized in Tables 5.1 and 5.2. Solid powdered antimony trioxide (0.2 g) was added directly to the cultures to give a saturated solution (~ 4 mg Sb/L).

The Erlenmeyer flasks were shaken horizontally (~ 135 rpm, 1.75 inch displacement) and maintained at 26 °C for 1 month (previous studies had shown that the production of trimethylantimony or trimethylarsenic species, in significant amounts, ceases after 1 month (Chapter 2, Reference 25)). After incubation, the cultures were autoclaved (19 psi, 121 °C, 20 minutes) before analysis.

Inorganic arsenic and antimony compounds were removed from the medium by using solid phase extraction (Section 2.2.5.1) and the eluate was analyzed for trimethylarsenic species or trimethylantimony species by using HG-GC-AAS procedures (Section 2.2.5.2). Quantification was performed, by using trimethylarsine oxide, or trimethylantimony dichloride, to perform standard additions.

5.3 RESULTS

5.3.1 Influence of Arsenic Compounds on the Biomethylation of Antimony Compounds (Experiments 8, 9 and 10)

Cultures of *S. brevicaulis* were prepared containing potassium antimony tartrate (either 10 or 100 mg Sb/L), and 0 (controls) to 100 mg As/L as sodium arsenite. After 1 month of incubation the concentration of trimethylantimony species in the cultures were determined, as trimethylstibine, by using HG-GC-AAS (Table 5.1). In the absence of sodium arsenite, between 0.0006 and 0.008% of the antimony substrate is biomethylated to trimethylantimony species. The final column in Table 5.1 gives the relative concentration of trimethylantimony species that is obtained by taking the average of the controls (i.e. cultures with no sodium arsenite added) in each experiment as being 100%.

Table 5.1. Influence of sodium arsenite on potassium antimony tartrate biomethylation: Concentration of potassium antimony tartrate and sodium arsenite substrates in cultures, and concentration of trimethylantimony species, after one month of incubation of cultures.

		Substrate Concentrations		Biomethylation Product Concentration	
Experiment	Culture	Concentration of Potassium Antimony Tartrate (mg Sb/L)	Concentration of Sodium Arsenite (mg As/L)	Trimethylantimony Species (μg Sb/L)	Relative[a] Concentration (%)
8	1-Control	10	0	0.8	100
	2	10	1	2.2	275
	3	10	10	4.0	500
	4	10	100	7.2	900
9	1-Control	10	0	0.5	100
	2	10	1	1.1	220
	3	10	10	3.0	600
	4	10	100	3.2	640
10	1-Control	100	0	0.6	100
	2	100	1	1.6	266
	3	100	10	3.0	500
	4	100	100	2.5	417

[a] Average concentration of trimethylantimony species in controls for each experiment taken as 100%.

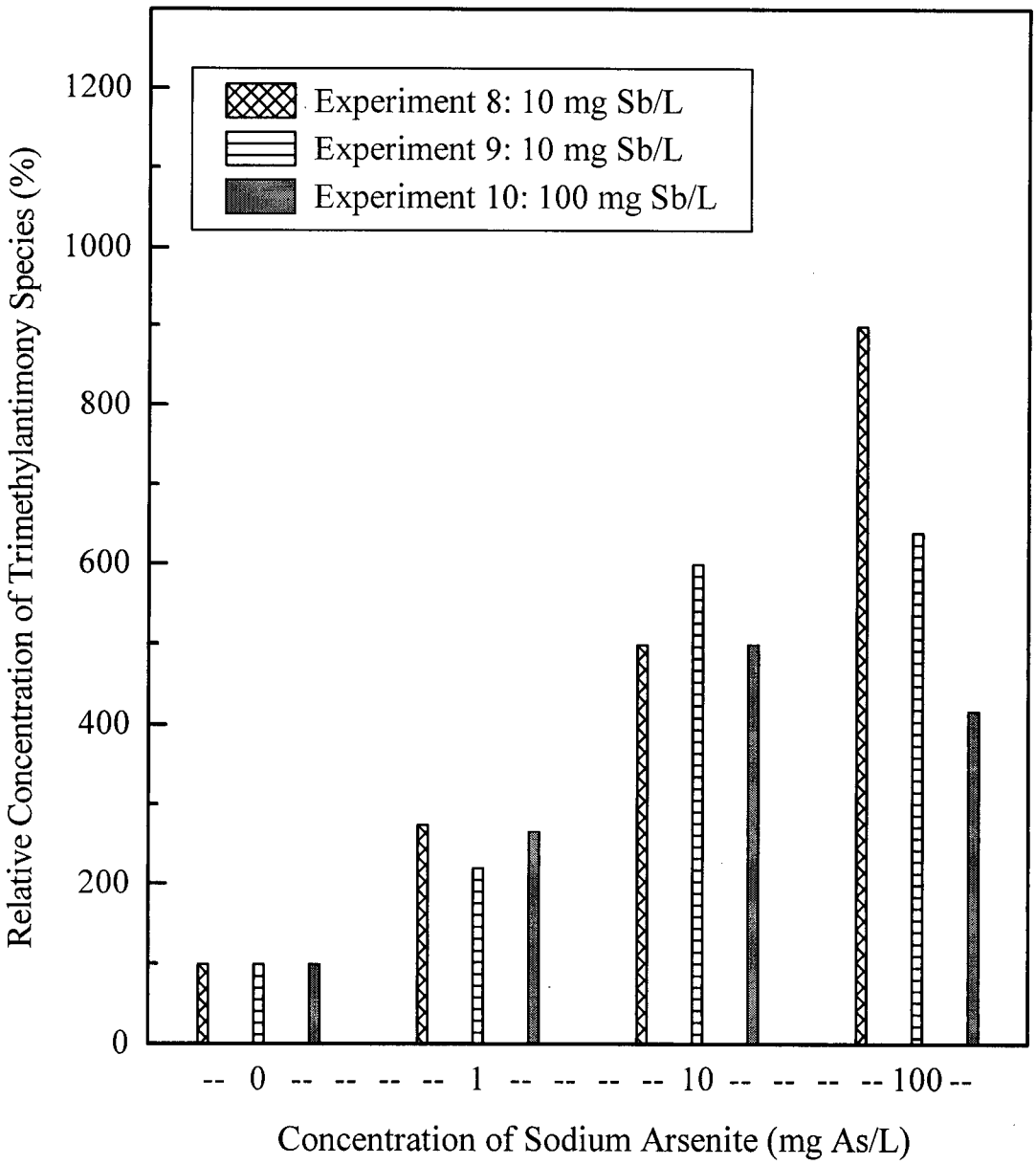

Figure 5.1. Relative concentration of trimethylantimony species (%) in cultures of *S. brevicaulis* after 1 month of incubation. Cultures contained either 10 mg Sb/L (Experiments **8** and **9**) or 100 mg Sb/L (Experiment **10**) as potassium antimony tartrate. Cultures contained between 0 (controls) and 100 mg As/L as sodium arsenite.

In these experiments, only the influence of arsenic(III) (as sodium arsenite) on the biomethylation of antimony(III) (as potassium antimony tartrate) was examined. The effects of arsenic(III or V) on the biomethylation of antimony(V) were not examined, because in previous experiments (Section 2.3.2.2) no evidence was found for the biomethylation of antimony(V) by *S. brevicaulis*. Also, the influence of arsenic(V) on the biomethylation of antimony was not examined because it has been previously shown that when arsenic(V) is added to cultures of *S. brevicaulis* it is reduced to arsenic(III) within a few days[25], whereas antimony biomethylation occurs over one month.

When the concentration of potassium antimony tartrate in the cultures was maintained constant either at 10 or 100 mg Sb/L, and the sodium arsenite concentration was increased (0, 1, 10, 100 mg As/L) the amount of trimethylantimony species produced increased (Figure 5.1). At 1000 mg As/L as sodium arsenite, *S. brevicaulis* did not grow.

5.3.2 Influence of Antimony Compounds on the Biomethylation of Arsenic Compounds

Cultures of *S. brevicaulis* were grown in media containing arsenic (either as sodium arsenite or sodium arsenate) and various antimony compounds. The concentration of trimethylarsenic species in the medium, after 1 month of incubation, was determined, as trimethylarsine, by using HG-GC-AAS. A representative series of HG-GC-AAS chromatograms is shown in Figure 5.2, these are from Experiment **14** (Table 5.2). The only significant peak in these chromatograms is that of trimethylarsine because almost all (>99.5%) of the inorganic arsenic was removed by using solid phase extraction. The concentration of trimethylarsenic species in each sample was determined by standard

additions and these results are reported in Table 5.2. In the controls (no antimony compounds added), between 1.2 and 5.3% of the arsenic substrate is biomethylated to trimethylarsenic species. The final column in Table 5.2 gives the relative concentration of trimethylarsenic species, obtained by taking the average of the controls (i.e. cultures with no antimony compounds added) in each experiment as being 100%. Some of these results are plotted in Figures 5.3 and 5.4. Error bars are not shown on the figures because the error in analysis is insignificant compared to the variation between culture replicates. The observed decrease in the levels of trimethylarsenic species is not a result of matrix (inorganic antimony) effects during the analysis because inorganic arsenic and inorganic antimony species were removed, before analysis, by using solid phase extraction, and quantification was done by standard additions.

