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Methylantimony Compound Formation in the Medium of *Scopulariopsis brevicaulis* Cultures: $^{13}CD_3$-L-methionine as a Source of the Methyl Group

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The filamentous fungus *Scopulariopsis brevicaulis* produces nonvolatile methylantimony compounds (found in the medium) when grown in antimony(III)-rich medium. To investigate the methyl source, $^{13}CD_3$-labelled L-methionine was added to the growth medium. After one month sodium borohydride reduction of media samples produced dimethylstibine and trimethylstibine. The methylstibines were separated on a packed GC column and obtained as gaseous fractions. Analysis of the methylstibines, in the gaseous fractions, by CGC–MS (ion-trap) established $^{13}CD_3$ incorporation in both the trimethyl-and dimethyl-antimony compounds. Copyright © 1999 John Wiley & Sons, Ltd.

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INTRODUCTION

The ability of the filamentous fungus *Scopulariopsis brevicaulis* to biomethylate inorganic antimony compounds to methylantimony compounds has been the subject of a number of recent publications.[1–4] Historically *S. brevicaulis* is well known for its ability to biomethylate arsenic. Since antimony and arsenic are both in Group 15 of the Periodic Table it is commonly assumed that their chemistry should be similar. Thus, if *S. brevicaulis* cultures readily produce trimethylarsine from inorganic arsenic, then cultures of *S. brevicaulis* supposedly should produce trimethylstibine from inorganic antimony. In order to test this hypothesis most research has focused on detecting trimethylstibine in the headspace of cultures, and in the majority of these studies no significant level of trimethylstibine has been detected.[1,3,4]

Recently, though, conclusive evidence for antimony biomethylation by *S. brevicaulis* was provided by analysis of medium samples (using HG–GC–AA),[4] and by analysis of headspace gas samples (using GC–AA) for the anaerobic stage of biphasic incubation.[2]

The yields of methylantimony compounds (especially volatile compounds) from cultures of *S. brevicaulis* containing inorganic antimony are relatively small[2,4] in comparison with analogous experiments performed with arsenic.[5,6] Some possible explanations for this are differences in rates of transport of substrate into the cell, differences in the mechanism of methylation, or inability to detect all the methylantimony compounds.

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To probe the mechanism of antimony methylation requires the same types of experiments as were performed to probe arsenic methylation.

The mechanism for arsenic methylation in biological systems, first proposed by Challenger,[7] involves a series of oxidative methylation and reduction steps. To support his scheme Challenger added [^{14}C-*methyl*]-D, L-methionine to a culture of *S. brevicaulis* and isolated trimethylarsine, which contained significant levels of ^{14}C, from the culture headspace, thus showing that the methyl groups came from methionine. Subsequently, Cullen *et al.* showed that the methyl group of L-methionine is transferred *intact* by addition of CD_3-L-methionine to a culture and trapping the evolved trimethylarsine, which was then introduced directly into a mass spectrometer.[8] These authors suggested that the biological methyl donor is *S*-adenosylmethionine. Most recently Cullen *et al.* performed experiments with cultures of *Apiotrichum humicola*, containing CD_3-L-methionine and arsenic. HG–GC–MS on the culture medium showed that CD_3 was present in the nonvolatile methylarsenic(V) compounds.[9] These nonvolatile compounds should be found in the medium according to Challenger's mechanism for arsenic methylation. Since L-methionine is the precursor for *S*-adenosylmethionine (SAM) these experiments imply that SAM is the source of the methyl groups. Involatile methylarsenic compounds were found in medium of *S. brevicaulis* cultures, but the experiments had not been set up in order to determine the methyl source.[6]

The experiments presented in this paper are analogous to Cullen's experiments with arsenic and *A. humicola*. We use hydride generation (HG) followed by gas chromatography (GC) and then mass spectrometry (MS) on collected fractions, to detect the $^{13}CD_3$-methyl group in nonvolatile methylantimony compounds found in the medium of *S. brevicaulis* cultures that had been incubated with potassium antimony tartrate and $^{13}CD_3$-L-methionine for one month.

EXPERIMENTAL

Reagents

All reagents were of analytical grade or better. Purified water was obtained by ion exchange (Barnstead). Solid-phase extraction (SPE) columns for sample clean-up were prepared using basic alumina (80–200 mesh Brockman activity I; Fisher Scientific). About 20 g of alumina was placed in a 60 ml syringe: a small glass wool plug was used to hold the alumina in place. The SPE column was primed by rinsing it with 50 ml of ammonium carbonate buffer (50 mM, pH 12, BDH). A pH 6 buffer (50 mM) was prepared by dissolving the appropriate amount of citric acid (BDH) in water and adjusting the pH with potassium hydroxide (Aldrich). Sodium borohydride (Aldrich) was prepared fresh daily by dissolving an appropriate amount of solid in deionized water. $^{13}CD_3$-L-methionine (methyl: ^{13}C, 90 atom%; D, 98 atom%) was obtained from BOC Prochem (Deer Park Road, London, UK).

