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INVESTIGATION OF THE BIOLOGICAL REDUCTION AND  
METHYLATION OF ANTIMONY COMPOUNDS

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A Thesis  
Presented to  
The Faculty of the Department of Chemistry  
Sam Houston State University

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In Partial Fulfillment  
of the Requirements for the Degree of  
Master of Science

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by  
Hakan Gürleyük  
May, 1996

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# ABSTRACT

**Gürleyük, Hakan**, Investigation of the Biological Reduction and Methylation of Antimony Compounds.

Master of Science (Chemistry), May, 1996, Sam Houston State University, Huntsville, Texas.

## Purpose

The purpose of this study was to investigate the biological reduction and methylation of antimony compounds by living organisms and to determine whether or not this process occurs in the cultures and soils examined.

## Methods

Experiments were carried out in which different bacteria (a culture of *Pseudomonas fluorescens*, bacterial mixed cultures isolated from soil samples from different places including an arsenic polluted site in Switzerland and a waste water plant and various places in Huntsville) were amended with different antimony compounds in various growth media. The bacteria were allowed to grow anaerobically in test tubes in the presence of various antimony compounds and the headspaces above the bacteria were then analyzed for reduced and methylated antimony compounds using a fluorine-induced chemiluminescence detector after separation by gas chromatography. A mass selective detector was also used to identify these compounds. Time course experiments were carried out to relate the production of the reduced and methylated compounds produced to the rate of bacterial growth. The growth of the bacteria was monitored by changes in the cultures' optical density.

Trimethyldibromoantimony, one of the antimony compounds used for amending the bacteria, was synthesized by bromination of Trimethylstibine which

was obtained by reacting antimony(III) chloride with a Grignard reagent, methyl magnesium iodide.

In addition, calibration curves and Henry's Law constants for four volatile sulfur and selenium compounds were determined in order to quantitate the total production of these compounds by bacteria.

## Findings

Trimethyldibromoantimony was successfully synthesized. The compound was identified by nuclear magnetic resonance spectroscopy, Fourier transform infrared spectroscopy and elemental analysis.

Reduction of trimethyldibromoantimony to trimethylstibine was easily achieved by *P. fluorescens* K27 in anaerobic cultures grown in a defined medium. This bacterium started producing detectable amounts of trimethylstibine during the lag phase of growth but the production increased almost exponentially when the culture went into the stationary phase after about 30 hours.

This thesis is the first unambiguous report of biological methylation of antimony compounds. In the headspace of the soil samples amended with potassium antimonyl tartrate and potassium hexahydroxo antimonate(V), trimethylstibine was found and identified by both its retention time compared to a standard detected by a chemiluminescence detector and its mass spectrum acquired by a mass selective detector. On the other hand, the blanks that contained no antimony compounds and the ones that were sterilized and yet had amended antimony did not produce any detectable trimethylstibine (detection limit = 2.1 parts per billion by volume).

The amounts of trimethylstibine found in the headspace above soils amended with potassium antimonyl tartrate and potassium hexahydroxo

antimonate(V) ranged from low parts per billion to low parts per million by volume within two weeks of amendment.

This thesis also reports Henry's Law constants of methyl sulfides and selenides in a biological defined medium. The Henry's Law constants of dimethyl sulfide, dimethyl disulfide, dimethyl selenide, and dimethyl diselenide in a well defined medium were calculated at 25 °C.

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Thomas G. Chasteen

Thesis Director

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# CHAPTER I

## Introduction

The first known case of biological methylation (biomethylation) goes back to the first quarter of the 19th century. People who were given bismuth carbonate to treat stomach disorders had foul smelling breath that was called “bismuth breath” at those times. In reality, the reason for bismuth breath was dimethyl telluride which was produced by biomethylation of the tellurium impurities in bismuth carbonate (Glockling, 1988). After several incidents of arsenic poisoning in Germany and England, Gmelin noted that a garlic smell was present in the rooms in which arsenic poisoning occurred (Gmelin, 1839). Following this clue, Fleck was able to show experimentally that molds were able to produce a gaseous arsenic compound from the arsenical pigment formulations (Fleck, 1872). He proposed this gaseous compound to be arsine ( $\text{AsH}_3$ ) but he was not able to identify it. Gosio isolated various cultures that produced this gas, which was called Gosio-gas for a long time (Gosio, 1893a, 1893b, 1897, 1901). In an attempt to identify Gosio-gas, Biginelli inoculated cultures of *Penicillium brevicaulis* on potato-mash with  $\text{As}_2\text{O}_3$  and collected the evolved gases in a solution of  $\text{HgCl}_2$  in dilute HCl (Biginelli's solution) by passing air through the cultures (Biginelli, 1901). He identified the resulting precipitate as  $(\text{CH}_3\text{CH}_2)_2\text{AsH}\cdot 2\text{HgCl}_2$  corresponding to diethylarsine for the gas he collected. Klason reconsidered Biginelli's analyses and came up with diethylarsine oxide as the Gosio-gas (Klason, 1914). These two identifications were later proven wrong by Wigren who synthesized diethylarsine and diethylarsine oxide, and showed that their behaviors towards  $\text{HgCl}_2$  were different from the so-called Gosio-gas (Wigren,

1924). The mystery of the nature of the Gosio-gas came to an end in 1933 when Challenger, using the same procedure as Biginelli, proved the Gosio-gas to be trimethylarsine (Challenger *et al.*, 1933). For his identification, he synthesized trimethylarsine and compared its behavior to that of the Gosio-gas when reacted with different reagents. He also compared the melting points of the synthetic and the experimental products he obtained. Soon, Challenger proved that methylation of selenium (Challenger and North, 1934), and tellurium (Bird and Challenger, 1939) is also possible and his findings started a wide range of research in biomethylation. Since then biomethylation of tin (Chau *et al.*, 1981; Guard *et al.*, 1981; Hallas *et al.*, 1982; Gilmour *et al.*, 1985), mercury (Yamada and Tonomura, 1971; Hamdy and Noyes, 1975; Pan-Hou and Imura, 1982), and lead (Wong *et al.*, 1975; Schmidt and Huber, 1976) has been detected and confirmed.

There have also been a few experiments (Knaffl-Lenz, 1913; Tiegs, 1925; Smith and Cameron, 1933; Challenger and Ellis, 1935; Barnard, 1947) with negative or inconclusive findings on the biomethylation of compounds containing antimony, an element in the same region of the periodic table. Challenger and Ellis (1935) inoculated molds on bread crumbs and added a solution of potassium antimonyl tartrate (tartar emetic-PAT). Aspiration of the evolved products through Biginelli's solution gave neither a precipitate nor an odor after 30 days. They then inoculated the molds in six flasks with an aqueous medium that only contained the necessary inorganic salts. Into three of the flasks, they added PAT and glucose and into the other three they added only PAT. Again, there was no precipitate and no odor after 33 days. They then put the flasks away and analyzed them 9 months later. The filtered medium contained no antimony whereas the washed mycelium contained opaque shiny crystalline particles. They proposed this compound to be antimony trioxide since it dissolved upon washing with tartaric acid. They concluded that the mold had consumed the tartrate and excreted antimony

trioxide. Barnard (1947) used the monosodium salt of the phenyl stibonic acid [NaHPhSbO<sub>3</sub>], and potassium antimonate [KSbO<sub>3</sub>] as substrates for *Scopulariopsis brevicaulis* and *P. notatum* (probably genus *Penicillium*). He aspirated the volatile products either into nitric acid to analyze the precipitate (Marsh test) or through a filter paper impregnated with silver nitrate solution and checked for a brown stain on the paper corresponding to trimethylstibine (Gutzeit test). *P. notatum* produced a small amount of a volatile antimony compound but the amount was too small to be able to draw any conclusions as to its identity. Samples of *P. notatum* amended with dimethylstibinic acid salt [(CH<sub>3</sub>)<sub>2</sub>SbO<sub>2</sub>Na] also produced positive results. However, this could have been because of the redistribution of the methyl groups or reduction to dimethyl stibine [(CH<sub>3</sub>)<sub>2</sub>SbH] by the inorganic salts rather than a biological activity. Craig (1986) states:

This latter work remains tantalizing and it is surprising that it has not been repeated with modern analytical methods for detecting and identifying the evolved products. There is evidence that methyl products were produced in this work but only on account of volatility and antimony content.

Reduction to stibine itself could account for the observations. This is an area which appears to offer rich dividends for a reinvestigation.

Considering the similarities between antimony and all the elements that are known to be biologically methylated, there seems to be no reason for this biological process not to exist for antimony compounds. Parris and Brinckman (1976) state:

There is no obvious thermodynamic or kinetic barrier to biomethylation and the chemical similarities between Sb and Sn, Pb, As, Se, and Te, which literally surround Sb in the periodic table, and all of which have been shown to be subject to biomethylation, would suggest biomethylation pathways for antimony.

Andreae *et al.* (1981) found methylstibonic acid,  $[(\text{CH}_3)\text{SbO}(\text{OH})_2]$  (MSA) and dimethylstibinic acid (DMSA), in marine and estuarine environments. Among various river waters and sea waters analyzed, the amounts of MSA ranged from 92.0 pptv (Sb) in Ochlockonee River, FL up to 2.3 ppbv (Sb) in Ochlockonee Bay estuary, FL. DMSA was not found in the rivers but its concentration ranged from 110.4 pptv (Sb) in Ochlockonee Bay estuary, FL to 588.7 pptv (Sb) in Gulf of Mexico. In a later work in Baltic Sea, Andreae and Froelich (1984) found MSA and DMSA at concentrations of 1.12 and  $<0.22$  ppbv (Sb) respectively. Although only water samples have been surveyed for methylantimony species, Craig (1986) proposed that these species could also be present in algae, marine invertebrates and fish, based on the arsenic precedents.

The presence of methylantimony compounds in the environment is a strong evidence for the biomethylation of antimony compounds. Parris and Brinckman (1976), in an attempt to hypothesize an oxidation scheme for trimethylstibine, suggested DMSA as one of the side products of this process. Therefore, it is a possibility that these methylantimony compounds could have originated from non-biological oxidation of trimethylstibine after its biological production.

Thayer (1984) suggests that Challenger's mechanism for the biomethylation of arsenic and selenium might also apply to antimony when the reduction potentials of these metalloids are considered:  $\text{H}_2\text{AsO}_4^-/\text{HAsO}_2$ , +0.662 V;  $\text{SbO}_3^-/\text{HSbO}_2$ , +0.678 V;  $\text{HSeO}_4^-/\text{H}_2\text{SeO}_3$ , +1.09 V;  $\text{HTeO}_4^-/\text{HTeO}_3^-$ , +0.813 V. The similarity in the reduction potentials for arsenic(V) and antimony(V) could suggest that antimony might also undergo biomethylation by the Challenger mechanism.

The most important problem in detection of trimethylstibine is its fast oxidation in gas phase. Parris and Brinckman (1976) found that the gas phase rate

constants for the oxidation of trimethylstibine and trimethylarsine to be  $10^3$  and  $10^{-6} \text{ M}^{-1} \text{ s}^{-1}$  respectively. Also, trimethylstibine is much less volatile compared to trimethylarsine: vapor pressures of trimethylstibine and trimethylarsine at 298 K are 103 and 322 torr respectively (Rosenbaum and Sandberg, 1940). This means that trimethylstibine would have a higher tendency than trimethylarsine to remain in solution if they were to exist in the same solution. These two facts point to the major downfall of the previous experiments in which the evolved gases were aspirated with sterile air into a solution to give a precipitate for further analysis. With such a fast oxidation rate, it is very probable that trimethylstibine was oxidized to trimethylstibine oxide  $[(\text{CH}_3)_3\text{SbO}]$ , before it reached the solution to give a precipitate. Barnard (1947) thought that this could be his problem and therefore, tried to decrease the amount of  $\text{O}_2$  in the aspiration air to 8% by adding  $\text{N}_2$ . Low oxygen content, on the other hand, obstructed the growth of the molds, and therefore, he could not find any volatile organoantimony compounds.

The possibility of biomethylation becomes more important when the current extensive use (*ca.*  $2 \times 10^7$  kg/y in U.S.A. and *ca.*  $1 \times 10^7$  kg/y in Japan) of inorganic and organic antimony compounds in conjunction with halocarbons in fire retardent systems is considered. Textiles, plastics, elastomers, paper, wood, paints and coatings are some of the products that contain antimony-based fire retardent systems and that we use daily (Maeda, 1994). If methylation is possible and if it occurs during the biodegradation of the discarded or poorly maintained consumer items, antimony could be converted into much more water-soluble forms. This can allow leaching of the antimony, reducing the flame retardency of the product and also cause pollution in waterways.

It is surprising that there has been no work on the biomethylation of antimony since 1947, in spite of the importance and potential of antimony for biomethylation and the developments in the technology available. Up to now,

many instruments and techniques have been developed to study metals and organometals found at extremely low concentrations in biological and environmental samples. These techniques include gas chromatography-flame photometric detection (Brinckman, 1981; Braman and Tompkins, 1979; Hodge *et al.*, 1979; Jackson *et al.*, 1982), gas chromatography-atomic absorption spectroscopy (Blair *et al.*, 1974; Brinckman *et al.*, 1977a; Chau and Wong, 1977; Parris *et al.*, 1977), high performance liquid chromatography-flameless atomic absorption spectroscopy (Brinckman *et al.*, 1977b; Stockton and Irgolic, 1979; Vickery *et al.*, 1979), and high performance liquid chromatography-inductively coupled plasma spectroscopy (Uden *et al.*, 1978; Fraley, *et al.*, 1979; Masatoshi *et al.*, 1980) all of which have proven successful for detection and analysis of various organo-tin, -mercury, -lead, -arsenic, and -selenium compounds. Flame (Ainsworth *et al.*, 1990a, 1990b) and flameless (Abbasi, 1989) atomic absorption spectroscopy, and high performance liquid chromatography-inductively coupled plasma spectroscopy (Fraley, *et al.*, 1979) have also been used for detecting and analyzing antimony(III) and antimony(V) in various samples. The only detection of methylantimony compounds, though, was reported by Andreae *et al.* (1981) using a graphite furnace atomic absorption spectrometer with hydride generation. Antimony(III), antimony(V), methylstibonic acid and dimethylstibinic acid were converted into the corresponding hydrides by using  $\text{NaBH}_4$  and separated by passing these hydrides through a column filled with 5% dimethyl dichloro silane in toluene. The separated stibines were then introduced into the graphite furnace by a glass connection tube and detected at 217.6 nm.