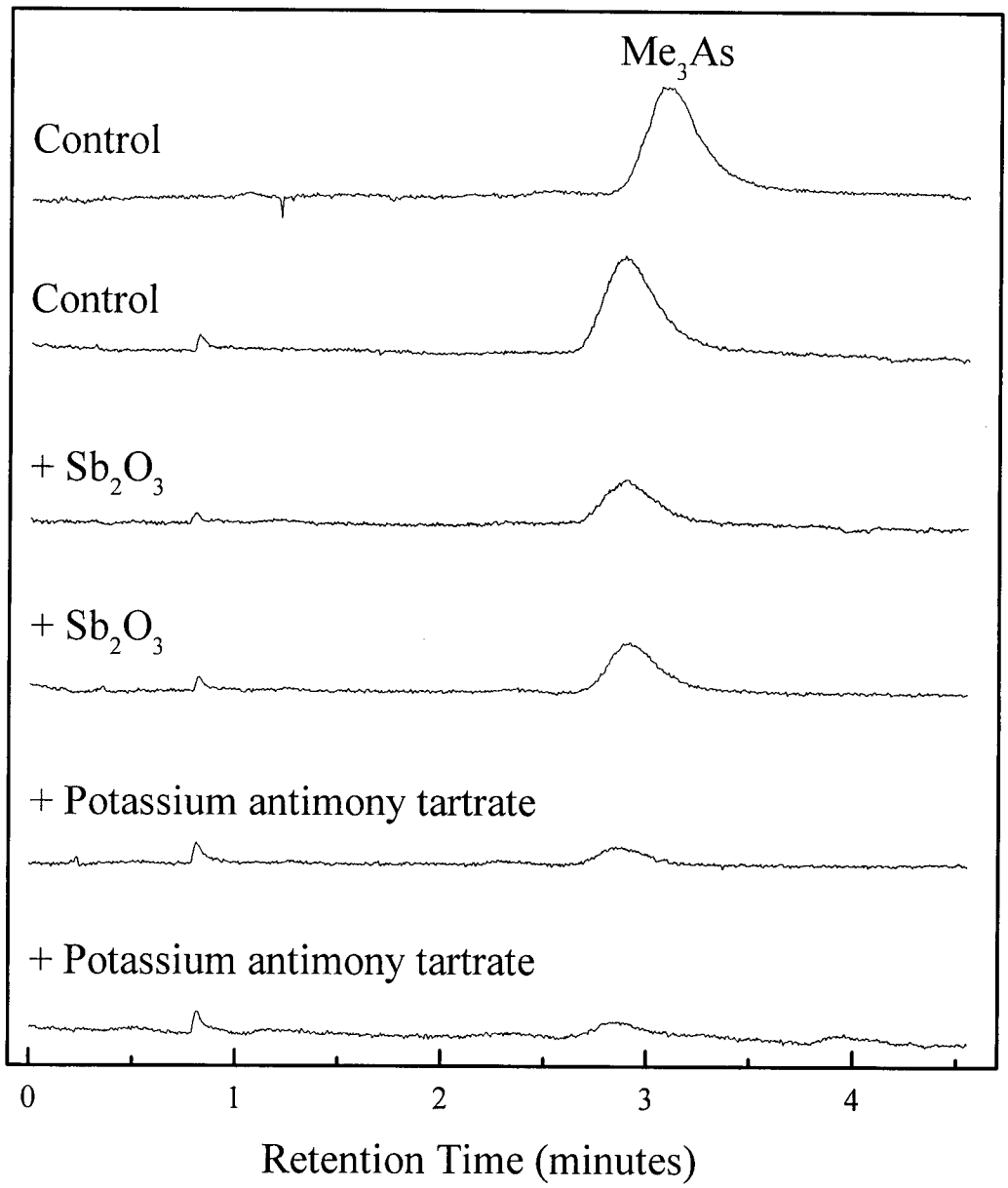

Figure 5.2. Chromatograms obtained when media samples (1 mL) from the cultures of Experiment **4** were analyzed by using solid phase extraction then HG-GC-AAS. Cultures were sampled and analyzed after 1 month of incubation.

Table 5.2. Influence of antimony compounds on arsenic biomethylation: Concentration of antimony and arsenic substrates in cultures, and concentration of trimethylarsenic species, after one month of incubation of cultures.

		Substrate Concentrations		Biomethylation Product Concentration	
Expt.	**Culture**	**Antimony Species (mg Sb/L)**	**Arsenic Species (mg As/L)**	**Trimethylarsenic species (μg As/L)**	**Relative[c] Concentration (%)**
11	1-Control	PAT[a] 0	$NaAsO_2$ 1	34	100
	2	PAT 1	$NaAsO_2$ 1	21	62
	3	PAT 2	$NaAsO_2$ 1	7	21
	4	PAT 4	$NaAsO_2$ 1	2	6
	5	PAT 6	$NaAsO_2$ 1	3	9
	6	PAT 8	$NaAsO_2$ 1	2	6
12	1-Control	0	Na_2HAsO_4 1	27	126
	2-Control	0	Na_2HAsO_4 1	16	74
	3	PAT 10	Na_2HAsO_4 1	9	42
	4	PAT 10	Na_2HAsO_4 1	8	37
13	1-Control	0	$NaAsO_2$ 1	38	100
	2	PHHA[b] 10	$NaAsO_2$ 1	45	118
	3	PHHA 100	$NaAsO_2$ 1	30	79

Table 5.2. Continued

14	1-Control	0	$NaAsO_2$ 10	153	110
	2-Control	0	$NaAsO_2$ 10	124	90
	3	Sb_2O_3 4	$NaAsO_2$ 10	64	46
	4	Sb_2O_3 4	$NaAsO_2$ 10	62	45
	5	PAT 50	$NaAsO_2$ 10	17	12
	6	PAT 50	$NaAsO_2$ 10	19	14
15	1-Control	0	$NaAsO_2$ 10	266	113
	2-Control	0	$NaAsO_2$ 10	205	87
	3-Control	0	Na_2HAsO_4 10	388	84
	4-Control	0	Na_2HAsO_4 10	536	116
	5	PAT 50	Na_2HAsO_4 10	187	40
	6	PAT 50	Na_2HAsO_4 10	229	50
	7	PAT 50	$NaAsO_2$ 10	27	11
	8	PAT 5	$NaAsO_2$ 10	88	37
	9	PAT 5	$NaAsO_2$ 10	161	68
	10	potassium tartrate 50	$NaAsO_2$ 10	286	121

[a] PAT = potassium antimony tartrate.

[b] PHHA = potassium hexahydroxyantimonate.

[c] Average concentration of trimethylarsenic species in controls for each experiment taken as 100%.

In each experiment, all cultures were prepared from the same batch of medium, antimony/arsenic substrates, and seed culture. Also, all cultures were incubated and analyzed under identical conditions. However, there were still large variations in the amounts of biomethylation products for replicate cultures. This variation is most likely the result of the inhomogeneity of the seed culture, but the observed effects, presented here, are greater than the variation from culture inhomogeneity.

In the presence of 1000 mg Sb/L, as potassium antimony tartrate, little inhibition of *S. brevicaulis* growth was visually observed. This is consistent with other work on two strains of *S. brevicaulis* where the EC_{50} (median effective concentration for inhibition of hyphal extension) values were determined to be greater than 300 mg Sb/L[27]. In the results presented below, reduction of arsenic biomethylation, in the presence of antimony, occurs at antimony concentrations significantly lower than the EC_{50} so the observed inhibition must be a result of more than just reduced growth.

5.3.2.1 Influence of Antimony(III) on the Biomethylation of Arsenic(III) (Experiments 11, 14 and 15)

In preliminary experiments (results not shown) involving sodium arsenite (all cultures: 1 mg As/L) and potassium antimony tartrate (0, 1, 10, 100, 1000 mg Sb/L) the cultures that contained 10 mg Sb/L, or greater, produced no detectable (< 5 ng As/mL) trimethylarsenic species.

To further examine this phenomenon, cultures were prepared containing 1 mg As/L as sodium arsenite and 0 - 8 mg Sb/L as potassium antimony tartrate (Experiment 11). The concentration of trimethylarsenic species in each culture after one month was

measured and there was a clear trend of inhibition of arsenic biomethylation as the antimony concentration increases (Table 5.2). Above 4 mg Sb/L the biomethylation of sodium arsenite is decreased to 10% of the controls.

In order to determine if the absolute amount of antimony, or the ratio of antimony to arsenic modulates arsenic biomethylation, cultures were prepared containing 10 mg As/L as sodium arsenite and 4 - 50 mg Sb/L (either as potassium antimony tartrate or antimony trioxide) (Experiments **14** and **15**). The concentration of trimethylarsenic species was measured (Figures 5.3a and 5.3b) and it was found that in the presence of approximately 5 mg Sb/L (either as potassium antimony tartrate or as antimony trioxide) the biomethylation of arsenic was decreased to approximately 50% of the controls. Since biomethylation in cultures containing 1 mg As/L is decreased to 10% of the controls in cultures containing 5 mg Sb/L or greater (i.e. 5 fold excess of antimony) it is clear that the ratio of antimony to arsenic is more important than the absolute amount of antimony present. Indeed, when the antimony concentration (in cultures containing 10 mg As/L) is increased to 50 mg Sb/L (i.e. 5 fold excess of antimony), arsenic biomethylation is decreased to approximately 15% of the controls.