S. brevicaulis culture

Submerged cultures of *S. brevicaulis* (ATCC 7903) mycelial balls were prepared by adding 100 ml of a seed culture to 300 ml of a glucose/minimal-salt medium[10] in 1-litre Erlenmeyer flasks. Potassium antimony tartrate was added to all cultures to give 100 mg Sb l^{-1} in the medium. Four flasks of live culture were prepared; to two of the flasks $^{13}CD_3$-L-methionine was added (0.1 g dissolved in 10 ml of water and added to the cultures via a 0.2 μm filter), whereas the remaining two flasks contained no methionine apart from that naturally produced. Two nonliving controls were prepared, containing CH_3-L-methionine, potassium antimony tartrate, medium and autoclaved *S. brevicaulis*. The Erlenmeyer flasks were shaken horizontally [*ca* 135 rpm, 4.5 cm displacement] and maintained at 26 °C for one month (previous studies had shown that the production of methylantimony compounds, in significant amounts, ceases after one month[4]). After incubation, the cultures were autoclaved before sampling [22 min, 121 °C, 19 psi (131 kPa)]. Previous experiments had shown that the carbon–antimony bonds of the methylantimony compounds produced are stable at the high temperatures encountered in the autoclave. All microbiological experiments were performed in the Biological Service Facility, Chemistry Department, University of British Columbia.

Analysis

The methylantimony compounds were separated from the large amounts of potassium antimony tartrate in the medium by solid-phase extraction (SPE). The medium (50 ml) was passed through a basic alumina SPE column and the eluate collected.

Hydride generation (HG) was performed on the

Table 1 Instrument parameters

IT–MS	
Mass range	*m/z* 100–200
Scan time	0.4 s
Segment Length	5 min
Peak threshold	0 counts
Mass defect	0 mm / 10
Background	*m/z* 150
Ion mode	Electron impact
Manifold temperature	260 °C
CGC	
Injector temperature	200 °C
Column temperature program	40 °C, 15 °C min^{-1}, 150 °C
Transfer line temperature	200 °C
Column	PTE™ −5, 30 m × 0.32 mm, 0.25 μm, Supelco 2-4143
HG–GC (HP 5890)	
GC temperature program	30–150 °C at 30 °C min^{-1}
Carrier gas flow	40 ml He min^{-1}
Column	2 m × 1/8 inch (0.3 mm) c.d. PTFE packed with Supelcoport SP 2100 45/60 mesh
Purge gas flow	100 ml min^{-1}

eluate from the SPE column by means of a previously described semicontinuous flow system.[6] The sample was buffered at pH 6 and 2% $NaBH_4$ was used to generate the hydrides. The volatile hydrides were trapped in a sample loop cooled in liquid nitrogen. They were then injected onto a packed GC column through a six-way valve, by rapidly heating the sample loop with hot water. The details of the HG–GC system are summarized in Table 1.

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 approximately 40 ml min^{-1}, so one fraction was taken every 20 s. The content of the gaseous fractions was analysed by capillary gas chromatography coupled with an ion-trap mass spectrometer (CGC–MS), as described elsewhere.[11] To achieve easily measurable levels of trimethylstibine and dimethylstibine in the collected fractions, generally 10–20 ml of culture medium was needed in an HG–GC run. One run was also performed in which the effluent from the GC column of the HG–GC system was analysed with an atomic absorption spectrometer as detector (HG–GC–AA), in order to measure the retention times of dimethyl- and trimethyl-stibine, to determine in which fractions these compounds would be found.

The CGC–MS consisted of a Star 3400Cx GC with a 1078 injector and a Saturn 4D ion-trap mass spectrometer (Varian Ltd). The operating conditions are given in Table 1; a volume of 1 ml, taken from the vial by using a gas-tight syringe (Precision Sampling Corp.), was injected using the split-injection technique (split ratio, 10:1).

RESULTS AND DISCUSSION

Preliminary remarks

The nonliving controls for these experiments consisted of: autoclaved *S. brevicaulis*, media, potassium antimony tartrate and CH_3-L-methionine. No methylantimony compounds were detected in these controls after one month of incubation. This indicated that biomethylation of antimony, with L-methionine as the methyl source, is not a passive process.

