Environ. Sci. Technol. **1998**, *32,* 882−885

Biomethylation of Inorganic Antimony Compounds by an Aerobic Fungus: *Scopulariopsis brevicaulis*

RICHARD O. JENKINS,† PETER J. CRAIG,*,† WALTER GOESSLER,‡ DAVID MILLER,† NAAMAN OSTAH,† AND KURT J. IRGOLIC‡

Faculty of Applied Sciences, De Montfort University, The Gateway, Leicester, LEI 9BH, U.K., and Institute for Analytical Chemistry, Karl Franzens Universität Graz, Universitätsplatz 1, 8010 Graz, Austria

Various metals and metalloids can be converted by a variety of microorganisms to their volatile methyl derivatives. These bioconversions are important from an environmental perspective because they take place over long time periods and the products have quite different properties (e.g., transportation, toxicological) as compared to the inorganic species from which they are derived. Whereas the biomethylation of arsenic is well established, that of the closely related element antimony is not, and there are no reports of antimony methylation by monoseptic microbial cultures. We report here, for the first time, the formation of trimethylantimony [$(CH_3)_3Sb$] by a characterized microorganism, *Scopulariopsis brevicaulis*, grown aerobically in the presence of inorganic antimony. Volatile antimony evolved into the headspace above the fungal cultures was quantified by remote trapping and analysis by inductively coupled plasma−mass spectrometry (ICP−MS). The existence of biogenic trimethylantimony was established, following exclusion of oxygen from cultures after growth, by remote trapping of volatile compounds and analysis by gas chromatography with compound-specific (mass spectrometry) or element-specific (atomic absorption) detection. No other volatile product containing antimony was detected in culture headspace gases.

Introduction

Much of the environmental chemistry of arsenic is governed by the existence of a wide range of methyl compounds, produced as natural products by microorganisms in the environment (see for example ref 1). Arsenic can be converted by a range of pure microorganisms to methylated arsenic species (*2, 3*). The phenomenon of the evolution of Gosio gas, trimethylarsine, by molds present on wallpaper containing Scheele's Green pigment [i.e., $Cu(AsO_2)_2$] was resolved by Challenger (*4*) in the 1930s. Challenger and Barnard also studied the interaction of several antimony compounds with various fungi. In a thesis (*5*) published in 1947, they report traces of coloration in a chemical test, suggesting that *Pencillium notatum* incubated aerobically in the presence of $KSbO_3$ produced a volatile form of antimony detectable in a remote part of the apparatus. The chemical test used (Gutzeit test) was by current standards primitive, and the molecular identification of volatile products was not possible at that time. Numerous workers over the past half-century have speculated on the possibility of antimony biomethylation. Several groups have reported on the detection of methyl antimony compounds occurring in the natural environment (*6, 7*). In the absence of any widespread industrial or other use of methyl antimony compounds, it must be presumed that these methyl antimony species are actually formed in the environment. In natural waters, methyl antimony species have been found (at the ng dm^{-3} level) (*8*). Cullen and co-workers reported the presence of methyl antimony compounds in freshwater plant extracts (*9*), which is the first report that such compounds have been detected in samples of biological origin. In all of these cases, the methodology for analysis has been based on hydride generation of a sample or extract to produce the antimony as a volatile species (reductive derivatization). Hydride generation of antimony tends to produce several products from a single analyte (dismutation) (e.g., a trimethyl antimony compound may produce mono- and dimethyl antimony species during hydride generation). Nevertheless, this imperfect analytical approach still demonstrates that a carbon to antimony linkage was present in the original sample.

Volatile antimony compounds have been shown to occur in landfill and sewage gases, but definitive compound-specific evidence was not available (*6, 10*). Very recently, it has been shown that a mixed culture of microorganisms present in soil samples can volatilize antimony into the headspace of anaerobic culture vessels (*11*). Although the volatile antimony was shown to be trimethylantimony, the producing organism(s) were not identified, and a chemical transmethylation route could have been possible. We now report, for the first time, the production of trimethylantimony by a pure aerobic microorganism: the filamentous fungus *Scopulariopsis brevicaulis*. This species is a known methylator of inorganic arsenic (*3, 12*).