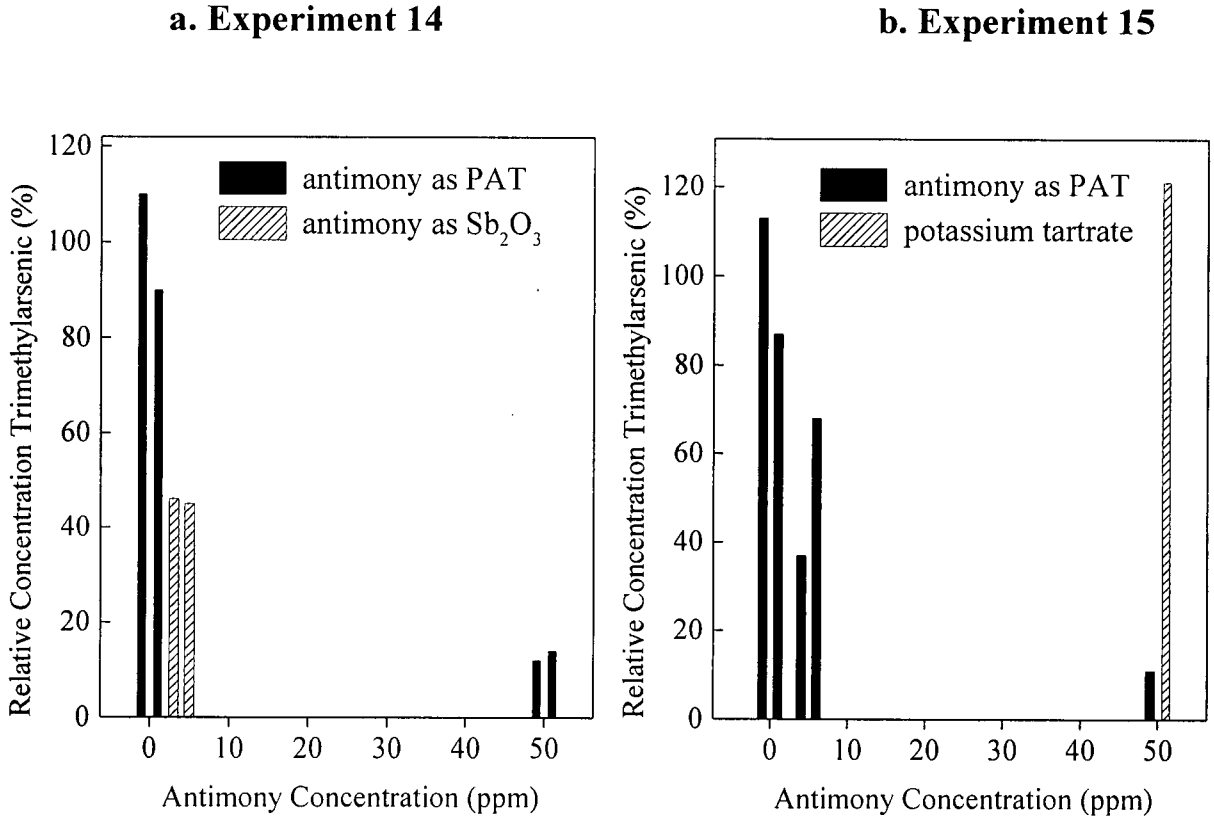

Figure 5.3. Influence of antimony(III) on the biomethylation of arsenic(III): Relative concentration of trimethylarsenic species (%) in cultures of *S. brevicaulis* after 1 month of incubation. All cultures contained 10 mg As/L as sodium arsenite. Cultures contained 0 (controls) to 50 mg Sb/L either as potassium antimony tartrate or as antimony trioxide (Figure 5.3a [////]). PAT=potassium antimony tartrate.

It is possible that the inhibition of sodium arsenite biomethylation by potassium antimony tartrate is not the result of the addition of antimony, but rather is due to the associated potassium tartrate that could complex arsenic, and thus decrease its bioavailability. However, as can be seen in Figure 5.3b, potassium tartrate (50 mg/L) did not decrease arsenic biomethylation. To further show that arsenic biomethylation is inhibited in the presence of antimony(III), antimony trioxide was substituted for potassium antimony tartrate. Although antimony trioxide is not very soluble (~ 4 mg Sb/L in the medium), it still proved to be just as effective at decreasing arsenite biomethylation (Figure 5.3a). That is, in Experiment **14** antimony trioxide (4 mg Sb/L) decreased arsenic biomethylation to 46% of the controls, whereas in Experiment **15**, a similar concentration of antimony (5 mg Sb/L) as potassium antimony tartrate decreased arsenic biomethylation to 53% of the controls.

5.3.2.2 Influence of antimony(III) on the biomethylation of arsenic(V) (Experiments 12 and 15)

Cultures were prepared containing 1 mg As/L as sodium arsenate and to two of these cultures was added 10 mg Sb/L as potassium antimony tartrate (10 fold excess of antimony) (Experiment **12**). The extent of arsenic biomethylation, after 1 month, was 40% of the controls (Figure 5.4a). Thus, a decrease in arsenic biomethylation in the presence of potassium antimony tartrate is not as pronounced for sodium arsenate (biomethylation: 40% of controls) as a substrate compared to sodium arsenite (biomethylation: 10% of controls). This trend was also evident when cultures containing 10 mg As/L as sodium arsenate had 50 mg Sb/L as potassium antimony tartrate added to

them (Experiment **15**, Figure 5.4b). Arsenate biomethylation was decreased to approximately 50% of the controls (versus 11% for arsenite).

These results might be an indication that the ability of antimony to inhibit arsenic biomethylation is achieved by regulation of arsenic transport into the cell rather than by regulation of arsenic biomethylation enzymes. After a reduction step, the biomethylation of arsenate proceeds by the same mechanism as arsenite (Section 1.4.2). So if antimony inhibits the biomethylation enzymes, the effect should be the same for arsenate or arsenite.

a. Experiment 12 **b. Experiment 15**

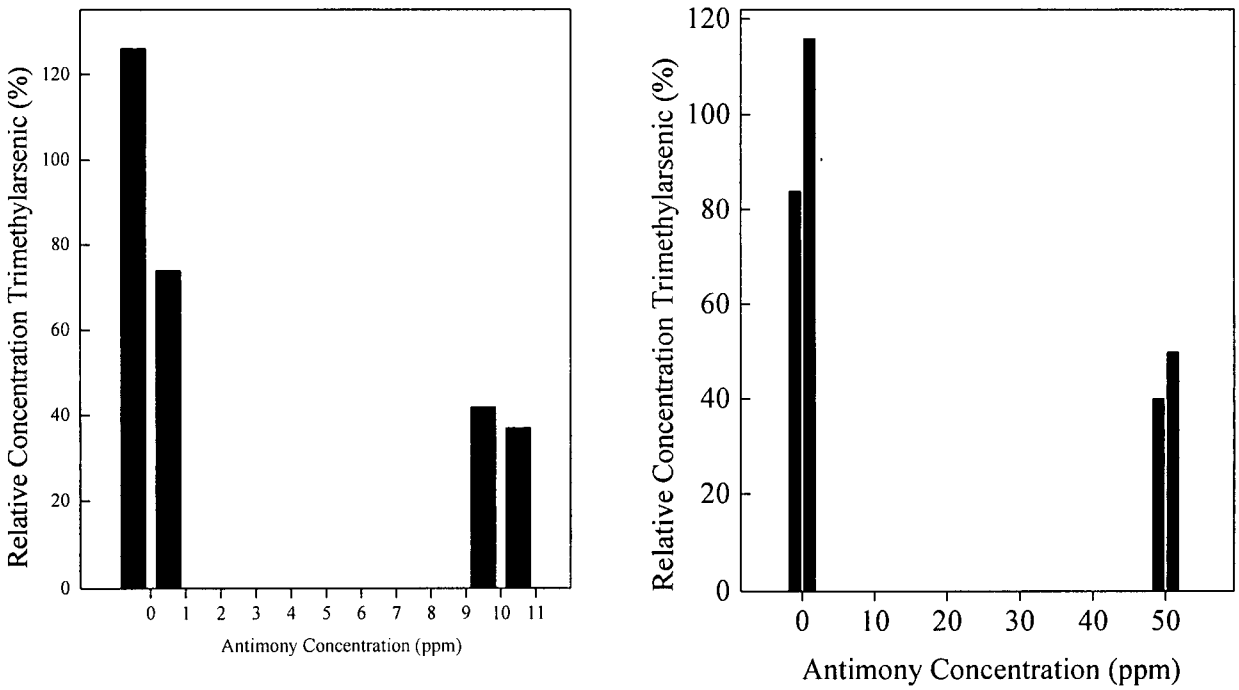

Figure 5.4. Influence of antimony(III) on the biomethylation of arsenic(V): Relative concentration of trimethylarsenic species (%) in cultures of *S. brevicaulis* after 1 month of incubation. Cultures contained 1 mg As/L (**a**), or 10 mg As/L (**b**) as sodium arsenate. Cultures contained 0 (controls) to 50 mg Sb/L as potassium antimony tartrate.

5.3.3 Influence of Antimony(V) on the Biomethylation of Arsenic(III) and Arsenic(V) (Experiment 13)

The presence of antimony(V) in cultures containing arsenic(III) (1 mg As/L) had no significant effect on the biomethylation of arsenic (Table 5.2). Even when an antimony concentration of 100 mg Sb/L was used (i.e. 100:1 ratio of antimony to arsenic), the trimethylarsenic concentration was still at 80% of the control concentration. The slight decrease in biomethylation observed is insignificant, compared to the variation in arsenic biomethylation between cultures (~ 30%). This result is not unexpected because in Chapter 2 it was found that antimony(V) is not taken up, or transformed, by cultures of *S. brevicaulis*, and for this reason the influence of antimony(V) on the biomethylation of arsenic(V) was not examined.

5.4 DISCUSSION

In this chapter it was demonstrated that when *S. brevicaulis* is incubated with arsenic compounds the production of trimethylarsenic species is significantly inhibited in the presence of antimony(III) compounds but not antimony(V) compounds. This phenomenon is more pronounced when using arsenic(III), as opposed to arsenic(V) as the substrate. In contrast, antimony(III) biomethylation by *S. brevicaulis* is not inhibited in the presence of arsenic compounds, in fact potassium antimony tartrate biomethylation is enhanced in the presence of sodium arsenite. This is in spite of the fact that arsenic(III), in the absence of antimony, is much more readily biomethylated (1.2 - 5.3% of added arsenic is biomethylated) by *S. brevicaulis* than antimony(III) (0.0006 - 0.008% of added antimony is biomethylated). These results were published in reference 28.