Since oxidation of trimethylstibine is probably the major problem for its detection, we propose that certain cultures of bacteria that are grown under anaerobic conditions would produce trimethylstibine in this reducing atmosphere and that it could be detected by headspace analysis using gas chromatography

with fluorine-induced chemiluminescence detection. Chemiluminescence detection after gas chromatographic separation has been proven to be highly selective and sensitive to alkyl sulfur, -selenium, -tellurium, -phosphorus and to a lesser degree to -tin, -lead, -mercury, and -arsenic compounds (Chasteen, 1990a; Chasteen *et al.*, 1990b). We also suggest that because of the similarities between the above mentioned elements and antimony, fluorine-induced chemiluminescence detector—or sulfur chemiluminescence detector (SCD) as it was commercially called before—which is available in our research laboratory, should also be able to detect trimethylstibine with a comparable selectivity and sensitivity. In addition to the identification by retention time of a trimethylstibine standard, some of the samples examined in this research were also analyzed by a gas chromatography-mass selective detector (GC/MS).

This thesis, therefore, describes the hunt for biological samples that can reduce and/or methylate antimony compounds. To that end, single cultures of known metalloid reducing and methylating bacteria were examined by amending them with various antimony compounds to determine whether they exhibited antimony reducing/methylating ability. Also, soil samples collected in and around Huntsville, Texas and a sample from an arsenic contaminated site in Switzerland were examined regarding the same question. Three compounds were used to poison the bacteria: trimethyldibromoantimony  $[(\text{CH}_3)_3\text{SbBr}_2]$  (TMDBA), synthesized in our lab with a modification of the method described by Doak *et al.* (1967), tartar emetic and potassium hexahydroxy antimonate,  $[\text{KSb}(\text{OH})_6]$  which is actually the hydrated form of the antimonate used by Barnard (1947). The headspace above the poisoned cultures was analyzed by GC/SCD and GC/MS for biologically produced organoantimony compounds.

Although headspace analysis with gas chromatography has been used as a powerful tool to quantitate the volatile compounds produced by a culture, it can

not go further than an estimation since some of these compounds remain dissolved in the medium. The dimensionless Henry's Law constant of a compound may be defined as the ratio of the equilibrium concentration of that compound in the headspace phase to that in the liquid phase at a specific temperature. Therefore, the total amount of a compound produced in a closed bacterial system of a liquid phase and a headspace phase can be evaluated if we can measure its Henry's Law constant at that specific temperature. As a part of this research and in conjunction with another project, the method described by Robbins *et al.* (1993) was modified in order to quantitate the total biological extracellular production of four volatile sulfur and selenium compounds: dimethyl sulfide, dimethyl disulfide, dimethyl selenide, and dimethyl diselenide by *Rhodobacter sphaeroides* 2.4.1 in Sistro minimal medium (Sistro, 1960).

# CHAPTER II

## Experimental

### Reagents

All chemicals used in this project were analytical reagent grade and used without further purification unless specified differently. Ethyl ether, methyl iodide, antimony(III) chloride, bromine, carbon tetrachloride, potassium antimonyl tartrate (PAT), potassium hexahydroxo antimonate(V) (PHA), dimethyl disulfide (DMDS), dimethyl diselenide (DMDS<sub>e</sub>), dimethyl sulfide (DMS), HPLC grade acetonitrile, potassium hydrogen phosphate, potassium dihydrogen phosphate, and sodium molybdate dihydrate were purchased from Aldrich Chemical Company, Inc. (St. Louis, MO USA). Dimethyl selenide (DMSe) and trimethylstibine (TMS<sub>b</sub>) were purchased from Strem Chemicals, Inc. (Newburyport, MA USA). Magnesium turnings, ethanol, sodium citrate, (ethylenedinitrilo)-tetraacetic acid disodium salt, sodium chloride and 10,000 ppm plasma emission standards of antimony, arsenic, selenium, tin and lead were obtained from EM Science (Gibbstown, New Jersey USA). Ultra high purity nitric acid was purchased from Curtis Matheson Scientific (Houston, TX USA). Ammonium sulfate and glycerol were obtained from J.T. Baker Inc. (Philipsburg, NJ USA). Nitrilotriacetic acid, magnesium sulfate heptahydrate, and succinic acid were acquired from Sigma Chemical Co. (St. Louis, MO USA) and potassium hydroxide, glutamic acid, calcium chloride dihydrate were obtained from Fisher Scientific (Pittsburg, PA USA). Iron(II) sulfate heptahydrate, boric acid, manganese sulfate, zinc sulfate, nicotinamide, thiamine, biotin, aspartic acid were contained in the vitamin, trace element and aminoacid and salt solutions, kindly

given to us by Professor Reinhard Bachofen at the University of Zürich, Institute für Plant Biology and Microbiology, Zürich, Switzerland.

Trimethyldibromoantimony (TMDBA) was synthesized as described below in our laboratory since it was not commercially available.

## Biological Media

All the media used in this project were derived from DM1 medium which was prepared according to our personal communications with Dr. Ray Fall at the University of Colorado (Boulder, Colorado USA).

- DM1 medium: DM1 medium was prepared by dissolving 7.0 g potassium phosphate dibasic, 3.0 g potassium phosphate monobasic, 1.0 g ammonium sulfate, 10.0 g of 50% glycerol, 0.5 g sodium citrate and 0.1 g magnesium sulfate heptahydrate in 1.0 L deionized water and by adjusting the pH to 7.4 by adding 0.1 M potassium hydroxide.
- DM2 medium: DM2 medium was prepared by adding 1.0 g/L potassium nitrate to DM1 medium.
- DMVTE medium: DMVTE medium contained everything DM2 medium contained plus 2.0 mL/L of vitamins solution (100 mg nicotinamide, 50 mg thiamine HCl, and 2 mg biotin in 100 mL deionized water) and 1 mL/L of trace elements solution (2.8 g boric acid, 2.1 g manganese sulfate monohydrate, 40 mg copper(II) nitrate trihydrate, 240 mg zinc sulfate heptahydrate, 750 mg sodium molybdate dihydrate and 6.0 g (ethylenedinitrilo)-tetraacetic acid disodium salt in 1000 mL deionized water).
- DMAATE medium: DMAATE medium was prepared by adding 20 mL/L amino acids and salts solution (10 g nitrilotriacetic acid, 2.0 g aspartic acid, 5.0 g glutamic acid, 11.0 g potassium hydroxide, 29.5 g magnesium sulfate

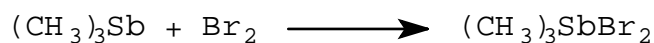
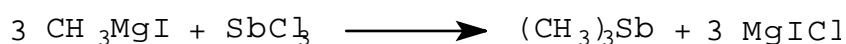
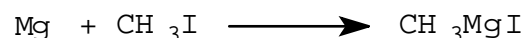
heptahydrate, 3.3 g calcium chloride dihydrate, 100 mg iron(II) sulfate heptahydrate in 1000 mL deionized water) and 1 mL/L trace elements solution (See DMVTE medium for trace elements solution components) to DM2 medium.

## Part 1.

### Synthesis of trimethyldibromoantimony

#### 1-1. Method

TMDBA was synthesized by bromination of TMSb which was obtained by reacting antimony(III) chloride with a Grignard reagent, methyl magnesium iodide (Doak *et al.*, 1967) (Figure 1).



**Figure 1.** The reactions for the synthesis of TMDBA.

Prior to the synthesis, every chemical, solution, and apparatus needed for the synthesis was placed in a model 2C1950 glovebox from Kewaunee Scientific Equipment Inc. (Adrian, MI USA) and the sealed box was purged with technical grade nitrogen gas (Bob Smith Gas Products, Huntsville, TX USA) for 15 minutes to get rid of oxygen. A slow flow of nitrogen was maintained during the course of the reaction to obtain positive pressure inside the glovebox and to carry out the moisture. A dry 500-mL round-bottom flask that contained 6.94 g of magnesium

turnings (0.285 moles) in 40 mL of ethyl ether was fitted with a reflux condenser upon which was mounted an addition funnel containing 26.8 g of methyl iodide (0.190 moles) and 130 mL of ethyl ether. The whole setup was then placed on a magnetic stirrer. Methyl iodide was added dropwise at a sufficient rate to maintain reflux. After the addition was complete and the Grignard reagent was generated, the reaction flask was cooled down to *ca.* -15 °C by inserting it into an ice/salt bath. Then the empty addition funnel was quickly replaced by a new one that contained 14.3 g of antimony(III) chloride (0.0633 moles) dissolved in 100 mL of ethyl ether. The reaction flask was placed back on the stirrer and the antimony(III) chloride solution was added dropwise to maintain reflux. At first, the addition produced a yellow precipitate which slowly dissolved in the solution and towards the end of the addition, two phases formed: a clear yellowish liquid and a brownish solid. Once the addition was completed, the reaction flask that contained the desired intermediate, TMSb, was inserted into the ice-bath and more table salt was added to the ice. The reflux condenser and the addition funnel were removed and a distillation head was fitted on the reaction flask. As a receiving flask, an empty 500-mL round-bottom flask was connected to the other end of the distillation condenser and was inserted into the ice-bath after the reaction flask was taken out and placed on a heating mantle. The heater was then turned on and ethyl ether and trimethylstibine were distilled into the receiving flask until the temperature reached 70 °C. When the distillation was complete, the receiving flask was disconnected from the distillation condenser, placed on the stirrer and fitted with an addition funnel that contained 10.0 g of bromine (0.0633 moles) in 50 mL carbon tetrachloride. Addition of bromine produced a white precipitate, trimethyldibromoantimony. Addition was continued until the slurry turned yellowish brown. The addition funnel was then removed and the flask was stoppered. After taking the flask out of the glovebox to a vent hood, it was filtered

with suction using a fritted Büchner funnel and washed several times with 200 mL of ethyl ether. The crude product was then recrystallized from 95% ethanol to give white crystals. The TMDBA crystals were then transferred to a vial for storage at room temperature.

## **1-2. Identifications**

### **Elemental analysis of TMDBA**

A 40 mg sample of the recrystallized product was sent to Galbraith Laboratories, Inc. (Knoxville, TN USA) for elemental analysis.

### **Fourier transform infrared spectroscopy of TMDBA**

The Fourier transform infrared spectrum of TMDBA was obtained by a BOMEM DA3 FTIR spectrometer which was controlled by a DEC PDP/11 computer that was also used to acquire data for display and storage.

Ten milligrams of TMDBA was mixed with 100 mg of dry potassium bromide and some of this mixture was transferred into a dye. The dye was pressed at 10,000 psi for 5 minutes to produce a thin sample film.

### **Nuclear magnetic resonance spectrum of TMDBA**

A 60 MHz Varian EM 360 NMR spectrometer was used to take the proton NMR spectrum of TMDBA.

Approximately 5 mg of TMDBA was placed in an NMR tube, obtained from Alltech (Deerfield, IL USA) and 10 drops of chloroform-*d* with 1% v/v TMS (Aldrich Chemical Company, Inc., St. Louis, MO USA) was added. The spectrum was obtained at room temperature.

### **Melting-point analysis of TMDBA**

A melting-point analysis could not be performed with TMDBA since at around 50 °C TMDBA decomposes into dimethylbromo antimony and methyl bromide (Doak *et al.*, 1967).

## Part 2.

# Microbiology

The first culture used in this research project was *Pseudomonas fluorescens* K27, isolated from Kesterson Reservoir, CA (Burton et al., 1987) by Dr. Ray Fall of University of Colorado, Boulder who also supplied the strains for this project. This bacterial strain is known to reduce and methylate selenate and selenite (Chasteen, 1990a, Zhang and Chasteen, 1994). To grow precultures, K27 was inoculated into DM1 medium previously sterilized using a 250-mL filter holder with 47 mm, 0.2  $\mu\text{m}$  membrane filter, both purchased from VWR Scientific (Sugarland, TX USA). The required concentration stock solutions of the three antimony compounds [Trimethyldibromoantimony, potassium antimonyl tartrate and potassium hexahydroxo antimonate(V)] were prepared in DM2 medium and sterilized by the same method. After the inoculation was made into required size sterile Erlenmeyer flasks and the mouth was closed with a sterile cotton, the cultures were incubated in a model R76 water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ USA) at 30 °C overnight to grow aerobically. After 24 hours of incubation, the cultures were diluted 1:1 with the desired stock antimonial DM2 solution in triplicate into autoclaved Hungate tubes (VWR Scientific) to give 10 mL of bacterial solution. The tubes were then tightly capped with open-top screw caps sealed with Teflon<sup>®</sup> septa that were also obtained from VWR Scientific and the cultures were then incubated anaerobically at 30 °C in a Precision Scientific (Chicago, IL USA) model 83 water bath for 24 hours until the headspace analysis (Chapter II, Part 4-2). Bacterial growth was monitored (by optical density) during incubation using a Klett-Summerson Photoelectric colorimeter (Klett MFG Co., NY USA) using a green filter ( $\lambda_{\text{max}} = 526 \text{ nm}$ ).

A set of controls for these experiments was prepared by diluting the aerobically grown pre-culture 1:1 with sterile filtered antimony-free DM2 medium. Another set was prepared by mixing the sterile antimony-free DM2 medium with the desired sterile stock antimonial DM2 medium 1:1. A third control contained just the sterile DM2 medium. All these controls were prepared in triplicate into Hungate tubes to give 10 mL solutions, capped with open top screw caps with Teflon septa for headspace analysis and incubated anaerobically at 30 °C in the water bath for 24 hours just like the samples that contained antimony.

