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Confirmation of the Aerobic Production of Trimethylstibine by *Scopulariopsis brevicaulis*

Paul Andrewes,[1] William R. Cullen[1]* and Elena Polishchuk[2]
[1]Environmental Chemistry Group, Department of Chemistry, University of British Columbia, Vancouver BC, Canada V6T 1Z1
[2]Biological Services Facility, Department of Chemistry, University of British Columbia, Vancouver BC, Canada V6T 1Z1

The filamentous fungus *Scopulariopsis brevicaulis* produces volatile trimethylstibine, found in the culture headspace, when grown in an antimony(III)-rich medium under aerobic conditions. The trimethylstibine was purged from cultures using a continuous flow of compressed air and trapped in a U-shaped tube containing Supelcoport SP 2100 at −78 °C. The trap contents were determined by using GC–ICP–MS methodology. Typically between 60 and 500 pg of trimethylstibine was trapped during sampling (12 h) from cultures containing 1000 μg Sb ml^{-1} as potassium antimony tartrate. The total production of trimethylstibine over 18 days of growth was estimated at 10 ng. Trimethylarsine was produced in greater quantities than trimethylstibine, even though no arsenic compounds were added to the medium.

Keywords: *Scopulariopsis brevicaulis*; biomethylation; potassium antimony tartrate; trimethylstibine

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INTRODUCTION

The fungus *Scopulariopsis brevicaulis* is well known for its ability to biomethylate arsenic.[1,2] Methylation of antimony by *S. brevicaulis* has been demonstrated recently.[3–5] The aerobic production of methylantimony compounds was established by using hydride derivatization of medium samples followed by GC–AAS;[3] Me_3Sb and Me_2SbH were the principle reduction products, and presumably originated from the appropriate antimony(V) precursors. Methylantimony compound production has also been demonstrated by trapping headspace gases of cultures under anaerobic conditions[4,5] (after a period of aerobic incubation with potassium antimony tartrate) even though *S. brevicaulis* is not an anaerobic micro-organism. This observation has been taken as evidence that trimethylstibine is produced in the aerobic phase of growth, but it is also possible that under anaerobic conditions involatile trimethylantimony(V) species in the medium (which are produced during aerobic growth) are reduced to trimethylstibine.

The presence of trimethylstibine in the headspace of *aerobic* cultures of *S. brevicaulis* has not been demonstrated directly, although its formation has been implied because of the measurement of antimony in remote nitric-acid traps.[4,5] However, antimony transport from cultures could be the result of the formation of other volatile species such as stibine (SbH_3).

In our earlier work with cultures of *S. brevicaulis* containing antimony, we were able consistently to determine methylantimony compounds in the medium (>300 ng), but we detected volatile trimethylstibine only occasionally at ultratrace levels.[3] For example, when a 14-litre fermenter (containing 9 l of medium) was used, we estimated that <1 ng of volatile antimony species were produced by the culture over 18 days.

In the present work, by refining the analytical technique used previously, and by using significantly higher doses of potassium antimony tartrate, we have been able to detect significantly higher levels of trimethylstibine in a repeatable manner. This is the first time that trimethylstibine has been directly detected in the headspace of aerobic

* Correspondence to: William R. Cullen, Environmental Chemistry Group, Department of Chemistry, University of British Columbia, Vancouver BC, Canada V6T 1Z1.
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Table 1 Sampling times, amounts of trimethylarsine and trimethylstibine trapped, and amounts trapped per hour of sampling, for the cultures in Experiment 2 containing 1000 μg Sb ml^{-1}

Elapsed time (days)	Sampling time (h)	Trimethylarsine in trap (pg As)[a]	Trimethylstibine in trap (pg Sb)[a]	Trimethylarsine (pg As/h^{-1})	Trimethylstibine (pg Sb/h^{-1})
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

[a] Data are incremental. Trimethylarsine (570 pg As) was produced between days 5 and 7, at the sampling times shown.

cultures, and confirms previous work implying its formation.