More research is required to establish how antimony inhibits arsenic biomethylation, and how arsenic enhances antimony biomethylation, but it is clear that studies of contaminated environments, such as mine-tailings, should consider potential interactions between metalloids.

Although *S. brevicaulis* is a crude model, the present results suggest that such interactions should also become part of studies of the human metabolism of arsenic species. Large variations in the amount of arsenic biomethylation in humans, and hence health effects, have been partially attributed to variations in diet[23]. However, these variations in biomethylation might be due to co-exposure to antimony. If arsenic biomethylation is considered a detoxification process then inhibition of this process by antimony(III) would increase arsenic toxicity. On the other hand it has been demonstrated that antimony(III) significantly reduces chromosome mutagenicity of arsenic(III)[20].

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CHAPTER 6

THE INTERACTION OF *SCOPULARIOPSIS BREVICAULIS*, AND OTHER MICROORGANISMS, WITH 10, 10'-OXYBISPHENOXARSINE (OBPA)

6.1 INTRODUCTION

In a submission to the *Expert Group to Investigate Cot Death Theories: Toxic Gas Hypothesis*[1] B. A. Richardson wrote as follows:

"It was recognized by Richardson in 1988 that microbial deterioration of plasticised polyvinyl chloride (PVC) containing arsenical preservatives might result in the generation of extremely toxic gaseous arsines. It was considered that this process might be a cause of sudden infant death."

This statement was based on the recognized phenomenon that volatile toxic arsines, usually trimethylarsine, are produced when some microorganisms, e.g. *Scopulariopsis brevicaulis*, interact with simple arsenic species such as arsenate or arsenite[2-5]. The assumption seems to have been made that microbial action on the arsenical preservatives used in the PVC might also produce toxic arsines. The *only* arsenical, used as a fungicide, in PVC is 10, 10'-oxybisphenoxarsine (OBPA, Figure 6.1). However, OBPA is not commonly used in cot mattress covers, which is probably why Richardson extended his hypothesis to suggest that: "the primary cause of sudden infant death syndrome (SIDS) is poisoning by gaseous phosphines, arsines and stibines, generated by deterioration of cot mattress material by microorganisms, particularly *Scopulariopsis brevicaulis* an otherwise harmless fungus that is normally found in all

domestic environments." The focus of most toxic-gas hypothesis research then shifted to production of stibines from antimony trioxide (present in bedding material as a flame retardant) by *S. brevicaulis*, and this was an impetus for the work presented in Chapters 2-4.

Although OBPA is not commonly used in cot mattress covers, it is marketed as Vinyzene® for use in many consumer products, such as shower curtains, wall coverings and carpets, which often become damp and dirty, and so could be exposed to microbial action. If OBPA is readily degraded by microorganisms to trimethylarsine, as originally implied by Richardson, this could be cause for concern.

Microorganisms could act on OBPA in a variety of ways (Figure 6.1). For instance, the biomethylation of OBPA might produce species **3** or **4**, and such a process for arylarsenicals is known[6]. However, these species would not be volatile at room temperature. Partial cleavage of the aryl-arsenic bond of OBPA and subsequent biomethylation could produce various species (**6**), but again these species would not be volatile at room temperature. The production of volatile species, such as trimethylarsine, would first require the biologically catalyzed cleavage of all aryl-arsenic bonds, an unsubstantiated process in 1988. Thus, there was no experimental foundation, at the time it was first proposed, for Richardson's toxic gas hypothesis based on arsine production from OBPA.

The currently accepted mechanism of trimethylarsine production by *S. brevicaulis* begins with oxidative methylation of arsenite and then there is a series of reduction and oxidative methylation steps (the Challenger mechanism[3]). Thus, for OBPA, trimethylarsine production can only occur after cleavage of all aryl-arsenic bonds.

Alkylarsenic(V) intermediates (Figure 6.1) in Challenger's mechanism have been detected[7]. Indeed, at low concentrations of inorganic arsenic species, volatile trimethylarsine is not produced in significant yields, although trimethylarsine oxide is produced[7]. Therefore, in most experiments, there is no need to directly determine trimethylarsine production, because the determination of intermediates on the pathway, the major products, will indicate if trimethylarsine production is likely.

The microorganisms most likely to degrade OBPA, by aryl-arsenic bond cleavage, are ones that have been exposed to OBPA, or similar compounds, in the environment. With this in mind, microorganisms were isolated from a soil contaminated with cyanodiphenylarsine (Figure 6.2), which is chemically very similar to OBPA, and was used in World War I as a chemical warfare agent (Clark II, Agent DC). If microorganisms from such an environment are unable to cleave aryl-arsenic bonds then it is unlikely that such cleavage could be accomplished by other common microorganisms.

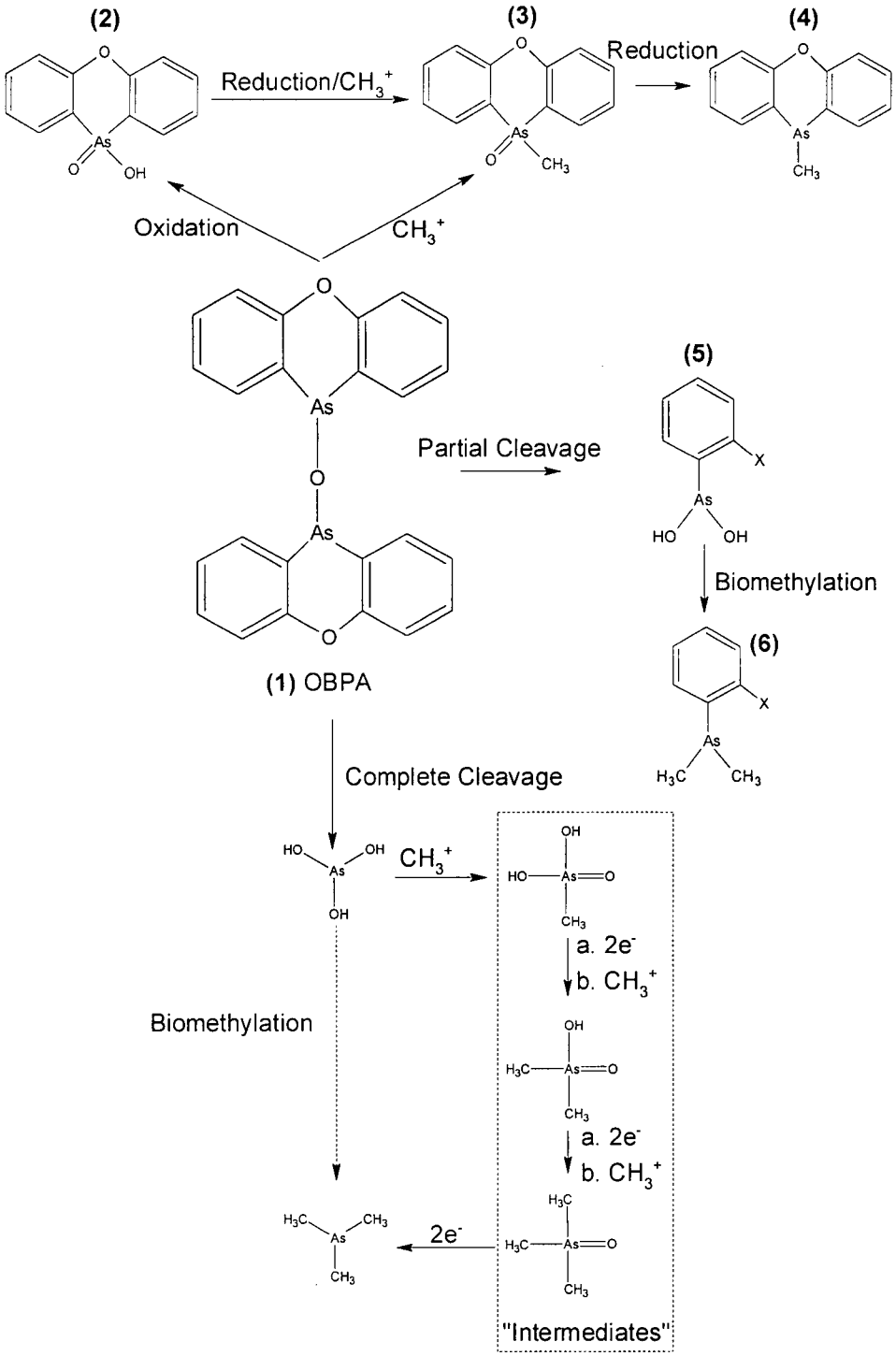

Figure 6.1. Structure of 10, 10'-oxybisphenoxarsine (OBPA) and some possible biotransformation pathways.

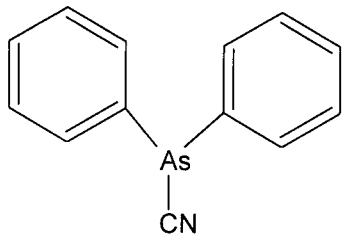

Figure 6.2. Cyanodiphenylarsine

Described in this chapter are results from two studies. In one study, *S. brevicaulis* was incubated with OBPA, and headspace gases and medium from these cultures was examined by using GC-ICP-MS (Section 3.2.5) and HG-GC-AAS (Section 2.2.5.2), respectively, to determine if OBPA is degraded by this microorganism to volatile arsenic species or their precursors. In another study, OBPA-tolerant microorganisms were isolated by Tina Liao from a soil contaminated with cyanodiphenylarsine. The OBPA-tolerant microorganisms were incubated with OBPA, and the medium examined for OBPA-degradation products and biomethylation intermediates, to assess if arsenic volatilization from OBPA is likely.