The time course experiments that followed were also carried out but only for cultures amended with TMDBA. For these experiments, a series of samples was prepared as described above and the production of TMSb was measured by GC/SCD in triplicate in parallel with the bacterial growth.

Three different soil samples were analyzed to find organisms that might reduce and/or methylate the above antimony compounds. Sample 1 (SS-Switzerland soil) was collected from an arsenic polluted leather tannery in Switzerland and was supplied by Professor Reinhard Bachofen at the University of Zürich, Institute Für Plant Biology and Microbiology, Zürich, Switzerland. Sample 2 (SPS-Sewage plant soil) was collected from the Sewage plant facility of Huntsville, Texas USA. Sample 3 (BSS-Body Shop soil) was collected from the backyard of an auto repair shop named “Body Shop” in Huntsville, Texas, after which we named the sample. Both of these U.S. soil samples were collected into plastic sterile sample bags and stored in our lab at room temperature.

These soil samples were inoculated into 3 different media: DM2, DMVTE, and DMAATE. Samples of 0.20 g from these soils were weighed and transferred to autoclaved Hungate tubes. Ten milliliter of a specific sterile antimonial stock medium with a specific concentration was added and the tubes

were then tightly capped with open-top screw caps with Teflon septa. The samples were incubated anaerobically at 30 °C in the water bath. After 2 weeks of incubation time, the headspace in each tube was analyzed.

Based on our preliminary results, we chose to continue with BSS as our soil sample and DM2 as our growth medium. Samples of 0.20 g from BSS were weighed and transferred to sterile Hungate tubes. These soil containing tubes were divided into 6 groups with 24 samples in each group. To the first group was added 10 mL of sterile DM2; the tubes were capped with open-top screw caps with Teflon septa. To the second and the third group were added 10 mL of sterile 0.01 mM PAT in DM2 and 0.01 mM PHA in DM2 respectively. Afterwards, these tubes were capped with open-top screw caps with Teflon septa. The fourth, fifth and the sixth group were capped and autoclaved at 121 °C for one hour. These soil samples were autoclaved twice more after a day and two days later to assure sterilization of outgrown spores. Then they were opened and sterile DM2, 0.01 mM PAT in DM2 and 0.01 mM PHA in DM2 were added to groups four, five and six respectively. All these controls were then incubated at 30 °C in the water bath and their headspaces were analyzed after 1 month.

## Part 3.

### Instrumentation

#### 3-1. Gas chromatograph-fluorine induced chemiluminescence detector (GC/SCD)

A Sievers 300 Sulfur Chemiluminescence Detector (Sievers Instruments, Boulder, CO USA), coupled with a Hewlett Packard (Norwalk, CT USA) 5890 Series II Gas Chromatograph was used for the analysis of headspaces above cultures. The chromatographic column used was a 30 m 0.32 mm i.d. capillary (Alltech Associates, Inc., Deerfield, IL USA) with 1  $\mu\text{m}$  5% phenyl, 95% methyl polysiloxane as the stationary phase. The carrier gas used was technical grade helium (Bob Smith Gas Products). The injector temperature was 275 °C, the inlet head pressure was 11 psi and the carrier flow was 1 mL/min. Through a transfer line heated to 150 °C the column was connected to a stainless steel reaction cell in the detector. A red sensitive photomultiplier tube in the reaction cell was used to count the photons emitted by the chemiluminescence reaction of the analytes coming out of the column with molecular fluorine ( $\text{F}_2$ ).  $\text{F}_2$  was produced by the electrical discharge of  $\text{SF}_6$  and excess  $\text{F}_2$  was collected by an ascarite trap. A vacuum pump (Sargent Welch Scientific Co., Skokie, IL USA) was used to maintain the pressure in the reaction cell below 1 torr (*ca.* 0.6 torr). The resulting signal from the SCD was recorded by a Hewlett Packard 3396 Series II Integrator. For all headspace analyses, the temperature program used consisted of: 1 min of cryogenic trapping at -20 °C (oven cooled by using liquid nitrogen supplied by Bob Smith Gas Products) followed by a temperature ramp of 20 °C/min until a final temperature of 250 °C. The temperature programs used for calibrations are in Table 1.

### **3-2. Gas chromatograph-mass spectrometer (GC/MS)**

In order to identify any biologically produced organoantimony compounds, a Hewlett Packard 5890 Series II Plus gas chromatograph, coupled to a Hewlett Packard 5972 mass spectrometer was used. Ultra high purity helium (Bob Smith Gas Products) was used as the carrier gas. The injector was kept at 275 °C and an electronic pressure control was used to maintain the inlet pressure at 11 psi and the carrier gas flow at 1 mL/min. A 30 m, 0.25 mm i.d. capillary column with a 1 µm stationary phase of 5% phenyl, 95% methyl polysiloxane was used for the chromatographic separation (J&W Scientific, Folsom, CA USA). The column was transferred to the detector by a heated transfer line (300 °C). The effluents from the column was ionized by a filament with an ionization energy of 50 eV. The resulting positively charged ions were focused into a stainless steel quadrupole. The masses from 35 to 500 amu were scanned 3 times every 2 seconds and were detected by an electron multiplier that worked at 2106 Volts. The signal was collected by a Hewlett Packard Vectra computer using Hewlett Packard Chemstation software which was also used to control the whole instrument. National Institute of Standards and Technology (NIST) mass spectra library (Hewlett Packard) was used to compare the resulting mass spectrum of the compounds.

### **3-3. Microwave digestion**

In order to digest the soil samples for analysis by inductively coupled plasma spectroscopy, an OI Analytical Microwave Digestion System (College Station, TX USA) with temperature and pressure feedback control was used. Microwave sample preparation for atomic absorption or inductively coupled spectroscopy is a fairly new technique (Accepted by United States Environmental

Protection agency (USEPA) in September, 1992) replacing the use of hot plates. In the OI Analytical Microwave Digestion System, the microwave output frequency is 2450 MHz. In the oven, there is a reversing 360° rotational platform to assure even energy exposure to samples and it can hold up to 12 vessels. The instrument is controlled totally by a personal computer using the WinWave software. For digesting our soil samples, the vessels were first taken up to 125 °C with 50% power and kept at the same temperature for 1 min. The temperature was then increased to 175 °C using 100% power and kept at that temperature for 4.5 min.

### **3-4. Inductively coupled plasma spectrometer**

In order to analyze the total antimony, arsenic, selenium, lead, and tin content of the soil samples, a Leeman Labs PS1000 axial inductively coupled plasma spectrometer was used. This instrument has a fixed Echelle grating and patented moving detector that can read the desired emission lines from a spectrum sequentially.

In this project, the argon plasma which was supplied as 99.999% liquid argon (Bob Smith Gas Products) was operated at 1.1 KW. The water coolant and the auxiliary argon flow were 18 and 0.5 L/min respectively. The nebulizer pressure was kept at 50 psi. The sample was aspirated into the nebulizer at 1.8 mL/min.

## Part 4.

### Procedures

#### 4-1. Calibrations

##### 4-1a. Calibrations of dimethyl sulfide, dimethyl disulfide, dimethyl selenide, dimethyl diselenide and trimethylstibine using

**GC/SCD:** The calibration of the response of the fluorine induced chemiluminescence detector to these five compounds was performed identically. DMDS will serve as an example: The preparation of the standards for DMDS was carried out under the hood. Ten serial dilutions of DMDS were made into acetonitrile from a neat solution, used as received from the vendor. Two-milliliter screw cap vials (Alltech Associates) were used to store the standards. Every bottle or vial was kept in an ice bath during dilutions and analyses by GC to prevent reagent loss. Triplicate splitless injections of 1  $\mu\text{L}$  from each standard vial using a 10- $\mu\text{L}$  syringe were made to the gas chromatograph. The GC/SCD analyses were performed as described in Part 3-1. Table I presents the temperature programs used for the calibration of DMDS and the other calibrated compounds. The densities used for concentration calculations were provided by the manufacturer. The peak areas from the integrator were transferred to a Macintosh IICI computer. The linear least squares fit for the plot of the logarithm of the peak areas versus the logarithm of the mass of DMDS injected and the error bars were calculated using KaleidaGraph 3.0 (Synergy Software, Reading, PA USA). A signal-to-noise ratio of 3 was used to calculate the detection limits.

The calibrations of DMS, DMSe, and DMDS<sub>2</sub> were carried out by the same procedure.

The preparation of trimethylstibine standards was carried out in a glovebox purged with technical grade nitrogen. Open top screw caps were used with Teflon septa (Alltech Associates) in order to prevent air oxidation of the standard while taking samples for injection into the GC. The rest of the procedure for calibration of TMSb was the same as DMDS.

**Table I.** Temperature programs used to calibrate GC/SCD.

	<b>DMS</b>	<b>DMDS</b>	<b>DMSe</b>	<b>DMDS<sub>e</sub></b>	<b>TMSb</b>
<b>Initial Temperature (°C)</b>	25	40	30	40	-20
<b>Initial Time (min.)</b>	1	1	1	1	1
<b>Rate (°C/min.)</b>	20	20	20	20	20
<b>Final Temperature (°C)</b>	100	125	100	150	150
<b>Final Time (min.)</b>	1	1	1	1	1

**4-1b. Calibrations of antimony, arsenic, selenium, tin, and lead using inductively coupled spectrometer:** Four serial dilutions from 10,000 ppm plasma emission standards of antimony, arsenic, selenium, tin and lead were made into ultra pure water supplied by a Millipore Milli-Q Plus system (Bedford, MA USA). Ultra pure water was used as the “0” level in the calibration. Unleaching materials were used during dilutions and sample handling. The data from ICP was exported to a Macintosh IIci computer and calibration curves using linear least squares fit were generated using KaleidaGraph 3.0.

## **4-2. Headspace analyses**

All the headspace samplings were performed using 1-mL gas tight syringes with push button valves, purchased from Alltech. The Teflon septum on the open top cap of a Hungate tube was pierced with the syringe needle. One milliliter of the headspace was pulled into the syringe and the push button valve was closed. The needle was then taken out of the tube and the headspace sample was immediately injected into the split/splitless injector of the GC. The push button valve was not opened before the needle was in the injector port completely. Sampled tubes were not sampled again since pierced septa were not considered to be gas tight anymore. The used syringe was then taken to the syringe cleaning device for cleaning. The syringe cleaning device was a simple filtration flask which was heated gently by a hot plate, while air was passed through the flask and the disassembled syringe to sweep out the volatile compounds left in the syringe. We used three syringes and two syringe cleaning devices at the same time which allowed around 40 min of cleaning per syringe. Although 40 min was more than sufficient for cleaning most of the samples, a syringe check run was performed by injecting 1 mL lab air to the GC. The syringes were never reused unless they produced no peaks in the syringe check run.

## **4-3. Elemental soil analysis**

The digestion procedure used in this research follows the USEPA Method 3051-microwave-assisted acid digestion of sediments, sludges, soils, and oils. Four samples of approximately 0.5 g were weighed to the nearest 0.0001 g and placed in PFA Teflon vessels (OI Analytical). Ten milliliter of ultra pure nitric acid was added to the vessels. The safety disks (OI Analytical) were placed on the vessels and the vessels were then capped. The blanks contained 0.5 mL of ultra pure water and 10 mL of ultra pure nitric acid. The control vessel that had the

temperature probe contained one of the blanks. After digesting the samples using OI Analytical Microwave Digestion System (Part 3-3), the vessels were allowed to cool to room temperature before they were opened. The digested samples were transferred to Teflon test tubes without further dilution and analyzed by inductively coupled plasma spectroscopy as described in Part 3-4. The data were then exported to a Macintosh IICI computer and the concentrations of antimony, arsenic, selenium, tin and lead in the digested solution was calculated. These solution concentrations were then converted into concentrations in the actual soil samples.

#### **4-4. Henry's Law constant determination of dimethyl sulfide, dimethyl disulfide, dimethyl selenide, dimethyl diselenide in a biological medium.**

In Siström minimal medium (SMM) (Siström, 1960), Henry's law constants were determined for DMS, DMDS, DMSe, DMDS<sub>2</sub> by adapting the method described by Robbins *et al.* (1993). Fifty milliliters of a culture of *Rhodobacter sphaeroides* 2.4.1 (ATTC #17023) was inoculated into *ca.* 450 mL of sterile filtered SMM in a 500-mL sterile bottle and then left in the dark overnight in order to deprive the bacteria of oxygen before being incubated in incandescent light (100 W tungsten light bulb; distance calibrated to 10 W/m<sup>2</sup> by a light meter, Zeiss, Stuttgart, Germany). The bacteria were then allowed to grow anaerobically at 25 °C in the water bath for one week. One week of growth time was chosen for consistency since in earlier experiments in our lab the concentrations of these compounds in the headspace over the bacteria was analyzed after a week of growth. The cells were centrifuged at 8000 rpm at 11 °C using a Sorvall RC-5 Superspeed Refrigerated Centrifuge (Dupont Instruments, Wilmington, DE USA) and the supernatant was sterile filtered using a 0.2 µm

membrane filter. Serial dilutions from pure DMS, DMDS, DMSe, and DMDS<sub>e</sub> standards into acetonitrile in separate 2-mL vials were made. The sterile filtered medium was transferred into a sterile Erlenmeyer flask and an appropriate amount of a standard solution that contained the appropriate amount of analyte (*ca.* 100 ng) which would produce a signal higher than the detection limit but that would not overload the detector was then added. During this process, the Erlenmeyer flask was kept in an ice bath. Using a 10-mL pipette, 4, 8 and 12 mL of the sterile filtered medium with the specific compound were transferred into  $16 \pm 0.1$  mL Hungate tubes in triplicate and the tubes were then closed with an open top screw cap with Teflon septa. The tubes were kept in a water bath at  $25 \pm 1$  °C for 24 hours and the headspace above the solution was then analyzed using GC/SCD as described in Part 3-1. The peak areas from the integrator were transferred into a Macintosh IIfx computer. The reciprocal of the peak areas were plotted versus the corresponding headspace phase to liquid phase volume ratios using KaleidaGraph 3.0. The linear least squares fit was calculated using the same software. The Henry's Law constant was calculated by dividing the slope of the linear least squares line equation to its y-intercept.