EXPERIMENTAL

A minimal-salts/glucose medium[6] (400 ml) was seeded with 40 ml of *S. brevicaulis* (ATCC 7903) mycelial balls (20–30 balls, ~1 mm diameter). The cultures were maintained in 1-litre Erlenmeyer flasks capped with cotton stoppers. After one month of growth the medium was decanted off the cultures and 400 ml of fresh medium was added. The antimony compounds were added (0.2 μm-filter sterilized) and each culture was transferred to a 1-litre Erlenmeyer flask topped with a ground-glass male joint (Pyrex no. 4980, stopper no. 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 inlet glass tube 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. Throughout the period of the experiment there was a constant flow of compressed air through each flask (~10 ml min^{-1}). At various stages (for example, see Table 1) of the experiment a U-shaped glass trap (22 cm × 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) and cooled in a large dry-ice/acetone-filled Dewar flask (−78 °C). The traps were left in place overnight (see Table 1 for typical sampling times). Gas flow rates were measured at the beginning and at the end of sampling and did not change significantly.

Two experiments were performed. In Experiment 1, two cultures contained potassium antimony tartrate (1000 μg Sb ml^{-1}), and one culture contained trimethylantimony dichloride (1 μg Sb ml^{-1}). In Experiment 2, performed one month later, two cultures contained potassium antimony tartrate (1000 μg Sb ml^{-1}), and one control culture contained no added antimony. The Erlenmeyer flasks were shaken horizontally [~135 rpm, 1.75-inch (7.8-cm) displacement], maintained at 26 °C, and kept in the dark to minimize 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 remove all medium completely) and drying at 150 °C. The average dry weight was 2.5 g (range: 2.2–2.9 g).

The sampling traps were capped with rubber septa and stored at −78 °C either in a dry-ice freezer or in a dry-ice/acetone bath. For work-up, the sampling trap was connected to a helium supply, and to an identical U-tube trap cooled in liquid nitrogen (−196 °C), the sample trap was allowed to warm, and the contents were volatilized by means of helium flow of 70 ml min^{-1} and gentle