6.2 MATERIALS AND METHODS

6.2.1 Materials

OBPA was synthesized by Changqing Wang by using a standard literature method[8]. Nutrient agar (NA) and nutrient broth (NB) were acquired from Difco Laboratories. A minimal-salts/glucose (Section 2.2.1) medium was prepared from reagent grade chemicals. The media were autoclaved at 121 °C, 19 psi, for 20 minutes. The OBPA was added to the NA and NB before autoclaving. Reagent grade chemicals, acquired from common distributors, were used for the hydride generation analysis.

6.2.2 Soil Sampling

A small quantity of cyanodiphenylarsine was spilt onto soil during a disposal operation in 1991 at the Defense Research Establishment Suffield (DRES), Alberta, Canada. The site (known as 14A) is easily located because it is completely devoid of vegetation. Two soil samples (0-10 cm depth) were collected on the 17th February 1998. The two soil samples were taken from randomly selected points within the area of the spill.

6.2.3 Details of Cultures

A small amount of each soil sample was streaked onto petri plates containing 1/10 strength nutrient agar saturated with OBPA (5 μg/mL). One set of petri plates was incubated at 17 °C and the second set at 30 °C. All plates were examined daily for the

appearance of colonies; those that grew are described in Table 6.1. The isolated microorganisms were then inoculated into nutrient broth (5 mL full strength NB in 16 × 100 mm test tube) saturated with OBPA (1 mg of OBPA per test tube), and placed on a rotary shaker (25 °C, ~135 rpm, 1.75 inch displacement) for a month. A control that consisted of nutrient medium and OBPA was also incubated on the rotary shaker. The medium from each culture was filtered (0.45 μm) after one month of incubation, and analyzed by using hydride generation-gas chromatography-atomic absorption spectroscopy (HG-GC-AAS) (Section 6.2.4).

A minimal-salts/glucose medium (400 mL, Section 2.2.1) was seeded with 40 mL of *S. brevicaulis* (ATCC #7903) mycelial balls (20-30 balls, ~1 mm diameter). Two replicate cultures were prepared. The cultures were made to be 1 ppm in OBPA by adding an appropriate volume of 1000 ppm OBPA in methanol, (in preliminary experiments it was observed that ≥ 2 ppm OBPA significantly inhibited the growth of *S. brevicaulis*). A control was prepared consisting of medium and OBPA. The cultures and the control were then incubated on the rotary shaker for one month. After one month of incubation, the medium from each culture and the control was filtered (0.45 μm) and analyzed by using hydride generation-gas chromatography-atomic absorption spectroscopy (HG-GC-AAS) (Section 6.2.4).

6.2.4 HG-GC-AAS Analysis.

Analysis was performed by using semi-continuous HG-GC-AAS (Section 2.2.5.2). Appropriate experimental conditions (1 M hydrochloric acid; 2% $NaBH_4$ in

water) were used, so that standards of sodium arsenite, sodium arsenate, methylarsonic acid, dimethylarsinic acid, and trimethylarsine oxide all readily formed volatile hydrides. The sample size was 5 mL, this volume corresponds to concentration detection limits of 1 ng As/mL and 2 ng As/mL for inorganic arsenic species and alkylarsenic species, respectively. OBPA does not form a volatile hydride under these conditions.

6.2.5 Details of Headspace Analysis of *Scopulariopsis brevicaulis* Cultures

Four cultures were prepared that contained 400 mL of minimal-salts/glucose medium (Section 2.2.1) and *S. brevicaulis*. Two of these cultures contained 1 mg OBPA/L (Cultures A and B), made by adding 0.4 mL of 1000 mg OBPA/L, in methanol, to each. Two of these cultures were controls (Cultures C and D), and had 0.4 mL of methanol added to them. The cultures were placed in sterile 1 L Erlenmeyer flasks topped with ground-glass male joints and capped with female ground-glass joints, which were fitted with inlet and outlet glass tubing. The cultures were continuously purged with sterile (0.2 μm filtered) compressed air and the headspace gases were sampled by trapping them in U-shaped glass traps (packed with 10% Supelcoport SP-2100 on Chromosorb) immersed in dry-ice/acetone. The contents of the traps were determined by using GC-ICP-MS (Section 3.2.5). Gas samples were taken 4, 9 and 12 days after preparing the cultures, over approximately 4 hours.

6.3 RESULTS AND DISCUSSION

6.3.1 Contents of Media from Cultures of *S. brevicaulis* and Microorganisms Isolated from Contaminated Soil

Inhibition of *S. brevicaulis* growth was not visually-observed in medium containing 1 mg OBPA/L. At ≥ 2 mg OBPA/L, growth was visually-observed to be inhibited. After 1 month of incubation of *S. brevicaulis* in medium containing 1 mg OBPA/L, the medium was analyzed by using HG-GC-AAS. No alkylarsenic species were detected (Table 6.2). Inorganic arsenic species were detected in the medium, but similar levels were detected in the controls and active cultures, i.e. the inorganic arsenic species detected arise from impurities in the medium and OBPA. Therefore, it appears that *S. brevicaulis* is unable to cleave all the aryl-arsenic bonds of OBPA.

Table 6.1. Microorganisms isolated from cyanodiphenylarsine contaminated soil. Microorganisms were isolated on 1/10 nutrient agar containing OBPA (5 ppm).

Micro-organism	Type	Macroscopic	Microscopic
OPBA-1	Fungus	Dark-green center, white rhizoid.	
OBPA-2	Bacteria	Shiny-white, convex, 2 mm diameter.	Gm (-)[a] coccoid, 0.5 μm, clusters.
OBPA-3	Bacteria	Shiny-white, convex, 1 mm diameter.	Gm (-) coccoid, clusters.
OBPA-4	Bacteria	Pale-pink, shiny, convex, 2 mm diameter.	Gm (-) rods, terminal or central spores, 1.2 μm, singlets and clusters.
OBPA-5	Bacteria	White, shiny, blob-like, various sizes.	Gm (-) coccoid, 0.7 μm, singlets and clusters.
OBPA-6	Bacteria	Yellow, shiny, convex, 2 mm diameter.	Gm (-) coccoid, 0.5 μm, clusters.
OBPA-7	Bacteria	Bright-yellow, shiny, irregular borders, 2 mm diameter.	Gm (-) rods in chains, 1 μm.
OBPA-8	Bacteria	Dark-pink, shiny, 2 mm diameter.	Gm (-) rods, 1.1μm, very big central or terminal spores.
OBPA-9	Bacteria	Dull-salmon-pink, 1 mm diameter.	Gm (-) rods, 1.2 μm, clusters very big terminal spores.
OBPA-10	Bacteria	Dull-white, central peak, 2 mm diameter.	Gm (-) rods in chains, 1 μm.
OBPA-11	Bacteria	Clear, flat, various sizes.	Gm (-) coccoids, in pairs and clusters 0.5 μm.

[a] – Gm (-) = negative gram stain.

About 80 microorganisms were isolated from the two cyanodiphenylarsine-contaminated soil samples by using standard microbiological methods. The actual number may be slightly less because of the possibility of duplicate isolation from more than one soil sample. This number is not as great as would be expected from a healthy soil but it is certainly an indication that the soil is not sterile. By using nutrient agar, saturated with OBPA, 11 macroscopically different microorganisms were isolated, 10 bacteria and 1 fungus, as pure cultures, described in Table 6.1. These microorganisms were subsequently grown for one month in a liquid medium saturated with OBPA. The medium was examined by using HG-GC-AAS and the results are reported in Table 6.2. Controls, containing medium and OBPA, contained ~5-10 ng As/mL as inorganic arsenic (either arsenite or arsenate). Similarly all cultures contained ~5-10 ng As/mL. No alkylarsenic species were detected. Therefore, microbial assisted cleavage of all aryl-arsenic bonds of OBPA did not take place. The 11 OPBA tolerant microorganisms either have some mechanism to exclude OBPA, or they detoxify it by some other means.

Table 6.2. Concentration of hydride forming arsenicals in cultures after one month of growth.

Microorganism	**Inorganic arsenic concentration (ng As/mL)**	**Organoarsenic concentration (ng As/mL)**
S. brevicaulis	2.9	< 2
S. brevicaulis	3.1	< 2
OPBA-1	8.3	< 2
OBPA-2	3.3	< 2
OBPA-3	6.4	< 2
OBPA-4	10.6	< 2
OBPA-5	6.8	< 2
OBPA-6	8.3	< 2
OBPA-7	8.1	< 2
OBPA-8	8.5	< 2
OBPA-9	7.5	< 2
OBPA-10	6.6	< 2
OBPA-11	8.5	< 2
CONTROLS		
Minimal-salts/ Glucose medium	4.5	< 2
Nutrient Broth Control	8.3	< 2

The solubility of OBPA in water (pH 7, 20°C) is 5 mg OBPA/L. Thus, for the cultures of OBPA tolerant microorganisms isolated from soils, *complete* degradation of only 5% of the dissolved OBPA to inorganic arsenic species would yield 75 ng As/mL and would be readily detected by using HG-GC-AAS. For the *S. brevicaulis* cultures, containing 1 mg OBPA/L, degradation of greater than 10% of the OBPA would be readily detected. However, if only partial aryl-arsenic bond cleavage occurred, the resulting aryl-arsenic species (Figure 6.1) would not be detected by using HG-GC-AAS methodology. Reverse phase HPLC with ICP-MS as a detector would enable the detection of such partial-breakdown products; however, only complete-breakdown products are relevant with respect to any hypothesis linking OBPA with poisoning by volatile arsines.

It was shown that the concentration of alkylarsenicals in the medium would have to exceed at least 40 ng As/mL before trimethylarsine production occurs[7]. The present results provide no evidence that microorganisms can breakdown OBPA to the extent where arsenic could be volatilized as trimethylarsine.