# CHAPTER III

## Data

### 1. Elemental analysis of trimethyldibromoantimony (Table II).

**Table II.** Results of the elemental analysis of a trimethyldibromoantimony sample.

Element	Theoretical	Experimental	Difference
Carbon	11.03%	10.88%	0.15%
Hydrogen	2.78%	2.64%	0.14%
Antimony	37.27%	36.47%	0.80%
Bromine	48.92%	50.14%	1.22%

### 2. Identification of trimethyldibromoantimony using Fourier Transform Infrared spectroscopy (Figure 2).

### 3. Nuclear magnetic resonance spectrum of trimethyldibromoantimony (Figure 3).

### 4. Calibration of dimethyl sulfide using F<sub>2</sub>-induced chemiluminescence detector (Figure 4).

**5. Calibration of dimethyl disulfide using F<sub>2</sub>-induced chemiluminescence detector (Figure 5).**

**6. Calibration of dimethyl selenide using F<sub>2</sub>-induced chemiluminescence detector (Figure 6).**

**7. Calibration of dimethyl diselenide using F<sub>2</sub>-induced chemiluminescence detector (Figure 7).**

**8. Calibration of trimethylstibine using F<sub>2</sub>-induced chemiluminescence detector (Figure 8).**

**9. Detection limits of the compounds calibrated using F<sub>2</sub>-induced chemiluminescence detector (Table III).**

**Table III.** Detection limits for dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl selenide (DMSe), dimethyl diselenide (DMDS<sub>e</sub>), and trimethylstibine (TMSb) calculated using a signal-to-noise ratio of 3.

	DMS	DMDS	DMSe	DMDS <sub>e</sub>	TMSb
Detection Limits (pg/μL)	31.9	16.2	14.2	19.5	15.2

**10. Calibration of Sb using inductively coupled plasma spectroscopy (Figure 9).**

**11. Calibration of As using inductively coupled plasma spectroscopy (Figure 10).**

**12. Calibration of Se using inductively coupled plasma spectroscopy (Figure 11).**

**13. Calibration of Pb using inductively coupled plasma spectroscopy (Figure 12).**

**14. Calibration of Sn using inductively coupled plasma spectroscopy (Figure 13).**

**15. Time course reduction of 0.01 mM trimethyldibromoantimony by *P. fluorescens* K27 (Figure 14).**

**16. Time course reduction of 0.1 mM trimethyldibromoantimony by *P. fluorescens* K27 (Figure 15).**

**17. The results of the headspace analyses of soil samples SS, SPS and BSS in different media amended with potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) (Table IV).**

**18. The amounts of dimethyl sulfide and dimethyl disulfide found in the headspace above the soil samples SS, SPS and BSS in different media amended with potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) (Table V).**

**19. The chromatogram of the headspace of BSS in DM2 (Figure 16).**

**20. The chromatogram of the headspace of BSS in DM2 amended with potassium antimonyl tartrate (Figure 17).**

**21. The chromatogram of the headspace of BSS in DM2 amended with potassium hexahydroxo antimonate(V) (Figure 18).**

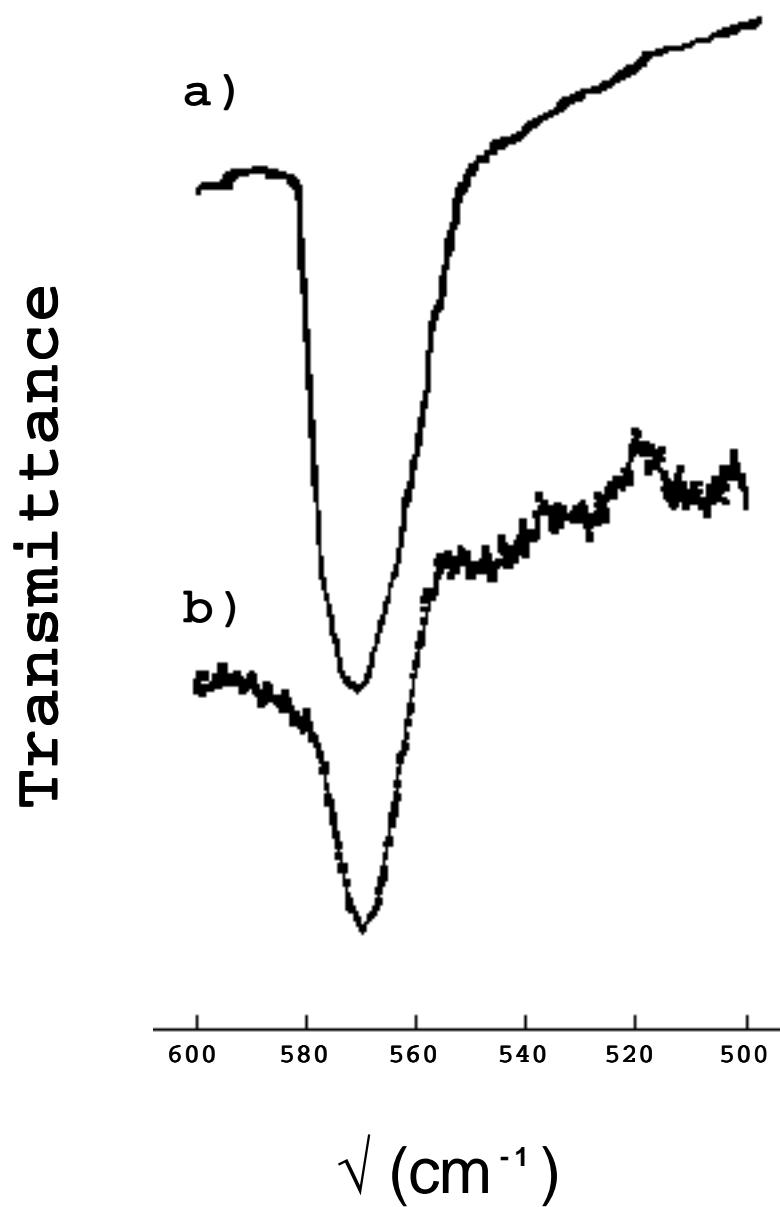
**22. The chromatograms of the headspace of sterilized BSS in DM2 amended with potassium hexahydroxo antimonate(V) (Figure 19a), potassium antimonyl tartrate (Figure 19b), and with no antimony compounds added (Figure 19c).**

**23. Total ion chromatogram and the mass spectra produced by the headspace analysis of a soil sample amended with potassium hexahydroxo antimonate(V) obtained by gas chromatography-mass spectrometer (Figure 20).**

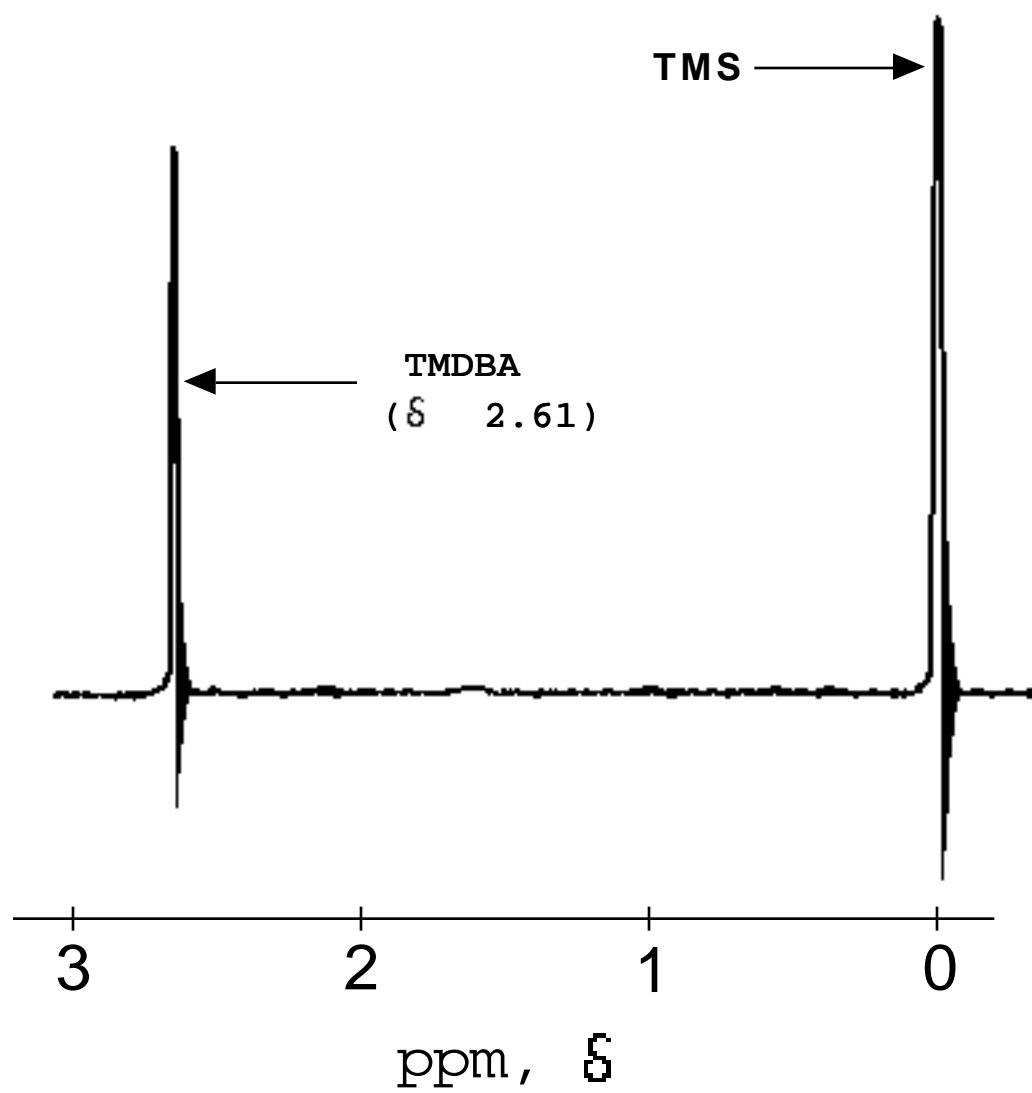
**24. Elemental analysis of the BSS soil sample using an inductively coupled plasma spectrometer (Table VI).**

**25. A representative plot of phase ratios vs. reciprocal peak areas that was used to calculate the Henry's Law constant for dimethyl sulfide in SMM medium at 25 °C (Figure 21).**

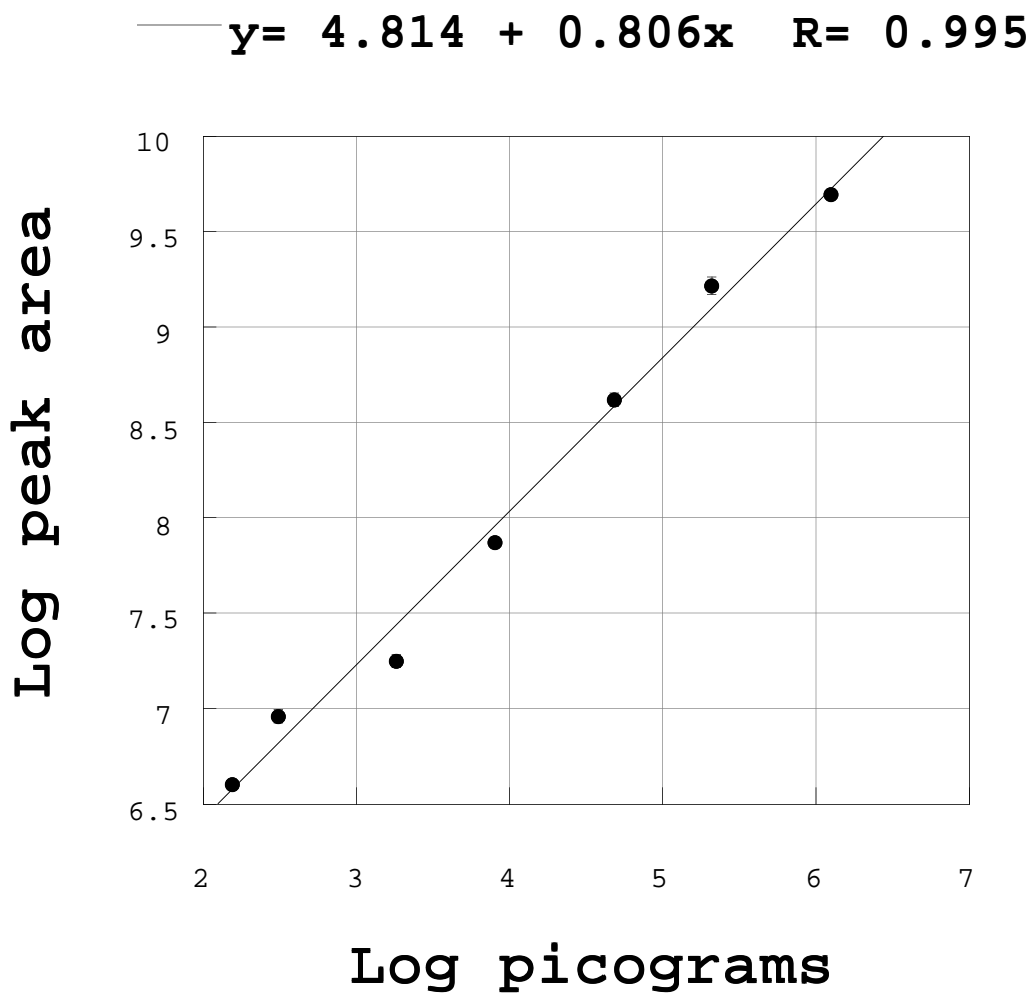
**26. Henry's Law constants for methyl sulfides and selenides in SMM medium at 25 °C (Table VII).**