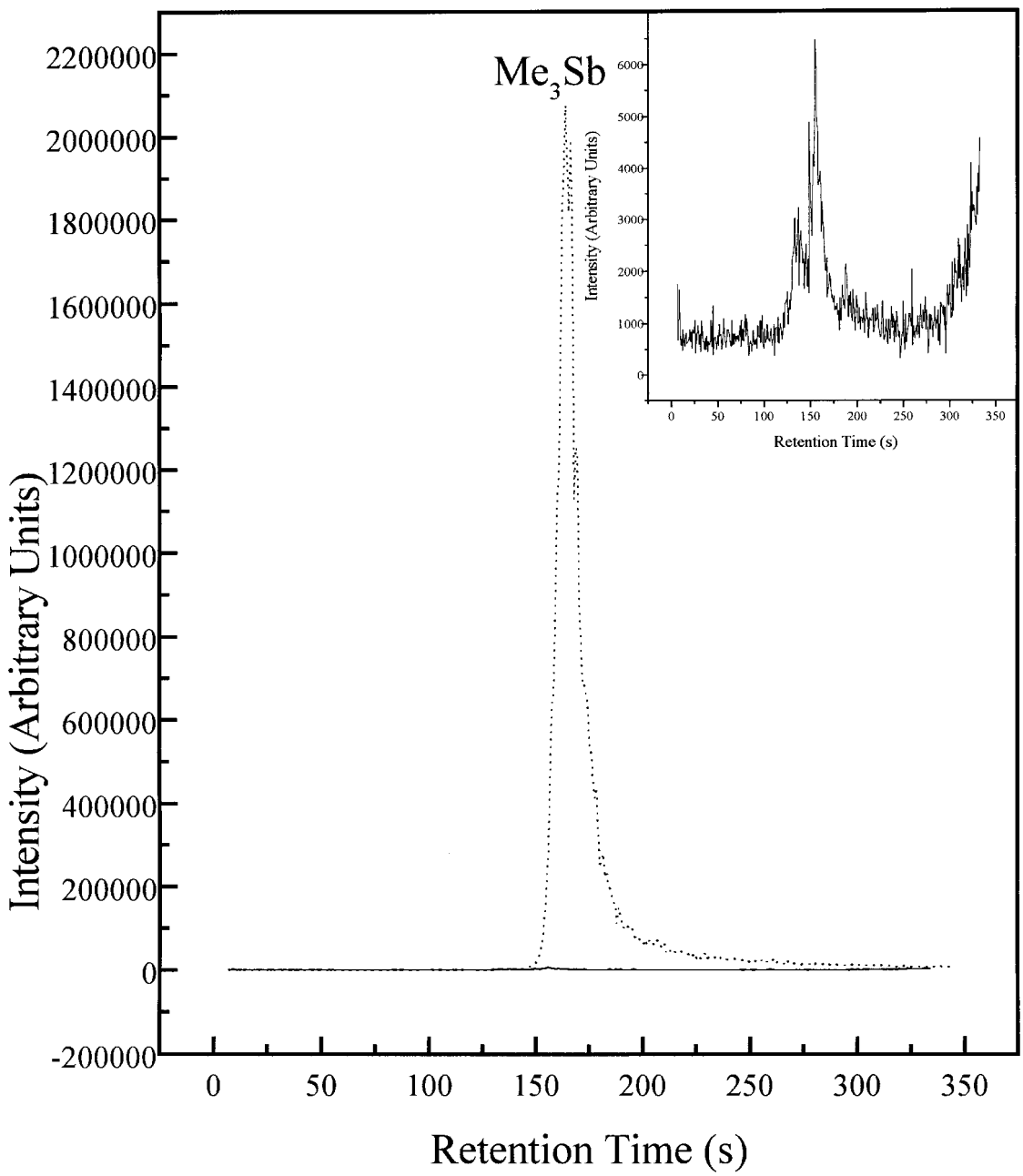

Figure 1 GC–ICP–MS single-ion chromatogram for antimony ($m/z = 121$) for culture headspace samples taken six days into Experiment 2. ·····, Sample taken from Culture 1, containing potassium antimony tartrate; —, control sample taken from Culture 3, which did not have potassium antimony tartrate added. Inset: zoom plot of control sample.

heating to 200 °C. In this way the gases were cryofocused in the second trap in order to reduce any previously broadened distribution of the volatile species in the sampling trap. After the cryofocusing step, the second trap was removed from the liquid nitrogen, connected to the ICP–MS, and heated to 200 °C. Details of the interface to the ICP–MS, and the ICP–MS operation, have been

described previously.[7] In Experiment 2, semiquantitative calculations of the amounts of gaseous species were made by comparing instrument responses for the gaseous samples against aqueous standards, as has been described elsewhere.[7]

Identification of the species was based on retention time. A sample of trimethylstibine was generated by sodium borohydride reduction of Me_3SbCl_2.

RESULTS AND DISCUSSION

In Experiment 1, the two cultures containing potassium antimony tartrate (1000 μg Sb ml^{-1}) and the culture containing trimethylantimony dichloride (1 μg Sb ml^{-1}) produced enough trimethylstibine to give a significant signal (average S/N $\simeq$ 1000) for all samples taken over the two-week period of the experiment. The peak areas for the trimethylstibine in these samples were approximately two orders of magnitude greater than typical values in any of our previously reported work. No attempt was made to quantify these preliminary data. The average response for trimethylstibine in the culture containing trimethylantimony dichloride (1 μg Sb ml^{-1}) was about six times more than that for the cultures containing potassium antimony tartrate (1000 μg Sb ml^{-1}).

In Experiment 2, the two cultures containing potassium antimony tartrate produced trimethylstibine which was detected in all headspace gas samples (Table 1). The GC–ICP–MS peak areas were similar to those seen in Experiment 1. In the control culture, where no potassium antimony tartrate was added, only traces of trimethylstibine were detected. A system blank was measured after compressed air was passed through the tubing and filters, and then through the cold (−78 °C) U-tube trap: the Erlenmeyer flask of culture was omitted. No antimony or arsenic compounds were detected in this blank. The absolute detection limit for the GC–ICP–MS was ~0.15 pg.