Experimental Section

Organisms and Culture Conditions. *Scopulariopsis brevicaulis* IMI 17297 was grown as a submerged culture in conical flasks (500 cm^3) containing 200 cm^3 of Oxoid malt extract broth (Unipath Ltd, Basingstoke, U.K.), pH 5.5, and a source of antimony. Flasks were inoculated with spores to a final concentration of $1-3 \times 10^5$ spores cm^{-3} and incubated at 25 °C and 100 revolutions min^{-1} for 8 days. After 24 h incubation, each flask was fitted with a Dreschel head (Fisher Scientific, Loughborough, U.K.), and the headspace gases were swept continuously with sterile air at a flow rate of around 30 cm^3 min^{-1}. Outlet gases from each flask were transferred along PTFE tubing to a nitric acid trap (40 cm^3 of concentrated HNO_3 held in a 100-cm^3 glass measuring cylinder); headspace gases were introduced to the base of the trap via a sintered glass sparger. Control incubations were prepared in an identical manner except that the medium was not inoculated (no fungus).

In other experiments, *S. brevicaulis* was cultivated in the liquid medium in foam-stoppered conical flasks in the presence of 0.1 g L^{-1} antimony (added as potassium antimony tartrate). After 6 days incubation, headspace gases and dissolved oxygen in the culture were removed by sparging with oxygen-free nitrogen gas for 15 min. The flasks were fitted with Dreschel heads, and during incubation for a further 3 days, headspace gases were transferred to Tenax-TA traps

* Corresponding author e-mail: pjcraig@dmu.ac.uk; tel.: +44-(0)116-2577102; fax: +44-(0)116-257728.
† De Montfort University.
‡ Karl Franzens Universität Graz.

S0013-936X(97)00824-9 CCC: $15.00
Published on Web 04/01/1998

(60/80 mesh) under a continuous stream of oxygen-free nitrogen gas (30 $cm^3\ min^{-1}$).

Analysis of Nitric Acid Traps. An inductively coupled plasma−mass spectrometer (ICP−MS) was used to determine element concentrations in the nitric acid traps. Solutions (1 in 10 dilutions) were delivered at 0.95 $cm^3\ min^{-1}$ to a Meinhard TR-30-A3 nebulizer. The aerosol was routed through a double-pass Scott-type spray chamber cooled at 2 °C to a Plasma Quad 2 Turbo Plus ICP MS (VG Elemental, Cheshire, U.K.).

Analysis by Antimony-Specific Atomic Adsorption Spectrometry. Volatile antimony compounds from *S. brevicaulis* culture incubations were also analyzed by purge-and-trap GC−ET−AAS (*13*). Tenax-TA traps from culture incubations were electrothermally heated to 125 °C over 5 min, and eluted compounds cryofocused on a chromatographic column (50 cm glass, OV-101 3%) immersed in liquid nitrogen. Following removal of liquid nitrogen, the column was heated electrothermally, and volatile compounds were eluted according to their boiling points. Helium at 40 $cm^3\ min^{-1}$ was used as the carrier gas, and detection of eluted compounds was by antimony-specific ET−AAS (Perkin-Elmer PE3100; S&J antimony lamp at 217.6 nm; quartz furnace heated to 850 °C). Identification was by retention time comparison with volatile antimony standards, produced by reduction of $(CH_3)_3SbCl_2$ (obtained from W. R. Cullen and I. Koch, University of British Columbia, Canada) with sodium borohydride. The reaction mix contained glacial acetic acid, 3 cm^3; Me_3SbCl_2, 4.9 μg; sodium borohydride, 10 mg; and Millipore Q water, 50 cm^3. Volatile antimony standards were purged from the solution using helium gas (40 $cm^3\ min^{-1}$) onto the cold trap and analyzed as for the culture headspace gases. The order of elution of stibine, mono-, di-, and trimethylantimony standards was confirmed by GC−MS.