6.3.2 Contents of *S. brevicaulis* Headspace

We believe that trimethylarsine would not be produced from OBPA without first cleavage of all aryl-arsenic bonds of OBPA. If this is the case, these degradation products (inorganic arsenic species and methylated intermediates on the pathway to trimethylarsine) should be readily detected if trimethylarsine is indeed an end-product. However, because of the remote chance that direct transformation of OBPA to trimethylarsine might be possible, it was deemed necessary to examine *S. brevicaulis*

headspace gases for trimethylarsine. Such a pathway would presumably require sequential cleavage and methylation reactions starting with the formation of **5** (Figure 6.1).

Two cultures were prepared that contained 1 mg OBPA/L (Cultures A and B) and two control cultures were prepared that did not contain OBPA (Cultures C and D). The cultures were continuously purged with compressed air over a 12 day incubation period. On days 4, 9 and 12 headspace gases were sampled by trapping the gases in U-shaped traps. At the end of the experiment, the contents of the traps were determined by using GC-ICP-MS. Representative GC-ICP-MS arsenic (m/z = 75) chromatograms obtained, on each sample day, for one of the control cultures (Culture C) and one of the cultures that contained OBPA (Culture A), are shown in Figure 6.3. There is a small spike in two of the control chromatograms (Day 4 and Day 9) that might be arsine. The only compound produced in significant quantities is trimethylarsine, although the amount produced was very small. All cultures, both controls and cultures with OBPA added, produced similar quantities of trimethylarsine (~ 1 pg As/hour).

The content of each trap was calculated (pg As) by using a one point calibration / internal standard method, described by Feldmann (Section 3.2.5.2)[9]. Also the amount of trimethylarsine produced over the sample time (pg As/hour) was calculated. By assuming the rate of trimethylarsine production between sampling is the same as that over the sample period, the total amount of trimethylarsine produced over twelve days of incubation was estimated to be 0.6 (Cultures A & B) and 0.4 (Cultures C & D) ng As.

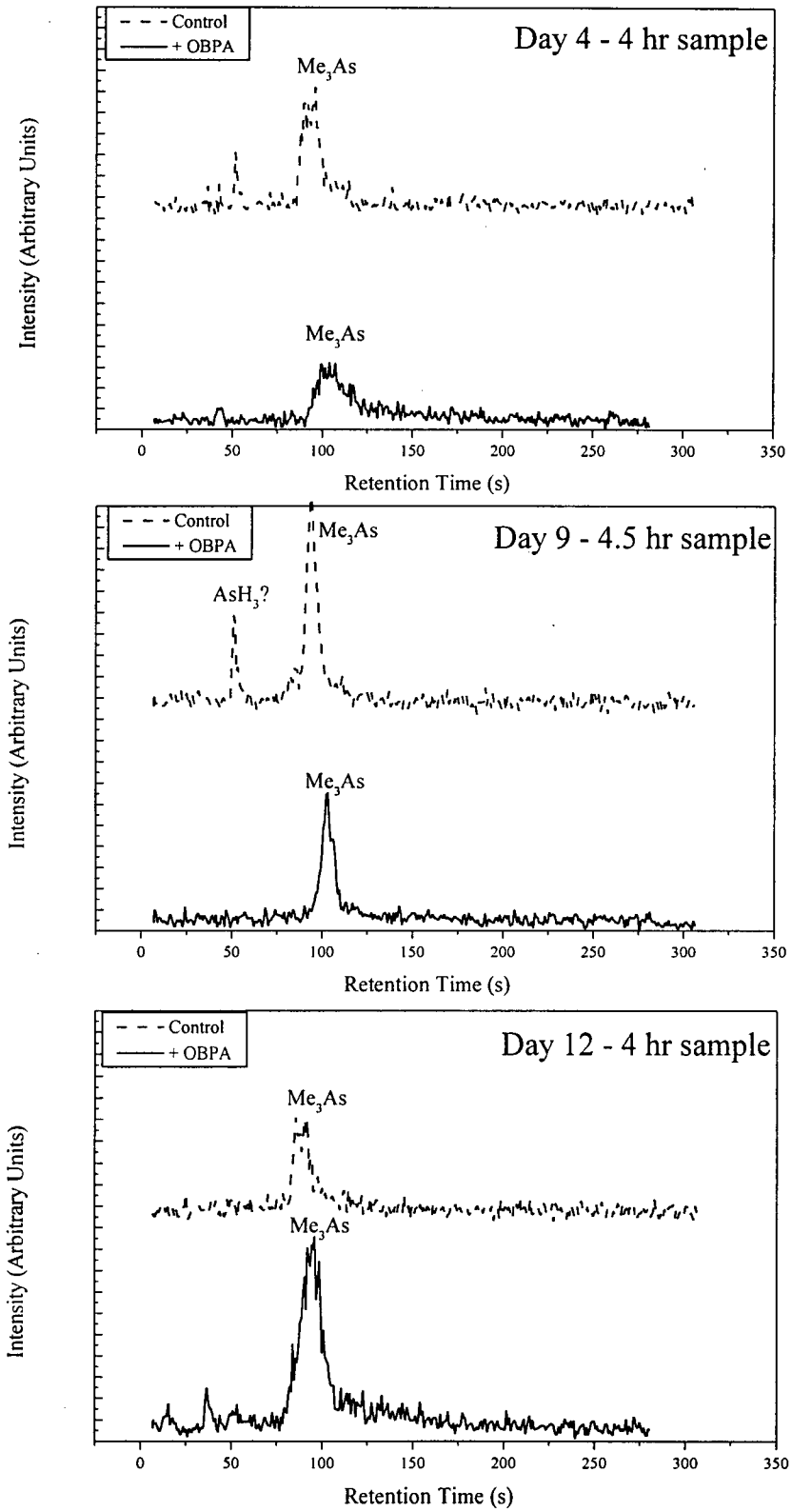

Figure 6.3. GC-ICP-MS chromatograms (m/z = 75) obtained by analyzing headspace gas samples taken on days 4, 9 and 12 from cultures A (*S. brevicaulis* + OBPA) and C (control: *S. brevicaulis*).

The amounts of trimethylarsine detected were extremely small, over the 12 day period < 0.0005% of the arsenic in OBPA was volatilized, which might be attributed to inorganic arsenic impurities in the OBPA. In contrast, 1% of arsenic is biomethylated, to trimethylarsine oxide, over 5 days when *S. brevicaulis* is cultured in 1 mg As/L as sodium arsenite or arsenate[7]. Thus, there is no evidence that *S. brevicaulis* can transform OBPA to significant amounts of trimethylarsine.

6.4 REFERENCES

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CHAPTER 7

CONCLUSION

Previous researchers have detected alkylantimony species in the environment[1-6], but only in a few cases were these species linked to a specific organism[1,2]. Laboratory cultures of microorganisms that biomethylate antimony were unknown at the commencement of this research. Because pure cultures of microorganisms would be useful in model studies of antimony biomethylation, a major goal in this research was to demonstrate the biomethylation of inorganic antimony compounds in laboratory cultures of the filamentous fungus *Scopulariopsis brevicaulis*, which is well known for its ability to biomethylate inorganic arsenic compounds to produce trimethylarsine[7,8] and nonvolatile alkylarsenic(V) species[9]. This goal was achieved and the results were published in references 10-14. Dimethylantimony and trimethylantimony species were detected in the media of *S. brevicaulis* cultures by developing a solid phase extraction technique to remove inorganic antimony(III) species from media samples before analysis by using HG-GC-AAS. Because SPE removed the inorganic antimony(III) species, large volumes of sample could be used for hydride generation analysis without interference from large quantities of stibine, and so the concentration detection limit of the technique was significantly lowered. Trimethylstibine was detected in headspace gases of *S. brevicaulis* by continuously trapping headspace gases in U-shaped traps at - 78 °C and analyzing the contents of the traps by using GC-ICP-MS[15]. This procedure avoids problems that might arise from the low stability of trimethylstibine at room temperature,

because the trimethylstibine is rapidly removed from the culture as it is produced and trapped in an environment where it is stable.

The major products of biomethylation (~95%) under aerobic conditions were nonvolatile dimethylantimony and trimethylantimony species, detected in media samples (Chapter 2). A minor product of biomethylation (~5%) was volatile trimethylstibine detected in headspace gas samples (Chapter 3).

Gradual abiotic oxidation of inorganic antimony(III) occurred in cultures of *S. brevicaulis*. Inorganic antimony(V) compounds were not biomethylated by *S. brevicaulis*, nor were they reduced to antimony(III) species, or taken up by the culture. The amounts of dimethylantimony and trimethylantimony species produced over one month did not significantly increase when the concentration of potassium antimony tartrate was increased by two orders of magnitude. Biomethylation products were not detected in biomass extracts, but this may be because these species are not readily extracted.

The nonvolatile dimethylantimony and trimethylantimony species were detected by using hydride generation techniques, so the actual precursor species are not known. In the future, these species should be identified, probably by combining a sensitive MS technique with a separation technique. The concentrations of nonvolatile dimethylantimony and trimethylantimony species were typically between 1 and 10 ng Sb/mL. However, these numbers should only be taken as an estimate because some major assumptions were made when performing the analysis. A key assumption was that the response of the standard, trimethylantimony dichloride, is the same as the response of the

analytes, trimethylantimony species produced by *S. brevicaulis*. Again, an HPLC-MS technique would be very useful to confirm the results obtained by using hydride generation.

Alkylantimony species were not detected in medium-only and nonliving-cell controls, even when these controls were supplemented with L-methionine. Thus, the biomethylation of inorganic antimony(III) compounds is not a passive process. Biomethylation may arise from the products of active metabolism acting upon arsenic and antimony (non-enzymatic biomethylation), or biomethylation may be an enzymatic process. Biomethylation was shown to be stereospecific (Chapter 4) suggesting that biomethylation is enzymatic.