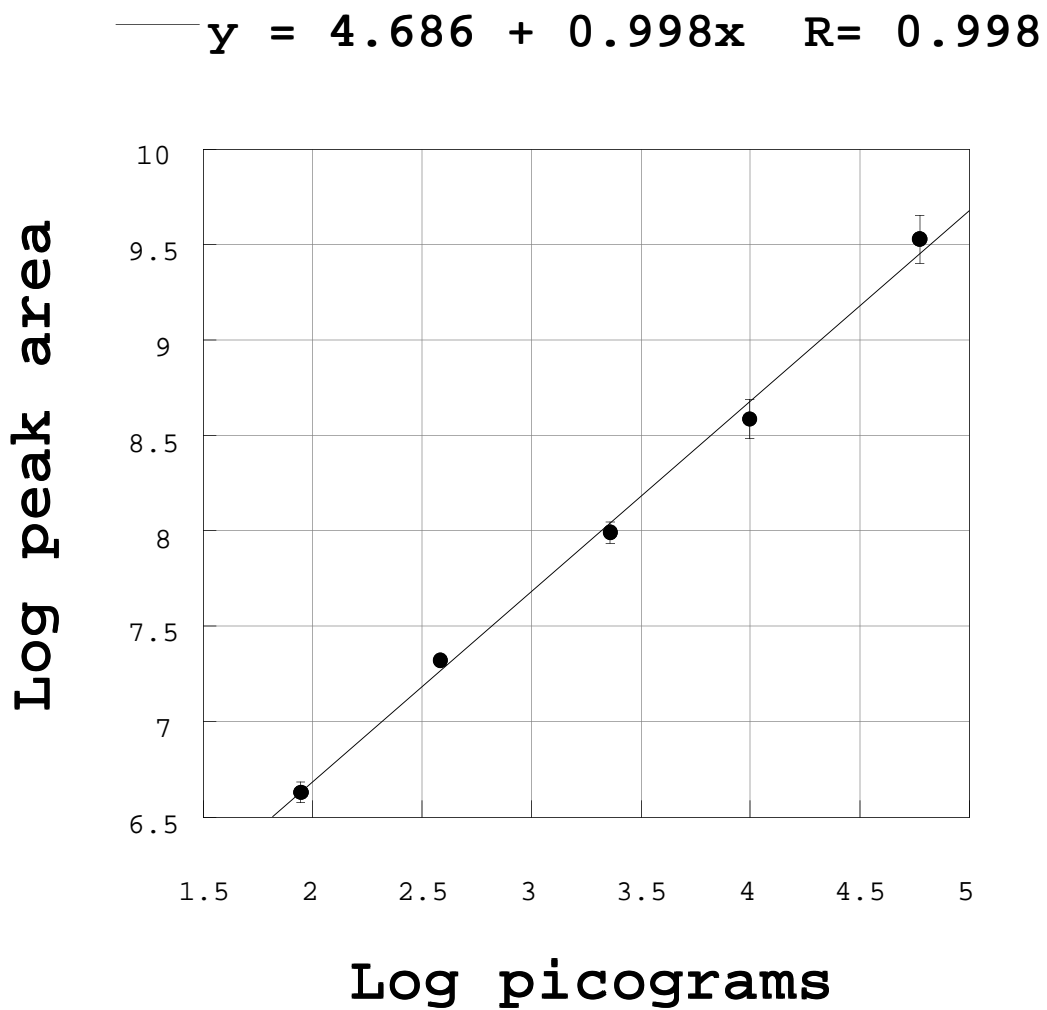
**Figure 2.** The spectrum of trimethyldibromoantimony between 500 and 600  $\text{cm}^{-1}$ . The peaks **a)** at 570  $\text{cm}^{-1}$  (Goel *et al.*, 1971), and **b)** 568  $\text{cm}^{-1}$  (experimental) are due to the asymmetric stretching of the carbon—antimony bonds.



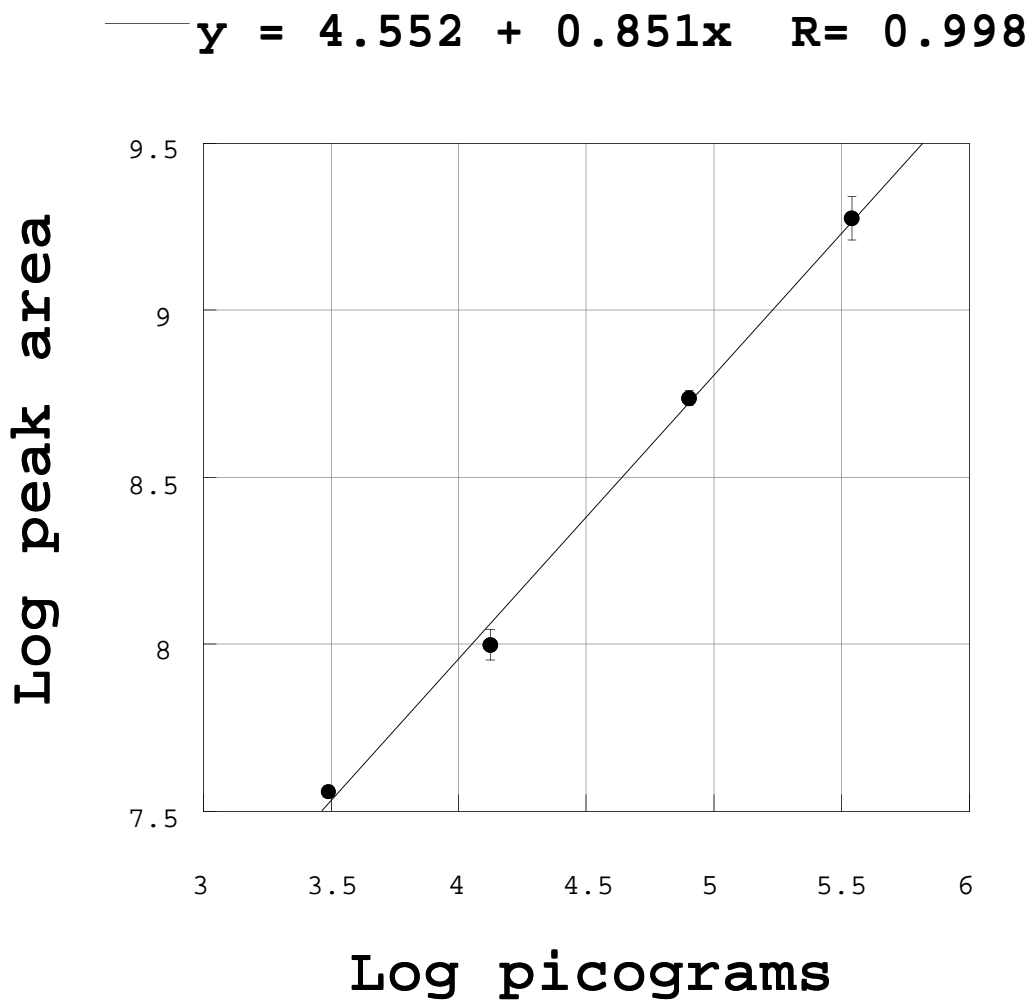
**Figure 3.** The nuclear magnetic resonance spectrum of trimethyldibromoantimony (TMDBA) with respect to tetramethyl silane (TMS).



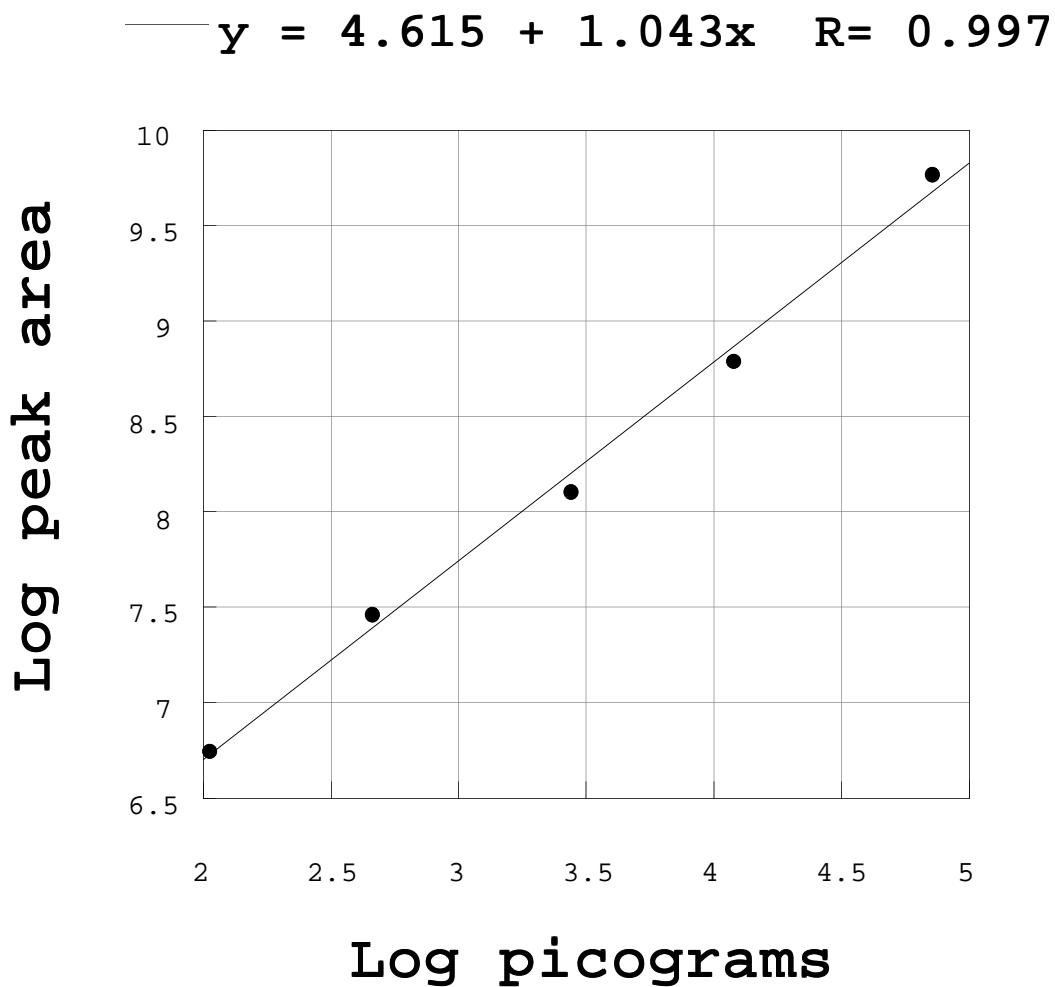
**Figure 4.** Calibration curve of dimethyl sulfide for GC/SCD. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



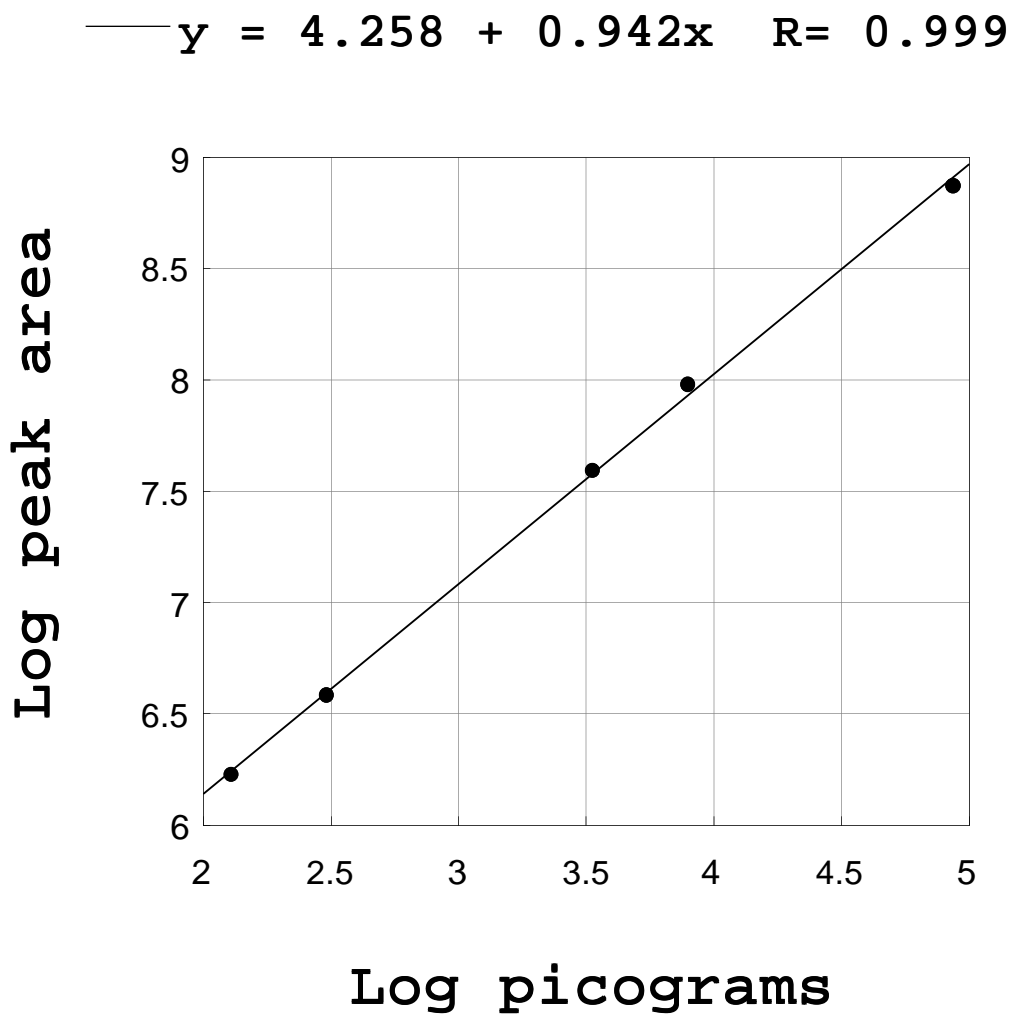
**Figure 5.** Calibration curve of dimethyl disulfide for GC/SCD. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