For all the active cultures of *S. brevicaulis*, dimethyl- and trimethyl-antimony compounds were detected in the medium samples after on month of growth. No attempt was made to measure volatile methylantimony compounds in the culture headspace because in previous work we have not been able to detect these compounds.[4] For the cultures

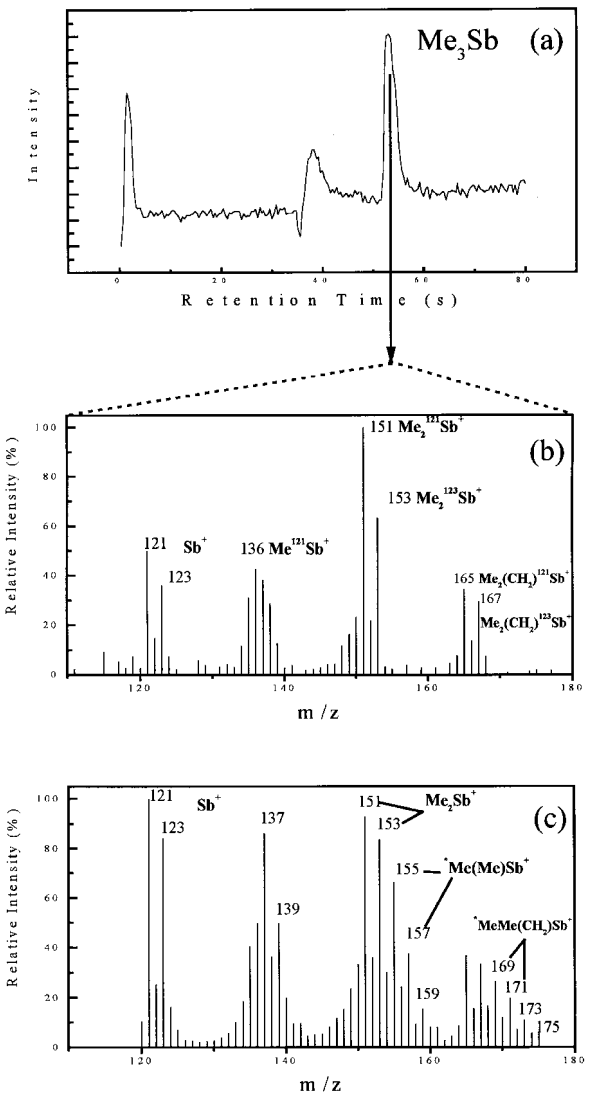

Figure 1 (a) CGC–MS (ion-trap) total ion chromatogram for the sixth fraction collected from the HG–GC system. (b) Mass spectrum corresponding to the trimethylstibine peak, for a medium sample from a culture of *S. brevicaulis* which had been grown in the presence of Sb(III) but without $^{13}CD_3$-L-methionine. (c) Mass spectrum for trimethylstibine formed by hydride generation on a sample of medium from a culture to which $^{13}CD_3$-labelled L-methionine had been added. *Me = $^{13}CD_3$.