The yields of biomethylation products (trimethylstibine, and nonvolatile dimethylantimony and trimethylantimony species) from cultures of *S. brevicaulis*, containing inorganic antimony compounds, are orders of magnitude lower than those obtained when inorganic arsenic compounds are used as a biomethylation substrate. This phenomenon may be specific to *S. brevicaulis*, or it may be that antimony is not readily biomethylated by most microorganisms.

Possible reasons why antimony compounds are not so readily biomethylated are:

1. Antimony compounds cannot readily access the cells.
2. Antimony compounds access the cells and are biomethylated in the same manner as arsenic compounds but at a reduced rate.
3. The mechanism of antimony biomethylation is completely different from the mechanism of arsenic biomethylation.

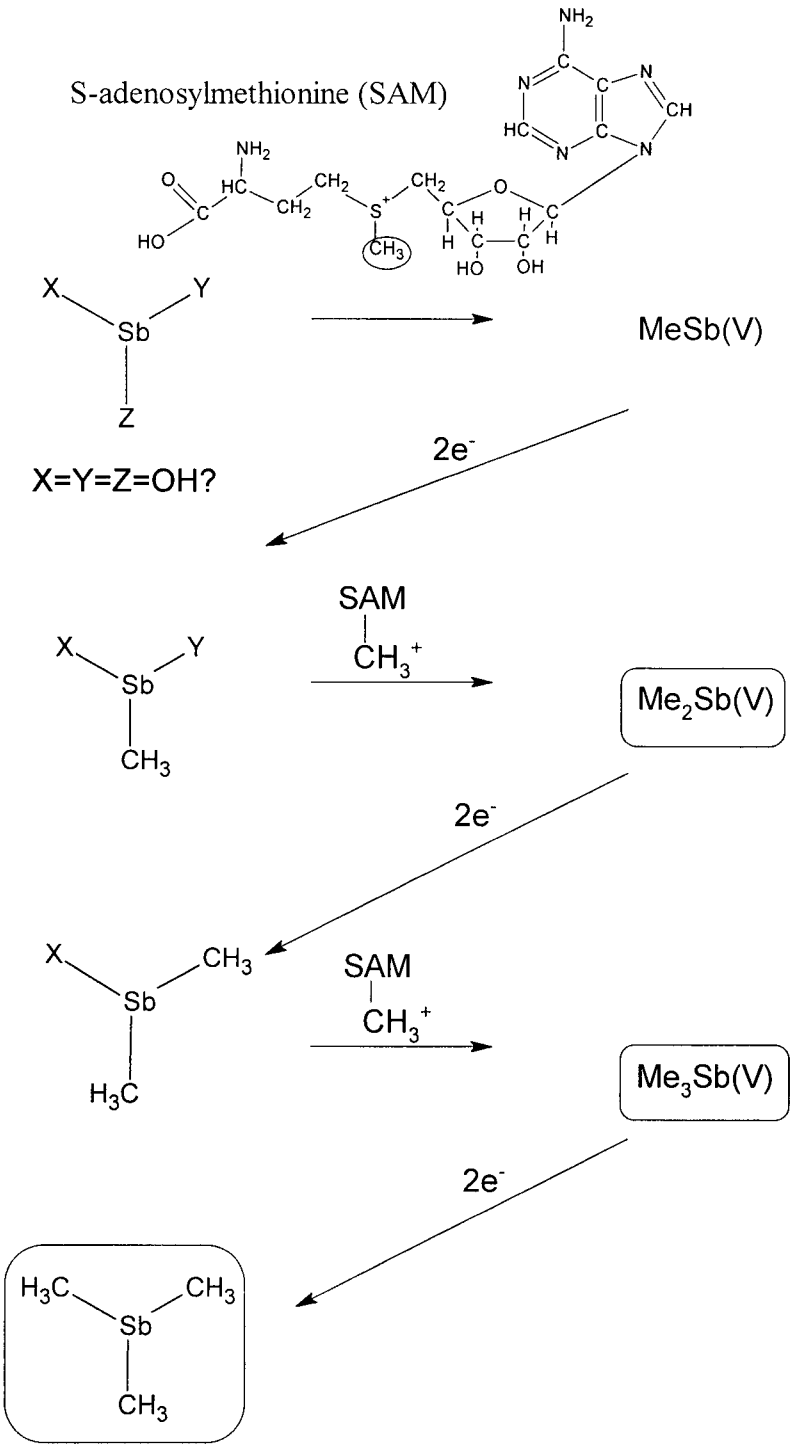

Figure 7.1. Proposed mechanism for the biomethylation of antimony. Species enclosed in boxes were detected in *S. brevicaulis* cultures.

Thus, another reason for performing the research described in this thesis was to examine the mechanism of antimony biomethylation in an attempt to explain why antimony is less readily biomethylated. The generally accepted mechanism of arsenic biomethylation, originally proposed by Challenger[8], involves a series of oxidative additions of a methyl carbocation to nucleophilic arsenic(III) and reduction of the arsenic(V) product so that the cycle can repeat. An analogous pathway can be proposed for antimony biomethylation (Figure 7.1). It is presumed that antimony trioxide and potassium antimony tartrate are sources of antimony species, such as $Sb(OH)_3$, that have a lone electron pair that makes the species sufficiently nucleophilic to react with a methyl carbocation. Unfortunately the solution chemistry of antimony species is poorly understood. Also, the nature of the inorganic groups in the nonvolatile alkylantimony species is not known. Thus X, Y and Z (Figure 7.1) represent unknown inorganic groups.

Table 7.1. Comparison of details of antimony and arsenic biomethylation for *S. brevicaulis.* E refers to either arsenic or antimony.

	Arsenic	**Antimony**	**References**
Reduction of E(V) to E(III)	✓	✖	Chapter 2, [9]
Oxidation of E(III) to E(V)	✖	✓	Chapter 2
Biomethylation of E(V)	✓	✖[1]	Chapter 2, 17
Biomethylation of E(III)	✓ High yield	✓ Low yield	Chapter 2, Chapter 3, 17-19
Biomethylation of RE(V), $R_2E(V)$	✓	No data[2]	9
Detection of intermediate: MeE(V)	✖	✖	Chapter 2, 9
Detection of intermediates: $Me_2E(V)$ and $Me_3E(V)$	✓ High yield	✓ Low yield	Chapter 2,[9]
Detection of volatile final product Me_3E	✓ High yield	✓ Low yield	Chapter 3, 17-19
L-methionine as a methyl donor	47%	47%	Chapter 4
D-methionine as a methyl donor	16%	20%	Chapter 4

[1] Results are uncertain, in this work no evidence was found for biomethylation of antimony(V). However Jenkins *et al.*[17,19] found evidence of antimony(V) biomethylation even though it was less than for antimony(III).

[2] Experiments have not been performed because compounds of the type RSb(V) and $R_2Sb(V)$ are not available.

At the heart of the Challenger mechanism is the reaction of the nucleophilic arsenic(III) with the methyl carbocation. In the laboratory, the model reaction of this process is the reaction of methyl iodide with sodium arsenite to form sodium iodide and methylarsonic acid (Meyer reaction). In the laboratory, antimony(III) does not appear to be nucleophilic enough to react with methyl iodide to form alkylantimony(V) derivatives[16]. In biological systems, methyltransferase enzymes should be able to surmount this barrier. The redox potentials for arsenic(III)/arsenic(V) and antimony(III)/antimony(V) are similar, so it is very likely that antimony biomethylation proceeds by the same mechanism as arsenic. It is likely that antimony biomethylation is a fortuitous process that occurs when arsenic methyltransferase enzymes, such enzymes have been isolated from mammalian systems, act upon antimony. The reason for believing that antimony biomethylation is a fortuitous process is that most researchers consider arsenic biomethylation to be a detoxification process but antimony is less toxic (as it cannot readily access the cell) and less common than arsenic, and so enzymes have probably evolved in response to high concentrations of arsenic rather than antimony.

Probing the mechanism of antimony biomethylation requires performing the types of experiments that were done to establish the mechanism of arsenic biomethylation (Table 7.1)[7,8]. The best evidence that Challenger presented to support his proposed mechanism is that methylarsonic acid and dimethylarsinic acid are biomethylated to trimethylarsine by *S. brevicaulis*; and compounds of the type $RAsO(OH)_2$, and RR'AsO(OH) are biomethylated by *S. brevicaulis* to $RAsMe_2$ and RR'AsMe,

respectively[8]. These types of experiments can not be done for antimony because compounds of the type $RSbO(OH)_2$, and RR'SbO(OH) are not available.

Cullen *et al.*[9] detected nonvolatile dimethylarsenic and trimethylarsenic species in the media of *S. brevicaulis* cultures. These species are expected intermediates on the Challenger pathway so their detection provides further support for the proposed mechanism of arsenic biomethylation. In the present research, dimethylantimony and trimethylantimony species were detected in the medium of *S. brevicaulis* (Chapter 2). The possibility existed that these putative intermediates on the pathway to trimethylstibine might arise from the oxidation of trimethylstibine or that they might even be analytical artifacts. This possibility was examined and there was no evidence to suggest that the dimethylantimony species is an analytical artifact or to suggest that it arises from the oxidation of trimethylstibine. It was not possible to differentiate between trimethylantimony species in the medium that might arise from trimethylstibine oxidation and those that are intermediates on the pathway to trimethylstibine. Indeed, this is also the case for arsenic. However, the oxidation of trimethylstibine and trimethylarsine in cultures of *S. brevicaulis* in these studies is unlikely (Section 3.1).

Challenger provided further evidence to support his proposed mechanism by suggesting the source of the methyl carbocation in arsenic biomethylation as "active" methionine (subsequently identified as S-adenosylmethionine). Similar experiments for antimony were described in Chapter 4 where L-methionine was established as a source of the methyl carbocation for antimony biomethylation. Furthermore, labeling studies revealed that the amounts of methyl groups from L-methionine incorporated into

trimethylantimony species were similar to the amounts incorporated into trimethylarsenic species.