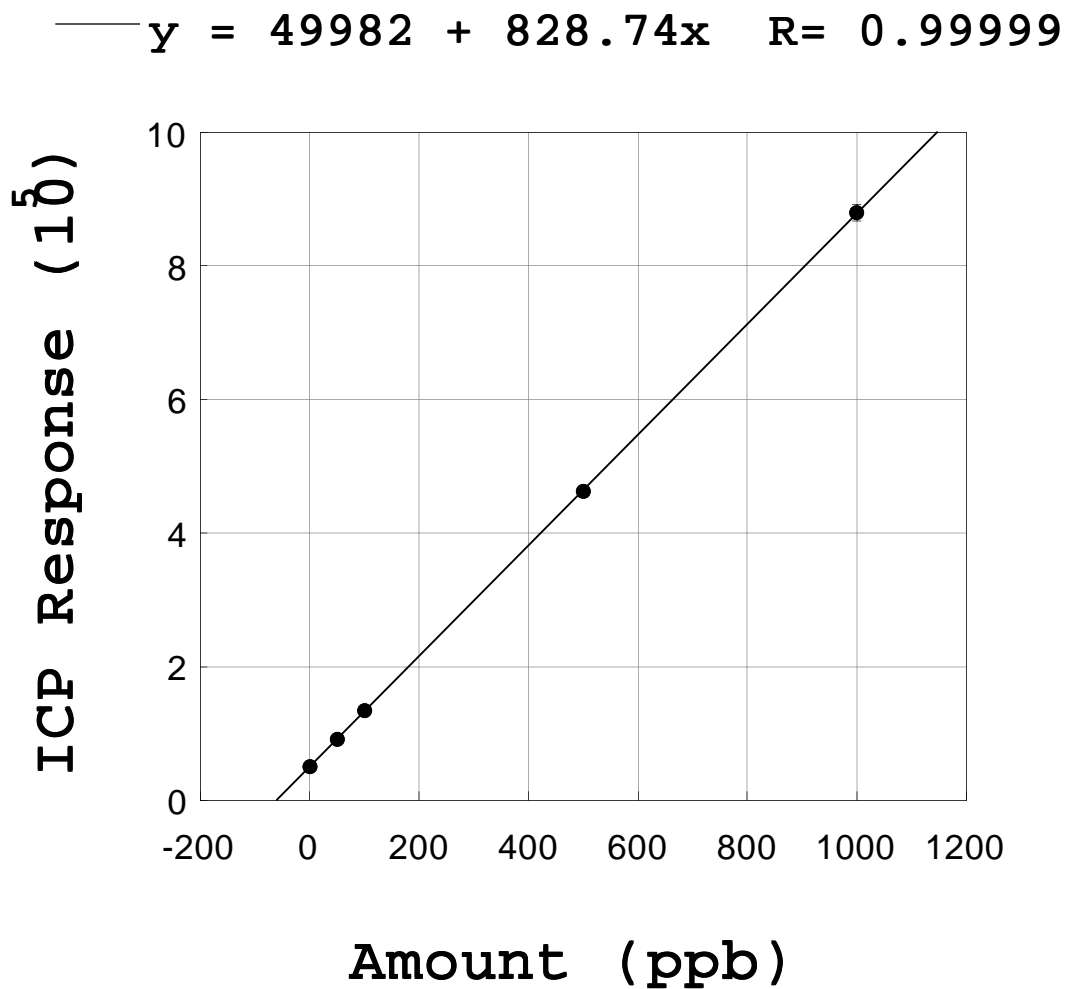
**Figure 6.** Calibration curve of dimethyl selenide for GC/SCD. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



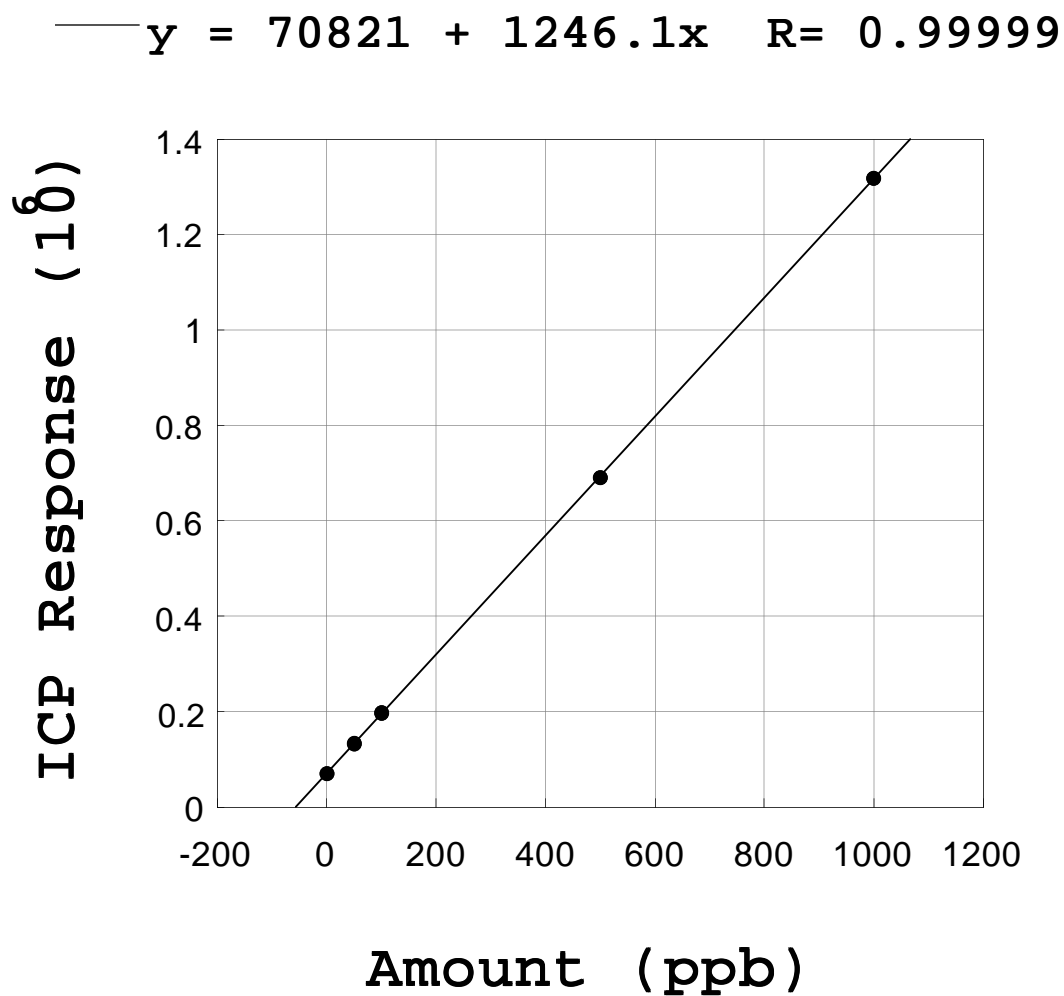
**Figure 7.** Calibration curve of dimethyl diselenide for GC/SCD. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



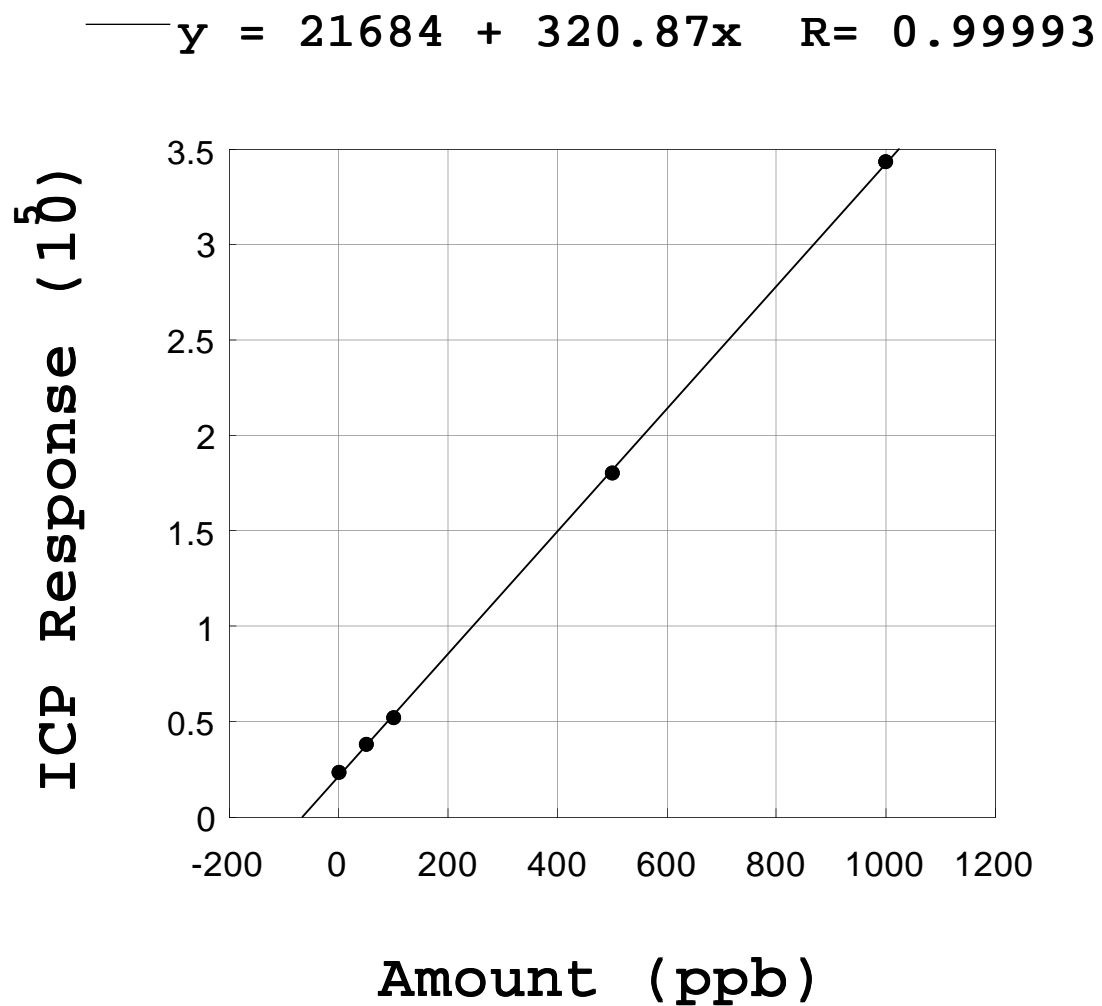
**Figure 8.** Calibration curve of trimethylstibine (TMSb) for GC/SCD. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