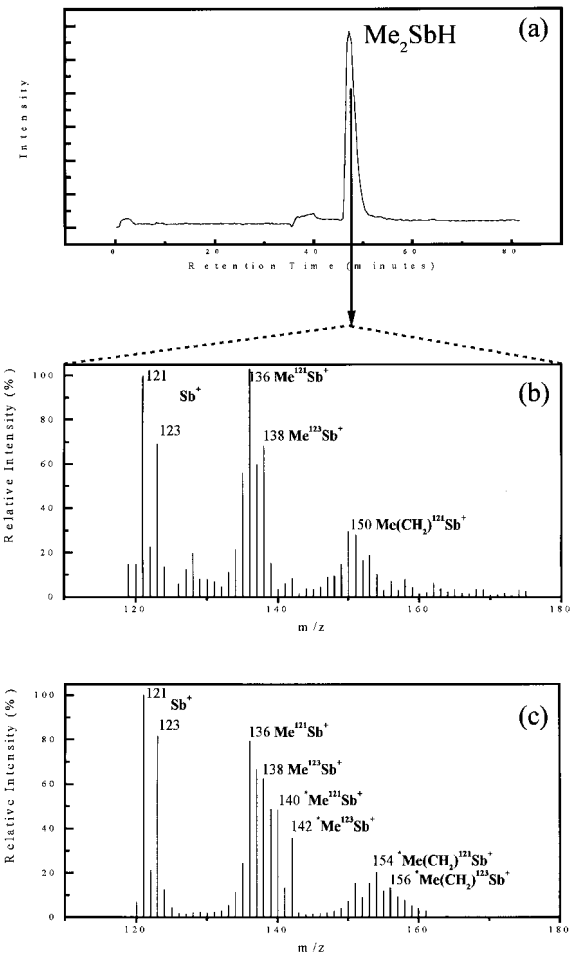

Figure 2 (a) CGC–MS (ion-trap) total ion chromatogram for the fifth fraction collected from the HG–GC system. (b) Mass spectrum corresponding to the dimethylstibine peak, for a medium sample from a culture of *S. brevicaulis* which had been grown in the presence of Sb(III) but without $^{13}CD_3$-L-methionine. (c) Mass spectrum for dimethylstibine formed by hydride generation on a sample of medium from a culture to which $^{13}CD_3$-labelled L-methionine had been added. *Me = $^{13}CD_3$.

containing L-methionine, the levels of trimethylantimony compounds were approximately 20 ng Sb ml^{-1}. For the cultures which contained no L-methionine, the levels of trimethylantimony in the media were approximately 5 ng Sb ml^{-1}, which is consistent with earlier work.[4]

Trimethylantimony species in the culture medium

A standard sample of trimethylstibine was generated from Me_3SbCl_2 by sodium borohydride reduction. The trimethylstibine retention time, when using the AAS detector, corresponded to the time window for the sixth fraction (the difference in dead volume for fraction collection and AAS detection was less than 1 ml, see above for details of fraction collection). When fraction collection

Table 2 Estimate of $^{13}CD_3$ (%) present in the trimethylantimony compound found in medium samples[a]

Culture no.	Analysis no.	$^{13}CD_3$ in trimethylantimony (%) 121	123	Average	Ion ratios 121/123	151/153	155/157	159/161
1 *S. brevicaulis* + labelled L-methionine + Sb(III)	1	35	28	31	1.32	1.02	1.52	1.81
	2	37	33	35	1.24	1.17	1.32	1.63
	3	41	33	37	1.17	1.00	1.41	1.95
	4	27	20	23	1.16	1.11	1.64	2.22
	5	34	28	31	1.19	0.93	1.44	1.40
	6	26	20	23	1.16	1.20	1.77	2.10
	7	38	27	33	1.19	0.89	1.44	2.60
2 *S. brevicaulis* + labelled L-methionine + Sb(III)	1	55	49	52	1.12	0.91	1.39	1.46
	2	50	42	46	1.06	1.25	1.57	2.30
	3	51	48	49	1.24	1.03	1.36	1.33
Controls								
3 *S. brevicaulis* + Sb(III)	1	<1	<1	<1	1.19	1.71	n/a	n/a
4 *S. brevicaulis* + Sb(III)	1	<1	<1	<1	1.25	1.50	n/a	n/a
	2	3	5	4	1.19	1.70	n/a	n/a
Me_3Sb standard from HG on Me_3SbCl_2	1	<1	<1	<1	1.12	1.54	n/a	n/a

[a] See text for discussion of the assumptions made in these calculations.

was performed, only the sixth fraction collected from the HG–GC system contained trimethylstibine, as measured by CGC–MS (ion-trap). Figure 1(a) shows a CGC–MS (ion-trap) total ion chromatogram for the sixth fraction. Figure 1(b) shows a mass spectrum for the trimethylstibine peak of Figure 1(a). This particular sample originated from a culture of *S. brevicaulis* to which *no* $^{13}CD_3$-L-methionine had been added. The parent ions are readily deprotonated ($Me_2CH_2Sb^+$ m/z = 165, 167). The fragment ions Sb^+ (m/z = 121, 123), $MeSb^+$ (m/z = 136, 138) and Me_2Sb^+ (m/z = 151, 153) arise from the loss of three, two and one methyl groups from trimethylstibine. In the mass range 135–139 the mass spectrum is complicated due to the presence of $MeSbH^+$ (m/z = 137, 139) and CH_2Sb^+ (m/z = 135, 137) fragment ions. Similar fragmentation patterns have been reported previously for both quadrupole MS[12,13] and ion-trap MS.[11]

A mass spectrum for the trimethylstibine originating from a culture which contained $^{13}CD_3$-L-methionine has a number of extra peaks (Fig. 1c). The extra peaks are consistent with a sample of trimethylstibine containing $^{13}CD_3$ methyl groups.

The fragmentation pattern for $^{13}CD_3$ containing stibines is unknown but it is unlikely to be much different from that of the unlabelled stibine, so for the purposes of quantification the fragmentation patterns are assumed to be the same.