GC−MS Identification of Volatile Antimony Compounds. Volatile compounds from *S. brevicaulis* culture incubations trapped on Tenax-TA were thermally desorbed at 200 °C for 10 min (TD4 Perkin-Elmer) and cryofocused at −40 °C. Subsequent transfer to a GC column took place under a flow of helium via a transfer line held at 180 °C. Separation of volatile compounds was achieved using a Carlo Erba 8000 gas chromatograph fitted with an OV1 column (30 m, 0.32 mm I.D.). Helium was used as carrier gas (2 $cm^3\ min^{-1}$), and the injector was held at 150 °C. After a 3-min delay, the column was heated from 40 to 100 °C at a rate of 10 °C min^{-1}. A Fisons MD800 mass spectrometer (EI, 70 eV) was used to identify eluted compounds, which was based on (a) an eight peak index of mass spectra and (b) the NIST library database of mass spectra. Identification of biogenic trimethylantimony was also based on retention time comparison with volatile antimony standards, produced by reduction with sodium borohydride. GC retention times (min) for volatile antimony standards were SbH_3, 1.55; CH_3SbH_2, 1.90; $(CH_3)_2SbH$, 2.46; and $(CH_3)_3Sb$, 2.83.

Results and Discussion

During aerobic cultivation in liquid medium, *S. brevicaulis* mobilized antimony from nonvolatile antimony substrates to remote nitric acid traps (Table 1). Under optimum cultivation conditions, up to 8 μg of antimony was mobilized from potassium antimony tartrate, based on a trap efficiency of 51% (determined by ICP−MS analysis of serial traps). There was no evidence of antimony transportation to the traps in the absence of fungus, even for high concentrations of antimony substrates in the culture vessels. The quantity of antimony mobilized from potassium antimony tartrate varied with antimony concentration in a disproportional manner: a 20-fold increase in antimony concentration enhanced mobilization around 2-fold. Antimony was mobilized from both the 3+ (potassium antimony tartrate and Sb_2O_3) and the 5+ (Sb_2O_5) oxidation states of the element and occurred less readily from the latter state. The most productive phase of antimony volatilization from potassium antimony tartrate (63% of total antimony volatilized) was a 2-day period toward the end of the linear growth phase in liquid culture, with relatively little occurring after growth had ceased (Figure 1).

TABLE 1. Antimony Mobilized to Remote Nitric Acid Traps during Cultivation of *S. brevicaulis* in Presence of Inorganic Forms of Antimony Substrate[a]

source of antimony (mg)	antimony mobilized to traps (ng)
PAT (400)	4107 ± 981[b]
PAT (20)	1723 ± 1008[b]
Sb_2O_3[d] (40)	404 ± 175[b]
Sb_2O_5[d] (400)	260 ± 50[b]
no fungus, PAT (400)	<40[c]

[a] The antimony content of nitric acid was determined by ICP−MS. Numbers in parentheses are total amounts of antimony (Sb) added to culture medium as potassium antimony tartrate (PAT), antimony trioxide (Sb_2O_3), or antimony pentoxide (Sb_2O_5). [b] Standard deviations for averages are based on $n = 3$. [c] Standard deviations are based on $n = 5$. [d] Present mainly as suspension of particles <250 μm diameter. Each culture flask contained between 1.12 and 1.98 g dry weight biomass after incubation. The nitric acid trap efficiency for volatile antimony species was 51%, so estimated yields are about 2-fold the values shown.