**Figure 9.** Calibration curve of antimony for ICP spectrometer. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.

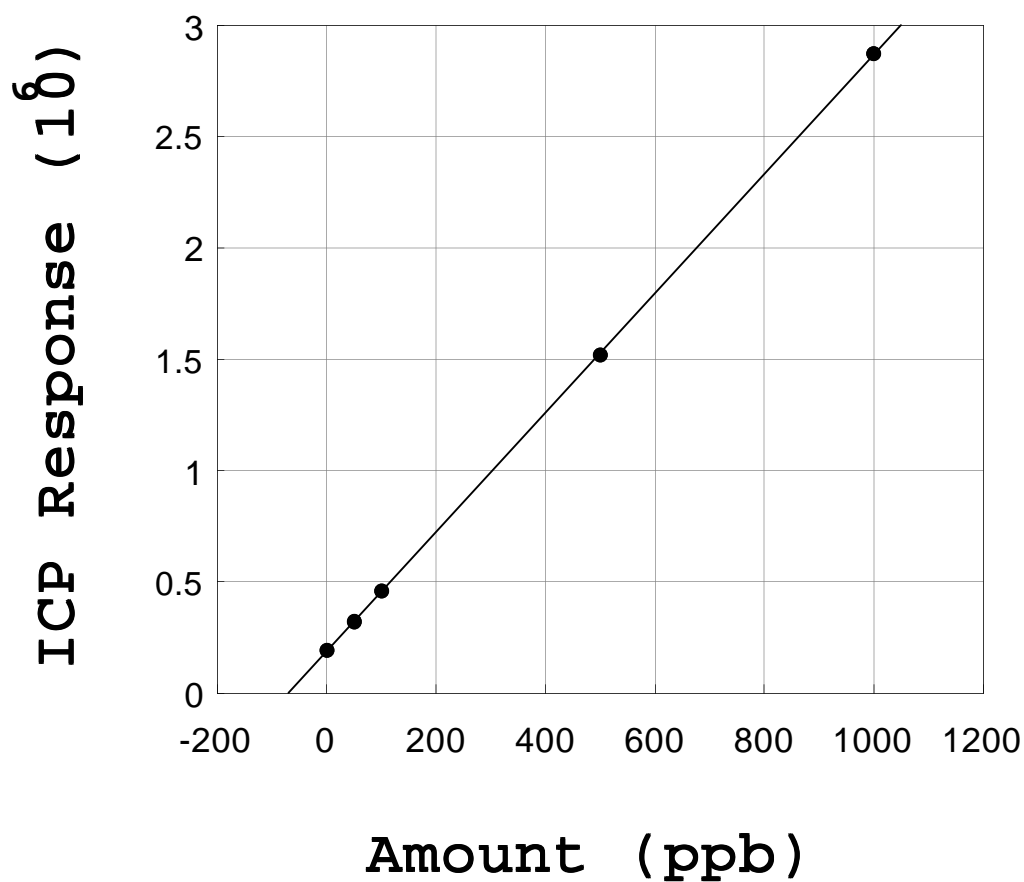


**Figure 10.** Calibration curve of arsenic for ICP spectrometer. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.

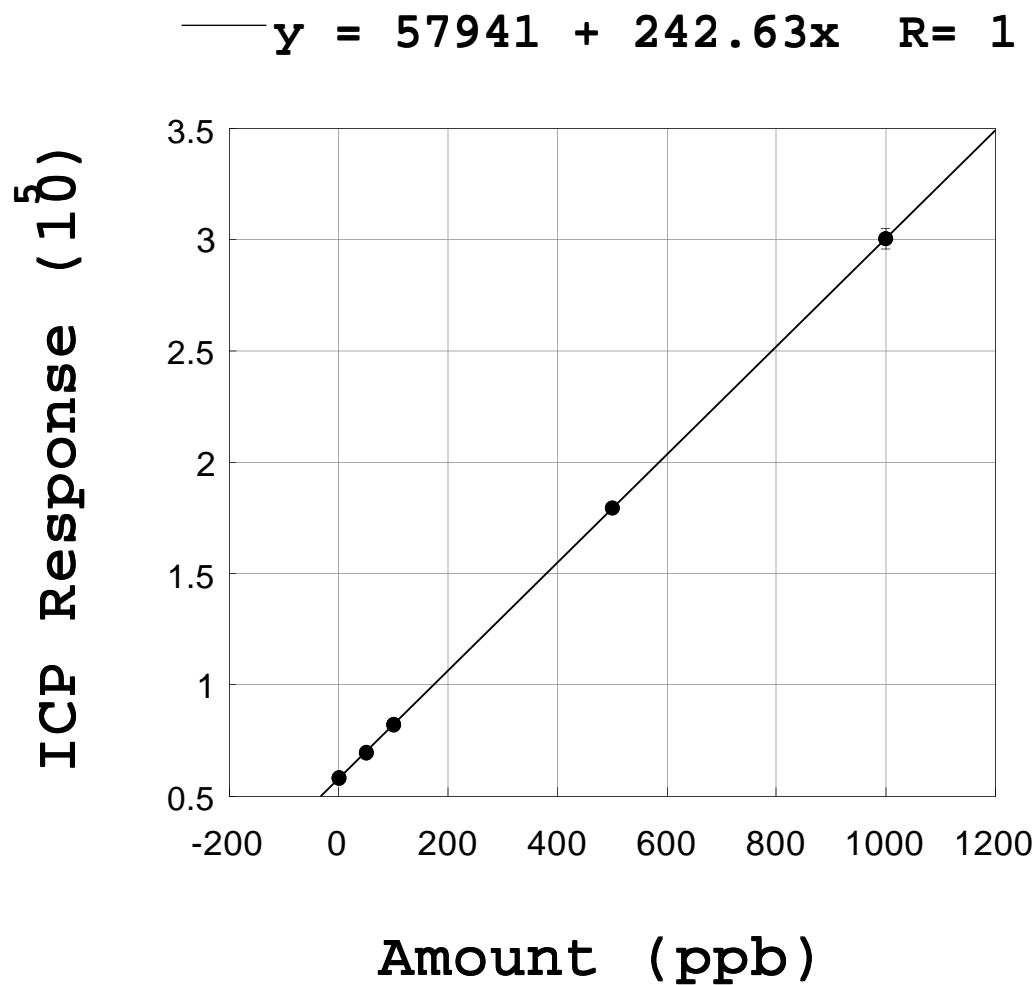


**Figure 11.** Calibration curve of selenium for ICP spectrometer. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.

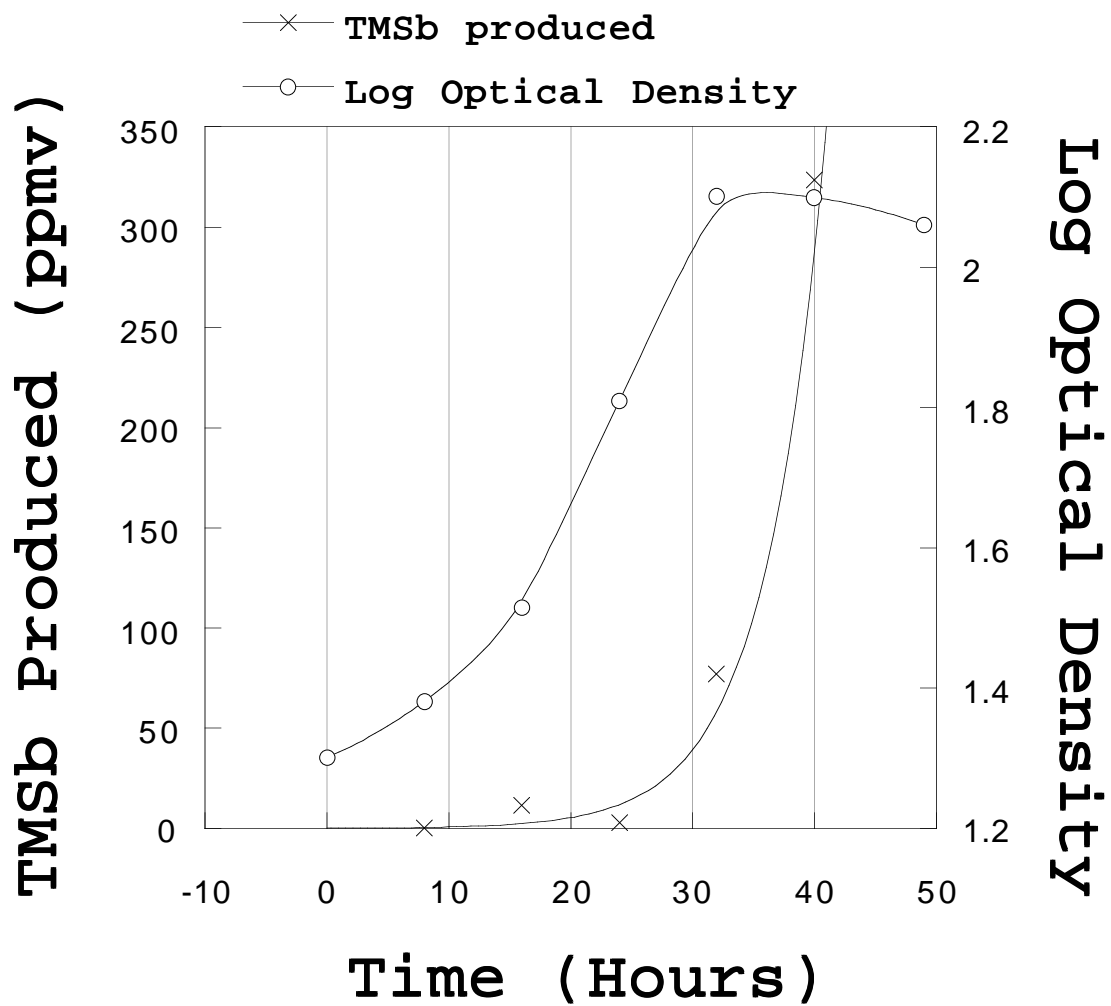
$$y = 1.8927e+05 + 2679.9x \quad R = 0.99999$$



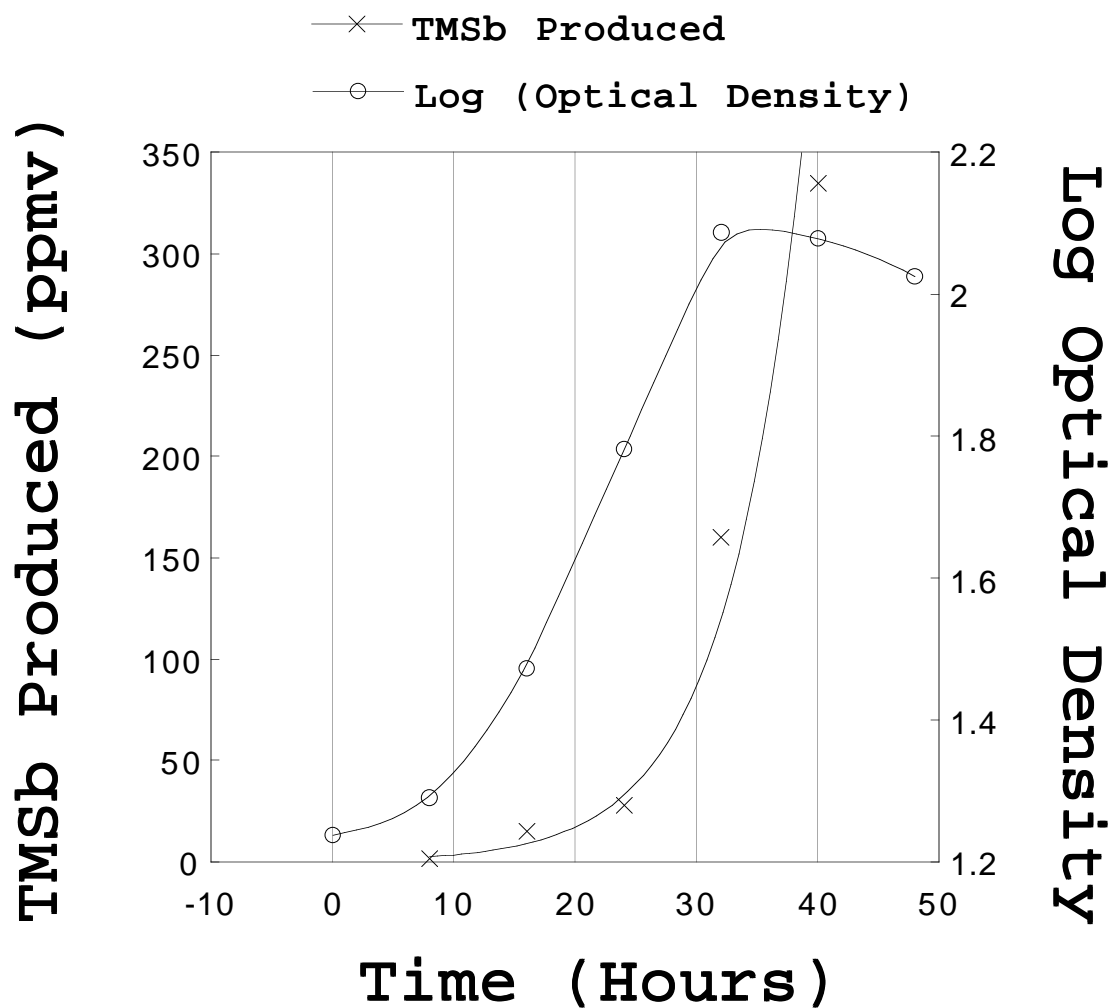
**Figure 12.** Calibration curve of lead for ICP spectrometer. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



**Figure 13.** Calibration curve of tin for ICP spectrometer. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.



**Figure 14.** Trimethylstibine (TMSb) production by *P. fluorescens* K27 amended with 0.01 mM trimethyldibromoantimony and the culture's growth in time.



**Figure 15.** Trimethylstibine (TMSb) production by *P. fluorescens* K27 amended with 0.1 mM trimethyldibromoantimony and the culture's growth in time.

**Table IV**

Results of the headspace analyses of SS, SPS, and BSS soil samples in various media amended with potassium antimonyl tartrate (PAT) and potassium hexahydroxy antimony (PHA).

	SS with PAT			SS with PHA		
	DM2	DMVTE	DMAATE	DM2	DMVTE	DMAATE
TMSb Produced	0	0	0	0	0	0

	SPS with PAT			SPS with PHA		
	DM2	DMVTE	DMAATE	DM2	DMVTE	DMAATE
TMSb Produced	+	+++	++++	++	++	++++

	BSS with PAT			BSS with PHA		
	DM2	DMVTE	DMAATE	DM2	DMVTE	DMAATE
TMSb Produced	++	+++++	++	+++++	0	++

TMSb production was scored as: 0, below detection limit (<12 ppbv); 1, 1,513-2,742 ppbv; ++, 2,743-4,002 ppbv; +, >4,003 ppbv. +, 1,513-2,742 ppbv; ++, 2,743-4,002 ppbv; +, >4,003 ppbv.

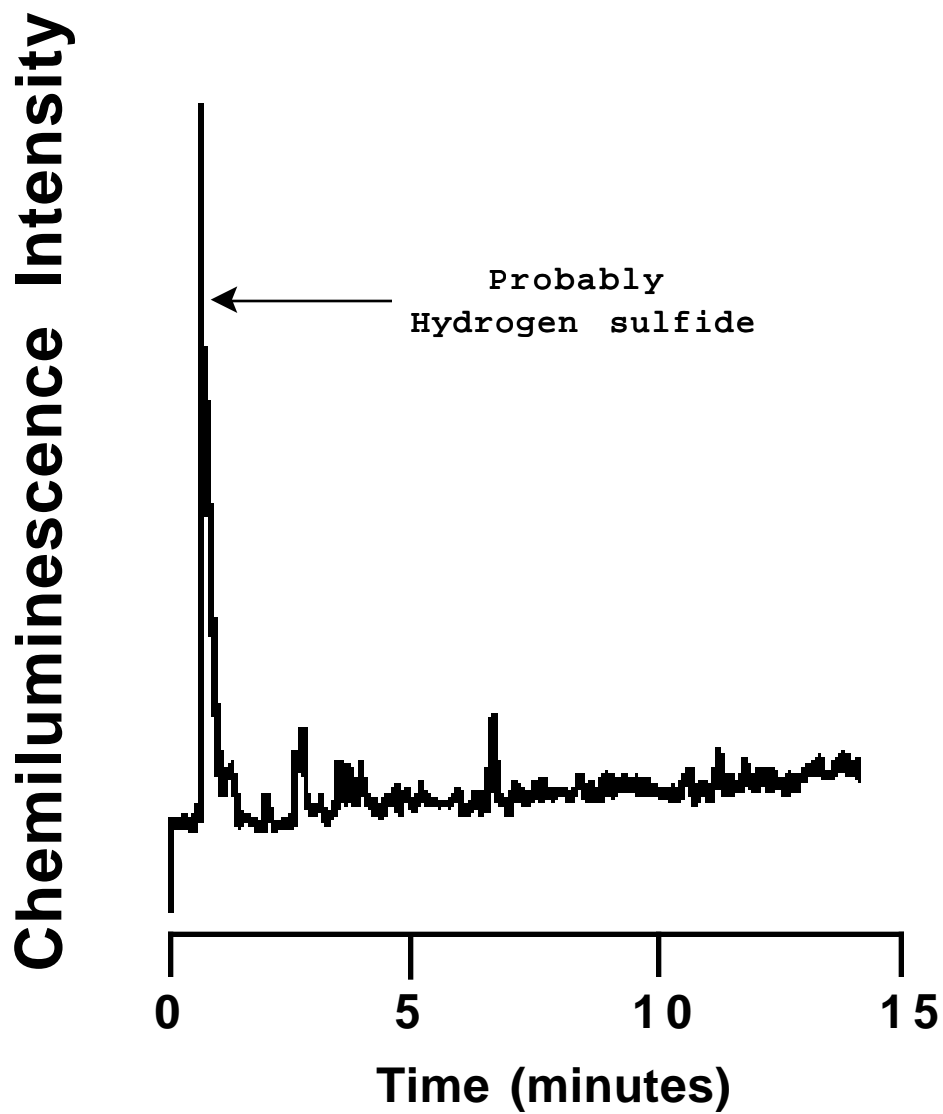
**Table V**

Dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) in the headspace of SS, SPS, and BSS soil samples amended with potassium antimonyl tartrate (PAT) and potassium hexahydroxy antimony (PHA).