Because the parent ions are not abundant in the mass spectrum and the $MeSb^+$ region of the mass spectrum is quite complex, only the Me_2Sb^+ fragment ions were quantified, assuming that the probability of finding a labelled methyl group in a fragment ions is the same for all fragments. We defined the fraction of $^{13}CD_3$ present in trimethylstibine for the ^{121}Sb isotope (f_{121}) according to Eqn [1], where A_{151}, A_{155} and A_{159} are the peak areas, at the trimethylstibine retention time, of the ion chromatograms for m/z = 151, 155 and 159 respectively. An analogous formula was used for the ^{123}Sb isotope. In using this procedure it is also assumed that other fragment ions are not significant. The results from the quantification are given in Table 2.

$$f_{121} = \frac{(A_{155}/2) + A_{159}}{A_{151} + A_{155} + A_{159}} \quad [1]$$

It can be seen from Table 2 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 shown in Table 2 represent the ratios of peak areas for the single-ion chromatograms. The natural isotope ratio for ^{121}Sb to ^{123}Sb is 1.34. The

Table 3 Estimate of $^{13}CD_3$ (%) in the dimethylantimony compound found in medium samples[a]

Culture no.	Analysis no.	$^{13}CD_3$ in dimethylantimony (%) 121	123	Average	Ion ratios 121/123	151/153	155/157	159/161
1 *S. brevicaulis* + labelled L-methionine + Sb(III)	1	38	29	33	1.29	1.01	1.29	2.32
	2	39	28	33	1.25	0.92	1.27	2.45
2 *S. brevicaulis* + labelled L-methionine + Sb(III)	1	46	40	43	1.26	1.21	1.08	2.08
Controls								
3 *S. brevicaulis* + Sb(III)	1	<1	<1	<1	1.31	1.16	n/a	n/a

[a] See text for discussion of the assumptions made in these calculations.

significant *measured* variations from the expected natural isotope ratio probably arise from instrumental error and from the presence of interfering fragments in the mass spectrum. Thus, the assumptions made above are not substantiated, and the results in Table 2 should only be taken as an estimate of the percentage of $^{13}CD_3$ present.

Cullen *et al.* found that cultures of trimethylarsine from cultures of *Apiotrichum humicola* grown in the presence of arsenite and CD_3-L-methionine contained 20% CD_3-labelled methyl groups.[9] In earlier studies Challenger found that cultures of *S. brevicaulis* grown in the presence of arsenite and ^{14}C-labelled D,L-methionine produced trimethylarsine containing 28.3% ^{14}C-labelled methyl groups.[7] Comparing methylarsenic and methylantimony compounds, it can be seen that there is no significant difference (given the large experimental uncertainties) in the percentage of labelled methyl groups, incorporated from L-methionine: for both arsenic and antimony there is significant incorporation of the methyl group of L-methionine, most probably after it has been converted to the active form, *S*-adenosylmethionine.

Dimethylantimony species in the culture medium

The cultures of *S. brevicaulis* also formed a dimethylantimony compound, from which, on hydride generation, dimethylstibine arises. The use of HG–GC–AA revealed that dimethylstibine should elute in the time window corresponding to the fifth fraction and indeed, this was found to be true when the fraction was examined by CGC–MS (ion-trap). Figure 2(a) shows the CGC–MS (ion-trap) total ion chromatogram for dimethylstibine. Dimethylstibine is almost baseline-resolved from trimethylstibine when this capillary GC method is used. 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 Fig. 2(b). The ion-trap MS fragmentation pattern for dimethylstibine is quite complicated. When *S. brevicaulis* was grown in the presence of $^{13}CD_3$-L-methionine, significant amounts of the labelled methyl group were detected in the dimethylstibine. This is evident in Fig. 2(c), where extra peaks can be seen in the mass spectrum attributed to $^{13}CD_3$-containing fragments. The fraction of $^{13}CD_3$ incorporated into the dimethylantimony compounds was estimated by the procedure outlined above for the trimethylantimony compound; the percentage incorporation was similar (Table 3).

In summary: both trimethylantimony and dimethylantimony compounds found in the medium of *S. brevicaulis* cultures, containing potassium antimony tartrate, contained $^{13}CD_3$ when $^{13}CD_3$-L-methionine was added to the medium. The mass spectra of the hydride derivatives showed that the methyl groups of L-methionine were transferred intact. Thus antimony is most probably methylated by *S*-adenosylmethionine in cultures of *S. brevicaulis*. These results suggest that the mechanism of antimony methylation is similar to the mechanism proposed by Challenger for arsenic.

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Note added in proof: *Apotricum humicola* is now named *Cryptococcus humicolus*.

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