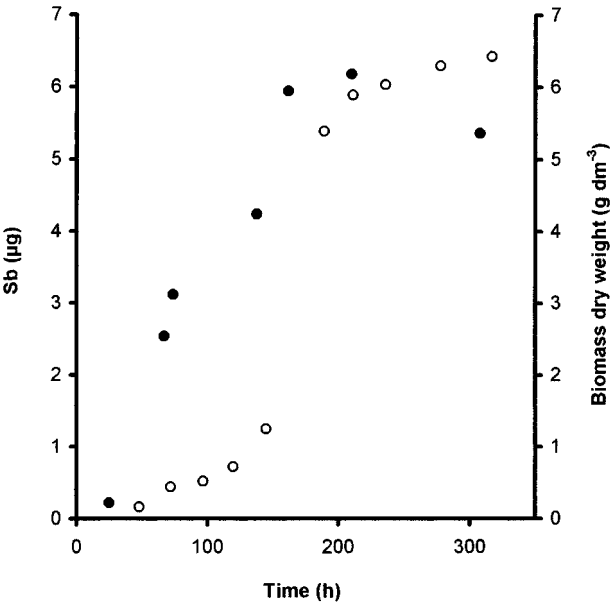

FIGURE 1. Mobilization of inorganic antimony during the cultivation cycle of *S. brevicaulis* IMI 17297. The culture medium contained antimony at 100 μg dm^{-3}, added as potassium antimony tartrate. Nitric acid traps were replaced at intervals and analyzed for antimony content by ICP−MS. Cumulative amounts of antimony mobilized (○) and biomass dry weight (●).

Initial attempts to identify the volatile antimony species produced by the fungus used a solid chromatographic adsorption material (Tenax-TA) in place of nitric acid traps to maintain antimony−carbon bonds. Under the entirely aerobic incubation conditions, volatile antimony compounds were not detected by gas chromatography−electrothermal−atomic absorption spectrometric (GC−ET−AAS) or by gas chromatography−mass spectrometric (GC−MS) analysis. By nitric acid extraction of these Tenax traps followed by ICP−MS analysis, however, antimony was shown to have been mobilized to these traps. Work with methylated antimony standards also demonstrated that methylated antimony compounds were not released from Tenax-TA after exposure to air. In subsequent culture experiments involving potassium antimony tartrate as antimony substrate, a biphasic