Aposhian *et al.*[20] isolated arsenic methyltransferase enzymes from various mammalian systems. It is expected that both arsenic and antimony biomethylation in *S. brevicaulis* involves some sort of methyltransferase enzyme. Almost all enzymatic processes are stereospecific, so if arsenic and antimony biomethylation does involve an enzyme (rather than being a purely chemical process), the methyl groups of L-methionine and D-methionine should be incorporated into alkylantimony and alkylarsenic species to different extents. This is indeed the case (Chapter 4). The methyl group of D-methionine is not incorporated as readily into alkylantimony and alkylarsenic species. The fact that there is some incorporation of the methyl group of D-methionine into alkylantimony species might be due to racemization of D-methionine.

Although many similarities were found to exist in the biomethylation of arsenic and antimony, some significant differences were also observed (Table 7.1). The significant differences are:

i. *S. brevicaulis* rapidly reduces arsenate to arsenite, but antimony(V) is not reduced to antimony(III).

ii. Arsenate is biomethylated, most likely after reduction within the cell to arsenite, but antimony(V) is not biomethylated.

iii. The yields of alkylantimony species are very much lower than the yields of alkylarsenic species.

The reduction of arsenate to arsenite is a well-known cellular process; arsenate is structurally very similar to phosphate so is actively taken up by cells; reduction of arsenate followed by elimination of arsenite then provides a means of detoxification. The structures of antimony(V) species, such as the octahedral hexahydroxyantimonate, are different from the tetrahedral phosphate and arsenate, so antimony(V) species are not taken up by the same active systems as phosphate, consequently there is no need for the cells to reduce antimony(V) species. Thus points i. and ii. are probably explained by cell transport phenomena.

Because the current evidence suggests that antimony biomethylation occurs via the same mechanism as arsenic biomethylation, then the minimal rate of antimony biomethylation, point iii., can be explained in terms of either a low rate of antimony turnover at the enzyme site or a low concentration of antimony at the site, as a result of low uptake, or both. To elucidate the reason for minimal antimony biomethylation more research needs to be performed. For instance, a useful experiment would be to obtain a cellular extract and examine how effective this extract is at antimony biomethylation.

It was shown that the mechanisms of antimony and arsenic biomethylation in cultures of the model microorganism *S. brevicaulis* are probably the same. This may also be the case for other organisms. Very little work on the biomethylation of antimony in mammals has been performed although one study claimed to show that antimony biomethylation in mammals does not occur[21], but detection limits for alkylantimony species were not given.

If antimony biomethylation does indeed proceed via the same mechanism as for arsenic then, like arsenic, byproducts of this process are expected to form in the environment. That is, when the adenosyl group of SAM, instead of the methyl group, attacks the nucleophilic antimony(III) then stibiosugars might form (Figure 7.2), Benson provided some evidence for stibiosugars in marine algae[22]. In the case of arsenic, the arsenosugars were first identified by using a natural-products approach[23]; arsenosugars were isolated by using preparative-scale chromatography and identified by using NMR and X-Ray crystallography. In the case of antimony, because of the low concentration of antimony in the environment, and the low rates of biomethylation indicated in this research, a natural-products approach is not practical. The most advanced analytical methodology will need to be used to detect such putative species; i.e. high-resolution chromatography and multiple stages of MS with high sensitivity. If antimony sugars do indeed exist, they are unlikely to have much influence in the environment, but their detection will allow greater understanding of the biotransformation of metalloids in general.

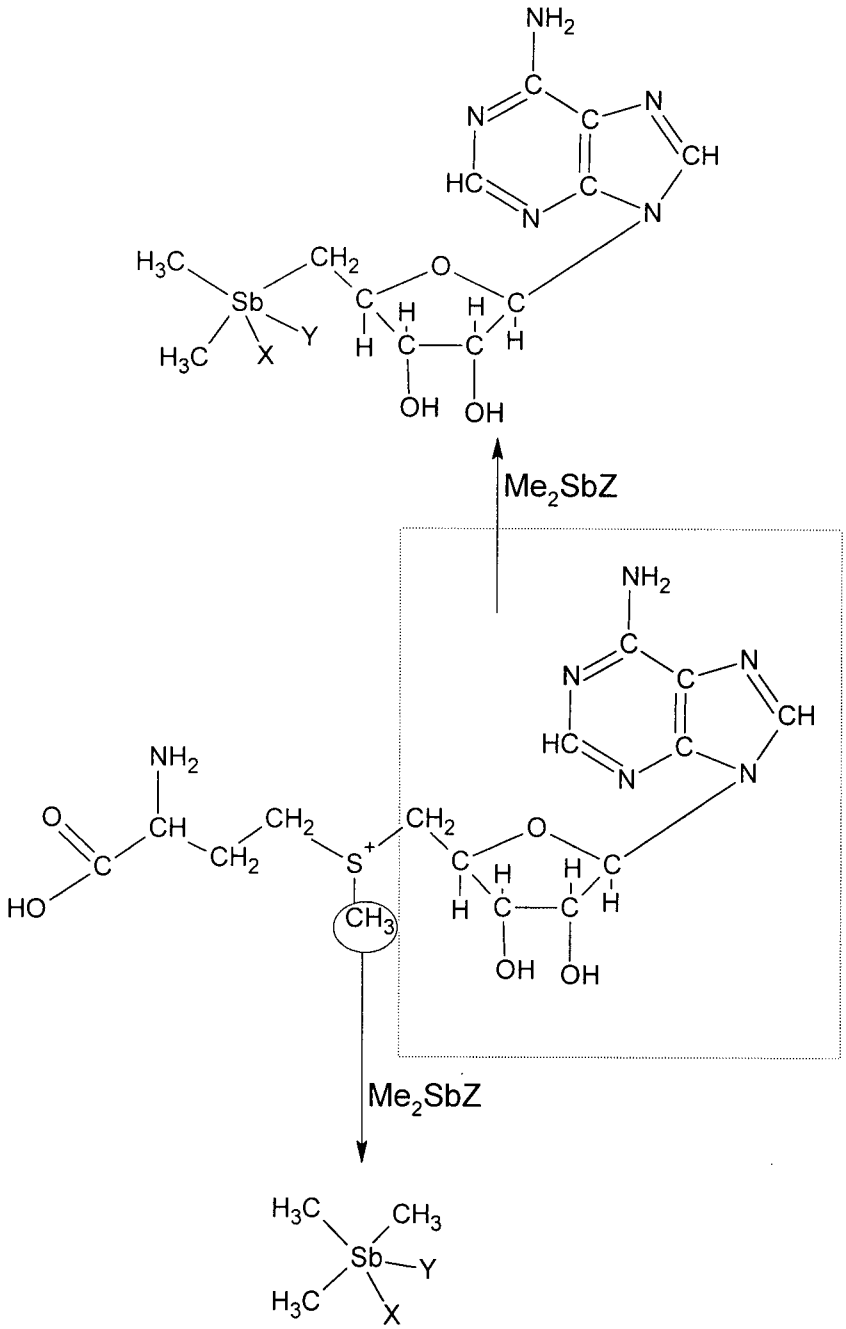

Figure 7.2. Possible products from the reaction of dimethylantimony(III) with SAM.

Because the evidence obtained throughout this research supports the notion that antimony biomethylation involves the same enzyme that performs arsenic biomethylation, it seemed reasonable that antimony and arsenic might compete in some manner for the enzyme. In the environment, where biomethylation modifies the effects of metalloid species, such a process might be very significant. This possibility was examined in Chapter 5 and it was found that inorganic antimony(III) compounds (potassium antimony tartrate or antimony trioxide) but not inorganic antimony(V) compounds (potassium hexahydroxyantimonate) inhibit the biomethylation of sodium arsenite by *S. brevicaulis*; less inhibition was observed for the biomethylation of sodium arsenate. The ratio of antimony to arsenic was more important than the absolute amount of antimony. The presence of sodium arsenite increased the biomethylation of potassium antimony tartrate. This work, and other research[24-27], indicates that it is worthwhile considering the interaction of co-contaminants when studying various environmental systems.

The primary aim of the research, presented in this thesis, was to demonstrate the production of trimethylstibine by *S. brevicaulis*. This aim was adopted because of concerns that the production of toxic volatile trimethylstibine by microorganisms in an infant's cot might be a cause of sudden infant death[28]. A logical extension of this work was to examine other possible sources of toxic gases. When the toxic gas hypothesis was originally proposed, Richardson suggested that the production of trimethylarsine from 10, 10'-oxybisphenoxarsine (OBPA), a germicide used in many consumer products, by microorganisms, especially *S. brevicaulis*, is possible. In Chapter 6, this possibility was examined and refuted. The results, presented in this thesis, do not preclude a toxic-gas

cause of SIDS because this work has focused only on very specific aspects of the toxic-gas hypothesis. However, these results do not support the specific case that antimony biomethylation by *S. brevicaulis* causes SIDS, a conclusion that was also reached by the Limerick committee[29].

The biomethylation of antimony by microorganisms, including *S. brevicaulis*, is an exciting field where much work still needs to be done. For instance, other species of fungi and different strains of *S. brevicaulis* should be studied to determine if there are any differences in the amounts of alkylantimony species produced. Anaerobic organisms also should be thoroughly investigated. Arsenic methyltransferase enzymes have been isolated, so it would be fascinating to determine if these enzymes can act upon antimony(III) species. The impact of these processes on the environment should be considered. For such studies to progress more selective and sensitive analytical techniques will probably need to be developed, and suitable standards (standard reference materials, stibiobetaine, stibiosugars, etc.) must be prepared.

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