A typical single ion chromatogram for antimony (m/z = 121), for a headspace gas sample, is shown in Fig. 1. The sample was taken from an active culture grown in the presence of potassium antimony tartrate. Also shown is the antimony chromatogram for a gas sample taken at the same time from the control culture to which no potassium antimony tartrate was added. It is interesting to note that the peak for the control culture gas chromatogram is about the same size as we have occasionally

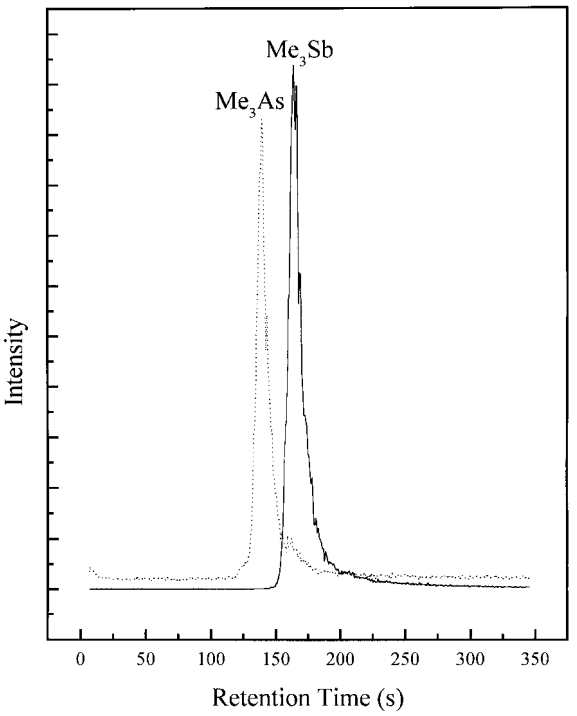

Figure 2 GC–ICP–MS single-ion chromatograms for antimony (m/z = 121, —) and arsenic (m/z = 75, ·····) from a headspace sample taken from Culture 1 of Experiment 2 after five days.

observed in the past in our trapping experiments from cultures containing potassium antimony tartrate.[3] Thus we have managed to increase the yield of trimethylstibine from our cultures significantly. The reasons for the increased yield we believe are: working at 1000 μg Sb ml^{-1} (compared with 10–100 μg Sb ml^{-1} in our previously reported experiments), using two to four times as much biomass as in our previous studies, and avoiding the use of latex tubing in our systems. Separate experiments revealed that trimethylstibine obtained by hydride generation is readily lost from systems containing latex.

In all these experiments we also detected significant levels of trimethylarsine, even though no inorganic arsenic was added to the medium. Typical single-ion chromatograms for a single trap for arsenic and antimony are shown in Fig. 2. Although similar amounts of trimethylstibine and trimethylarsine are present in the gas chromatogram, it is important to realize that no arsenic was added to these cultures and the concentration of antimony in the cultures was 1000 μg Sb ml^{-1}. The

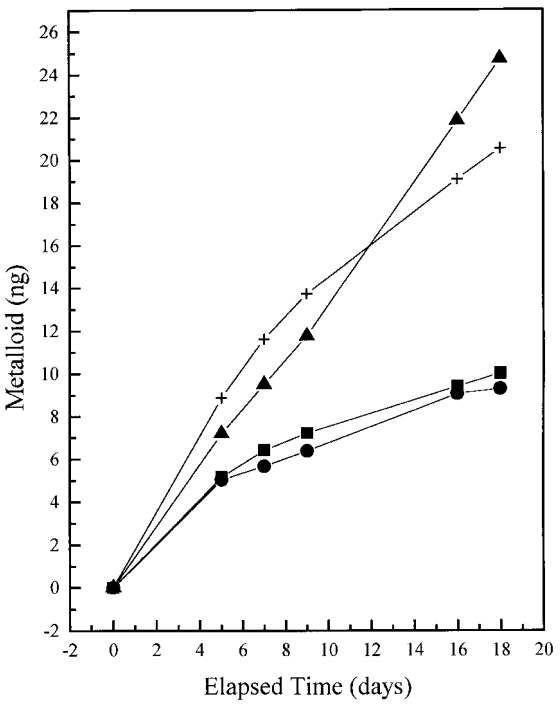

Figure 3 Time course for the production of trimethylstibine and trimethylarsine, in Experiment 2, from two cultures of *S. brevicaulis* containing 1000 μg Sb ml^{-1} as potassium antimony tartrate. The estimated total amount of volatile metalloid produced (between $t = 0$ and the time of sampling) is plotted against time. It is assumed that the rate of production of volatile species during the period between taking samples is the same as during sampling. +, Trimethylarsine flask 1; ▲, trimethylarsine flask 2; ●, trimethylstibine flask 1; ■, trimethylstibine flask 2.

arsenic that is volatilized must arise from impurities in the medium (the concentration of arsenic in the medium was less than 2 ng ml^{-1}). 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).