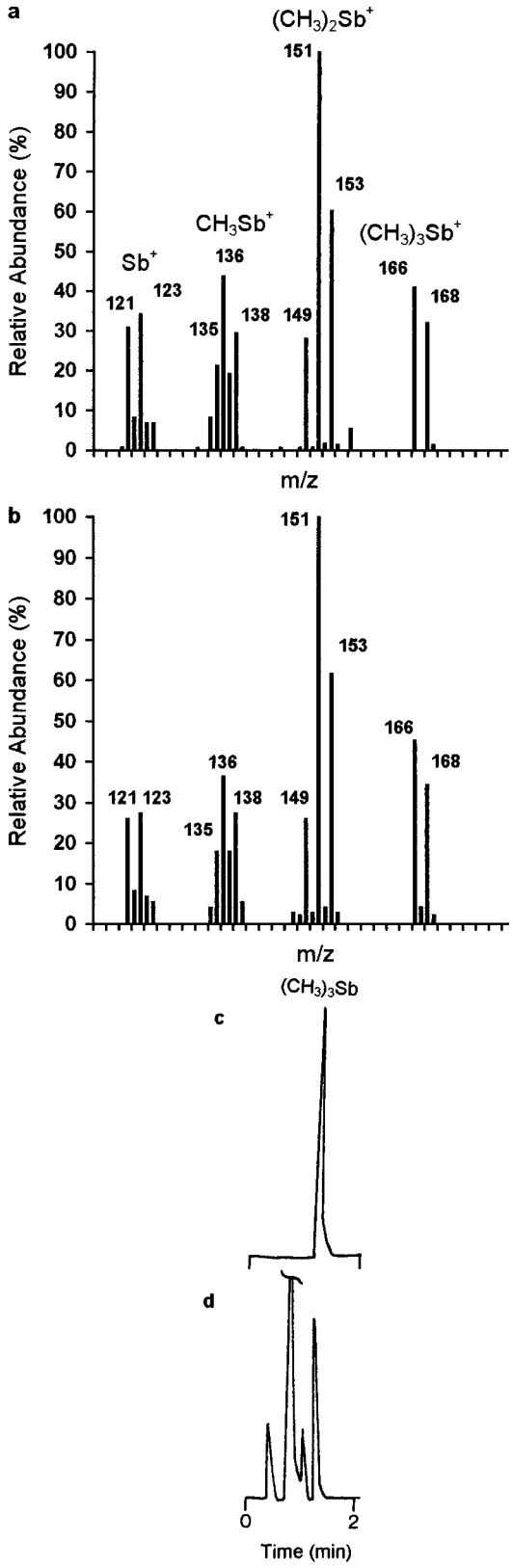

FIGURE 2. Identification of volatile antimony compounds produced by *S. brevicaulis* IMI 17297 from inorganic antimony substrate. (a) Mass spectrum of biogenic trimethylantimony. (b) Mass spectrum (reference) of trimethylantimony from NIST library. (c) Typical GC–ET–AAS chromatogram of volatile antimony compounds from culture headspace gases (peak RT = 1.36 min). (d) Typical GC–ET–AAS chromatogram of volatile antimony compounds (standards) produced by reduction of Me_3SbCl_2. Retention times are 0.41, 0.88, 1.14, and 1.36 min for stibine, mono-, di, and trimethylantimony, respectively.

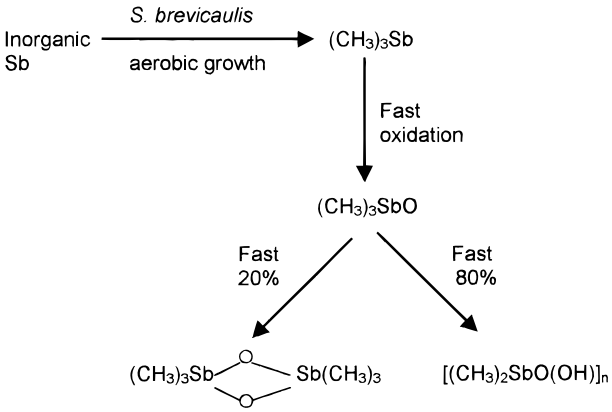

FIGURE 3. Proposed fate of biogenic trimethylantimony. Based on ref 14.

(aerobic/anaerobic) incubation was adopted in which the fungus was cultivated aerobically to the commencement of the antimony volatilization phase (aerobic phase); headspace gases were then transferred to Tenax-TA traps under anaerobic conditions (anaerobic phase). Under these conditions, a volatile antimony compound was released from these traps by electrothermal desorption, and biogenic trimethylantimony was identified by GC–ET–AAS analysis, based on retention time comparison with known compounds (Figure 2). Formation of trimethylantimony in the biphasic incubations was confirmed by GC–MS (Figure 2). In each circumstance, only trimethylantimony was observed. We hypothesize therefore that, in the entirely aerobic experiments involving nitric acid traps (i.e., Table 1), mobilization of antimony was caused by biogenic trimethylantimony. This latter compound oxidized rapidly to a less volatile species (see Figure 3), which was more tightly bound and was not released electrothermally from our solid-phase traps. We do not exclude the possibility that volatile antimony compounds formed under entirely aerobic conditions are not identical to those formed during biphasic incubations. However, since *S. brevicaulis* does not grow in the absence of oxygen, the trimethylantimony trapped during the anaerobic phase was a product arising from aerobic growth.

The rate constant for reaction of trimethylantimony with oxygen in a gaseous phase has been estimated to be 2×10^3 M^{-1} s^{-1}, which is around 10^9 higher than that for trimethylarsine (*14*). If pseudo-first-order reaction kinetics are assumed for oxidation of trimethylantimony in air, the half-life of the volatile antimony is around 50 ms. Since the residence time of a molecule in the headspace of our culture incubations was approximately 8 min, we would expect complete oxidation of biogenic trimethylantimony in aerobic cultures before it reached the traps. The oxidation products (see Figure 3) are less volatile than trimethylantimony, which accounts for our inability to desorb trimethylantimony from Tenax-TA traps in our entirely aerobic experiments. We reconcile oxidation of trimethylantimony in the culture headspace with mobilization of microgram quantities of antimony to nitric acid traps by assuming that the products of trimethylantimony oxidation continue to move with the bulk flow of air.