For Experiment 2, by determining the amounts of trimethylstibine in each trap we were able to estimate the production rates of trimethylstibine over the time of sampling (Table 1). Hence by extrapolating this information over the entire experiment we were able to construct a time course for cumulative trimethylstibine produced (Fig. 3). The period of maximum trimethylstibine production was near the beginning of the experiment. This is probably because use of a large amount of biomass rapidly depleted the medium, and so the rate of growth decreased significantly after a few days. For both experimental replicates, approximately 10 ng of trimethylstibine was produced over the course of the entire experiment (18 days). The actual amount produced may be higher than this if sampling is not 100% efficient or if trimethylstibine is being oxidized. The oxidation of trimethylstibine is not likely to be significant at these low concentrations, and in previous studies,[3] where we measured recoveries of a trimethylstibine standard from our system, we found little evidence of oxidation at such concentrations; these recovery studies also showed trapping to be efficient. Thus we believe the amounts of trimethylstibine produced are not likely to be much greater than our estimate. However, it is possible to verify the quantity of trimethylstibine produced. The most reliable method of doing this would be to spike the continuous flow of compressed-air culture purge gas with an ultratrace 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 <100% trapping efficiency. It would also eliminate errors that may arise from the use of aqueous solutions to calibrate gaseous samples.

Previous researchers have been unable to detect trimethylstibine reproducibly in the headspace of antimony-rich *S. brevicaulis* cultures.[8,9] In these studies the detection limits (50 ng) for the methodology used have been much higher than what we have found to be required (10 pg).

In a study where 'trimethylstibine' was detected by trapping in remote nitric-acid traps, the levels produced ranged from 260 to 4107 ng over approximately 12 days, depending on the substrate.[4] This quantity is significantly higher than the 10 ng we report. Differences in fungal strain and medium make comparisons of results difficult. However, the high levels of volatile antimony measured may be a result of the production of stibines other than trimethylstibine (e.g. SbH_3).

In a previous publication we reported that we were unable to detect significant amounts of trimethylstibine;[3] we have now been able to trap and analyse much greater levels of trimethylstibine because of analytical improvements and by 'stimulating' the culture with high substrate concentrations. In our previous publication we concluded that: 'methylantimony species present in the media are the final biotransformation products of antimony(III) from aerobic cultures.' Although this work shows that the final biotransformation product

for antimony is in fact trimethylstibine, it does not show it to be a very significant product. In earlier experiments with an identical fungal strain, incubation conditions and medium, we reported the production of involatile methylantimony compounds to be typically around 300–3000 ng over one month. Thus, significantly greater amounts of involatile species are produced. In this respect arsenic and antimony seem similar, but the ability of *S. brevicaulis* to methylate arsenic is much greater. For arsenic (at substrate concentrations of 1 μg As ml^{-1}) the dominant product is trimethylarsine oxide.[10] At higher concentrations trimethylarsine oxide is readily reduced by *S. brevicaulis* to trimethylarsine.[11] The culture containing trimethylantimony dichloride (1 μg Sb ml^{-1}) produced approximately six times more trimethylstibine than the cultures containing potassium antimony tartrate (1000 μg Sb ml^{-1}). The low production of trimethylstibine, in cultures containing 1000 μg Sb ml^{-1}, is probably a result of low rates of biomethylation.

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.[12] The present results show that trimethylstibine is produced by cultures of *S. brevicaulis* but detection of the levels produced requires the most sensitive analytical methodology available. In our experiments it is 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 to the notion that enough trimethylstibine could be produced in a cot environment to be harmful to a child. It should be remembered that *S. brevicaulis* is not commonly found in large amounts on cot mattresses.[13]

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