We believe our demonstration that trimethylantimony can be produced by an aerobic microorganism has much significance to the environmental chemistry of inorganic antimony compounds. Antimony from man-made material—such as textiles, drugs, plastics, paper, wood, paints, and fire-retardant systems—could be volatilized to trimethylantimony and converted by oxidation to more mobile forms (*14*) (see Figure 3), leading to increased interaction of this element with biological food chains at high concentra-

tions. Our demonstration of antimony biomethylation also provides a plausible mechanistic account for the origin of the forms of methyl antimony observed in the natural environment in biota (*9*) and water (*8*). The possibility therefore exists of an environmental chemistry of antimony similar in richness to that known to exist for arsenic (*3, 6*−*10*). Since trimethylantimony is not stable in air, assessment of the environmental properties of antimony should include a consideration of the physical chemical properties of the products of $(CH_3)_3Sb$ oxidation rather than of $(CH_3)_3Sb$ alone.

It has been suggested that the action of *S. brevicaulis* on antimony present in poly(vinyl chloride) cot mattress covers generates the highly toxic SbH_3 and that this trihydride, if produced, could act as an anticholinesterase leading to fatality in infants by inducing cardiac or respiratory failure (*15*). Our work doers not substantiate this claim in that we never saw any SbH_3 in our experiments, although we were able to detect it in standards (Figure 2).

Acknowledgments

This work was supported by grants from The Foundation for the Study of Infant Deaths and from the Department of Health (to P.J.C. and R.O.J.). We thank W. R. Cullen and I. Koch of the University of British Columbia for provision of $(CH_3)_3SbCl_2$.

Literature Cited

(1) Andreae, M. O. In *Organometallic compounds in the environment*; Craig, P. J., Ed.; Longman: London, 1986; pp 198−229.

(2) Byrne, A. R.; Slejkovec, Z.; Stijve, T.; Fay, L.; Göessler, W.; Gailer, J.; Irgolic, K. J. *Appl. Organomet. Chem.* **1995**, *9*, 305.

(3) Cullen, W. R.; Reimer, K. J. *Chem. Rev.* **1989**, *89*, 713.

(4) Challenger, F. *Chem. Rev.* **1945**, *36*, 315.

(5) Barnard, K. Ph.D. Thesis, University of Leeds, U.K., 1947, pp 9−29.

(6) Hirner, A. V.; Feldman, J.; Goguel, R.; Rapsomanikis, R.; Fischer, R.; Andreae, M. O. *Appl. Organomet. Chem.* **1994**, *8*, 65.

(7) Andreae, M. O.; Froelich, P. N. *Tellus* **1984**, *36B*, 101.

(8) Andreae, M. O.; Asmodé, J.-F.; Foster, P.; Van't dack, L. *Anal. Chem.* **1981**, *53*, 1766.

(9) Dodd, M.; Pergantis, S. A.; Cullen, W. R.; Li, H.; Eigendorf, G. K.; Reimer, K. J. *Analyst* **1996**, *121*, 223.

(10) Feldmann, J.; Hirner, A. V. *Int. J. Environ. Anal. Chem.* **1995**, *60*, 339.

(11) Gürleyük, H.; Van Fleet-Stalder, V.; Chasteen, T. G. *Appl. Organomet. Chem.* **1997**, *11*, 471.

(12) Cullen, W. R.; Froese, C. L.; Lui, L.; McBride, B. C.; Patmore, D. J.; Reimer, M. *Appl. Organomet. Chem.* **1977**, *139*, 61.

(13) Craig, P. J.; Mennie, D.; Needham, M.; Ostah, N. *J. Organomet. Chem.* **1993**, *447*, 5.

(14) Parris, G. E.; Brinckman, F. E. *Environ. Sci. Technol.* **1976**, *10*, 1128.

(15) Richardson, B. A. *J. Forensic Sci. Soc.* **1994**, *34*, 199.

Received for review September 15, 1997. Revised manuscript received January 5, 1998. Accepted January 5, 1998.

ES970824P