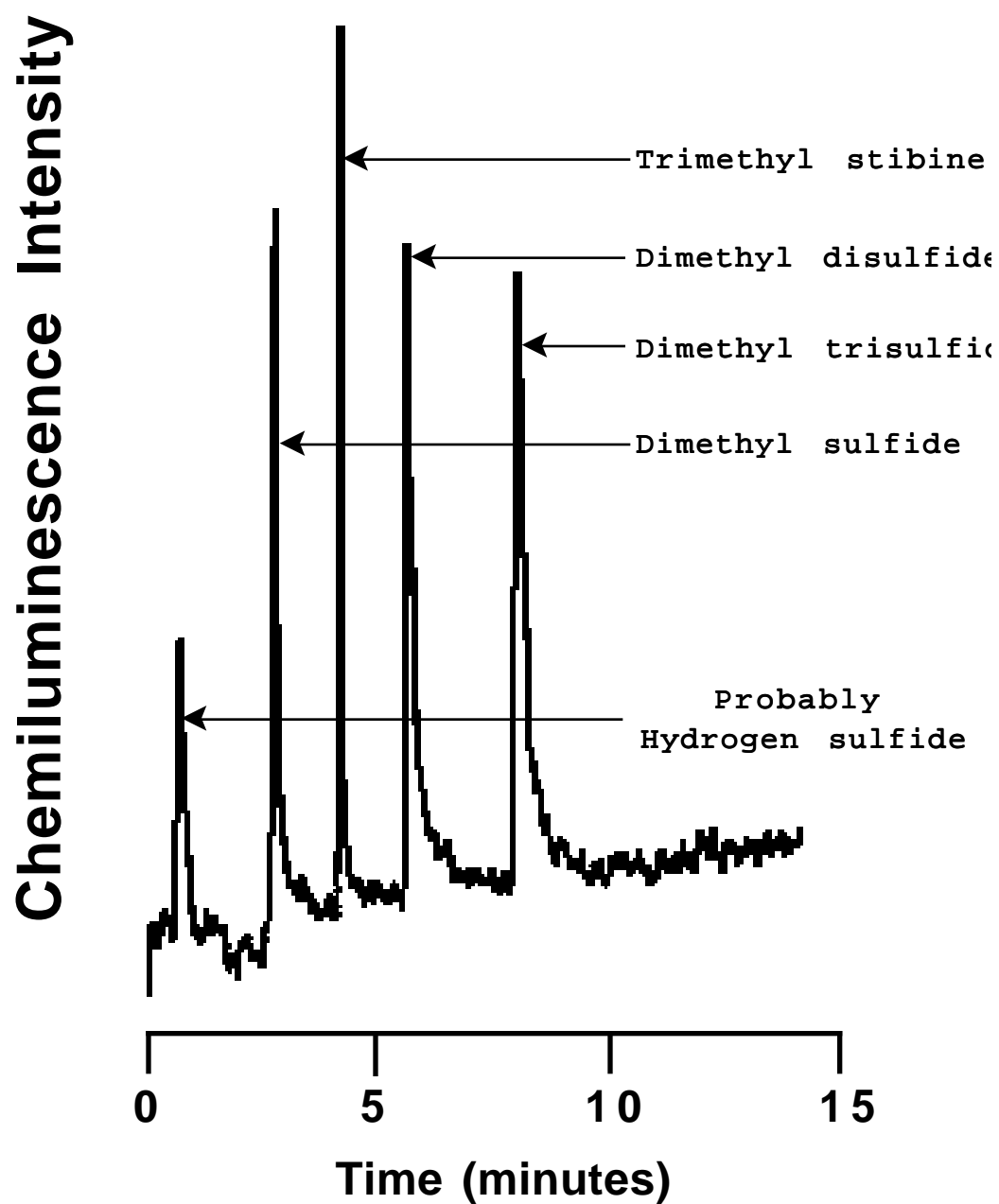
Soil sample from Switzerland (SS)		SS with PAT		SS with PHA	
	DM2	DMVTE	DMAATE	DM2	DMAATE
DMS	+	+	+	+	+
DMDS *	++	+	+	+	+
Soil sample from a sewage plant in Huntsville, TX USA (SPS)					
SPS with PAT		SPS with PHA			
	DM2	DMVTE	DMAATE	DM2	DMAATE
DMS	+++	++	++++	+++	+++
DMDS *	+++++	+	+	+	0
Soil sample from the backyard of an auto repairshop in Huntsville, TX USA (BSS)					
BSS with PAT		BSS with PHA			
	DM2	DMVTE	DMAATE	DM2	DMAATE
DMS	++	+++	++	+	++
DMDS *	+	+++++	+++++	+++++	+

DMS production was scored as: 0, not detected (<31.9 ppbv); 9-396.5 ppbv, 396.5-819.3 ppbv; +, 819.3-1,472 ppbv; ++, 1,472-2,138 ppbv; +, >2,138 ppbv.

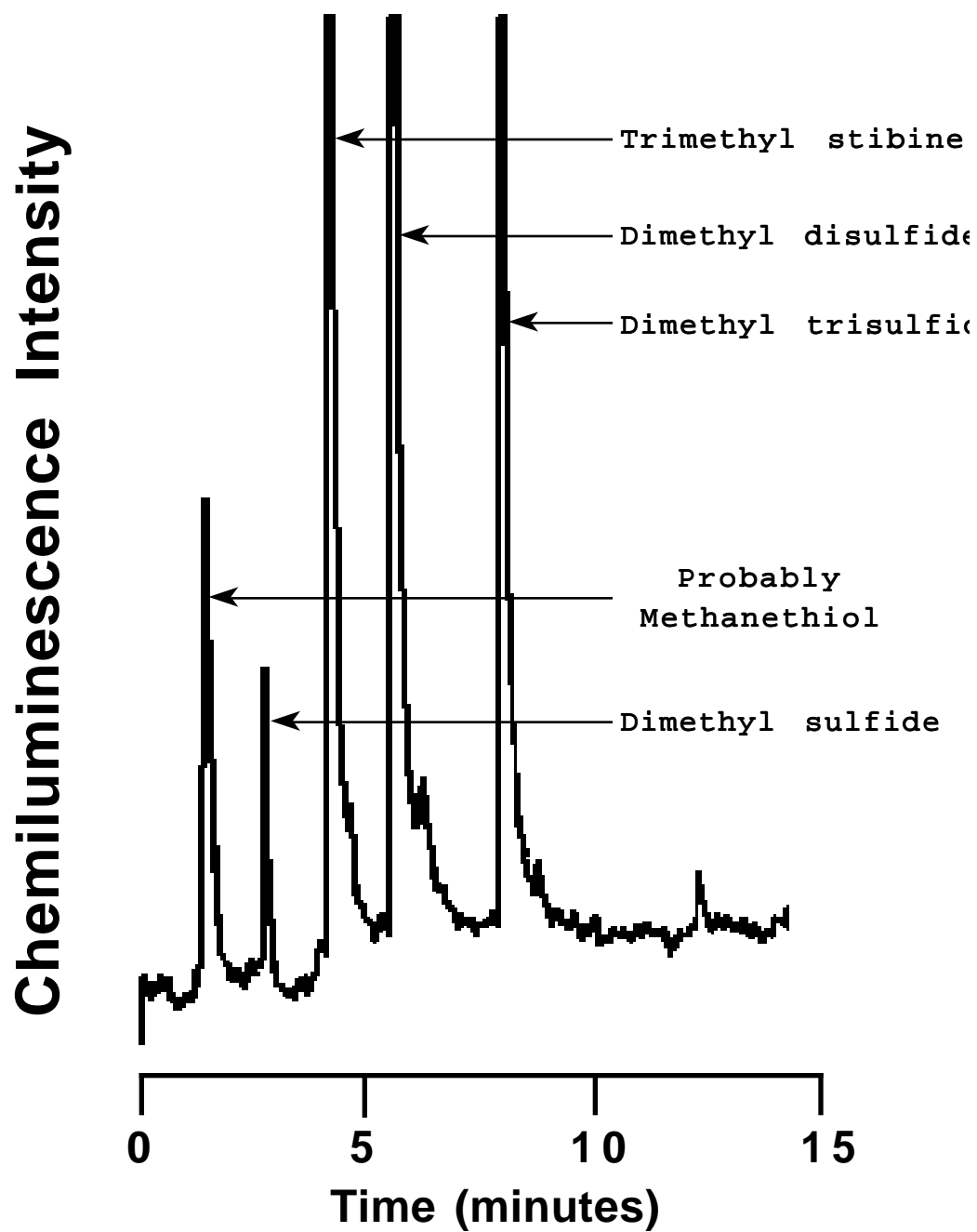
\*DMDS production was scored as: 0, not detected (<16.2 ppbv); 16.2-55.6 ppbv, 55.6-111.2 ppbv; +, 111.2-194.4 ppbv; ++, 194.4-277.6 ppbv; +, >277.6 ppbv.



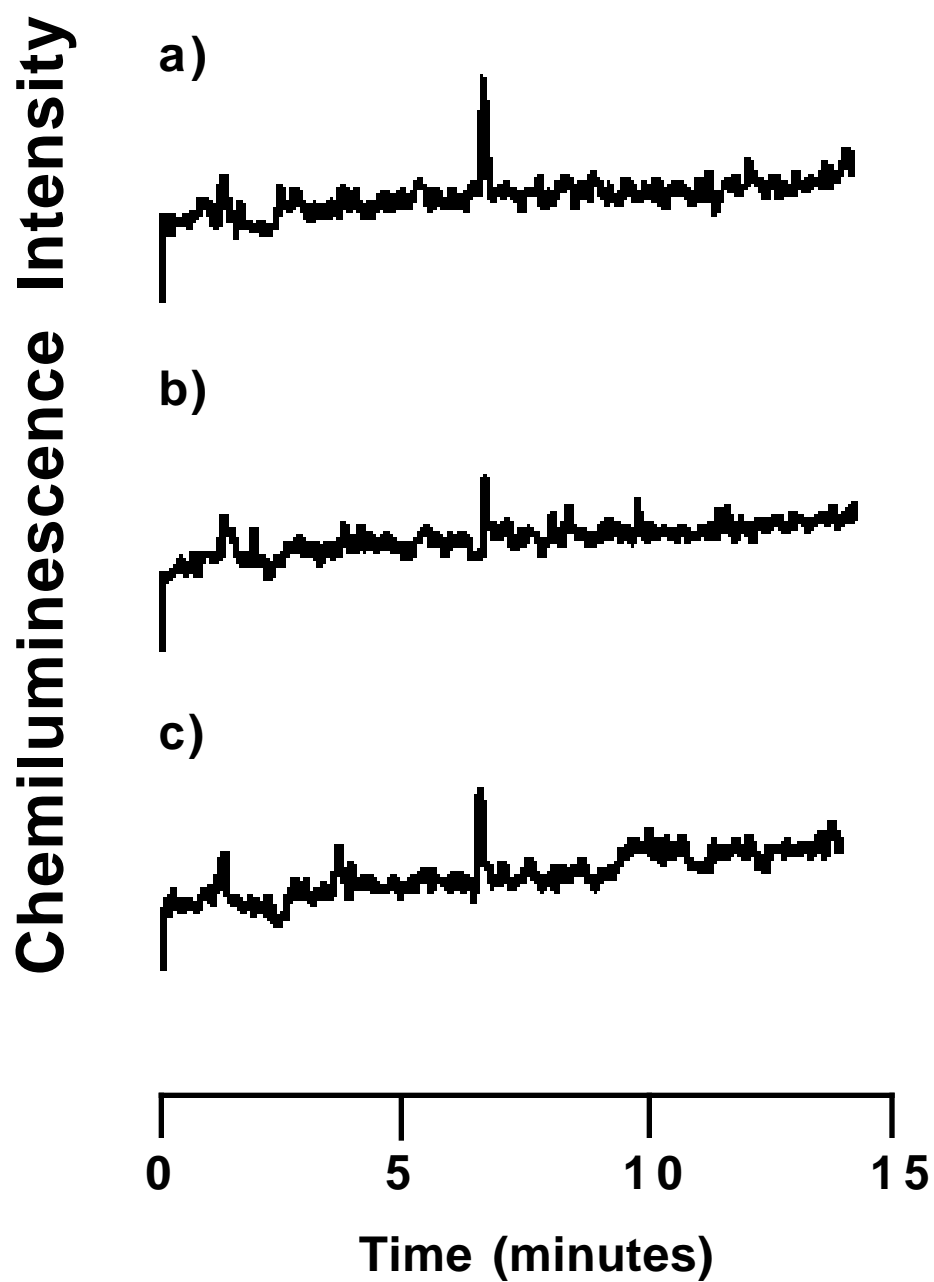
**Figure 16.** The chromatogram of the headspace of BSS soil in DM2, 1 month after inoculation (no antimony added).



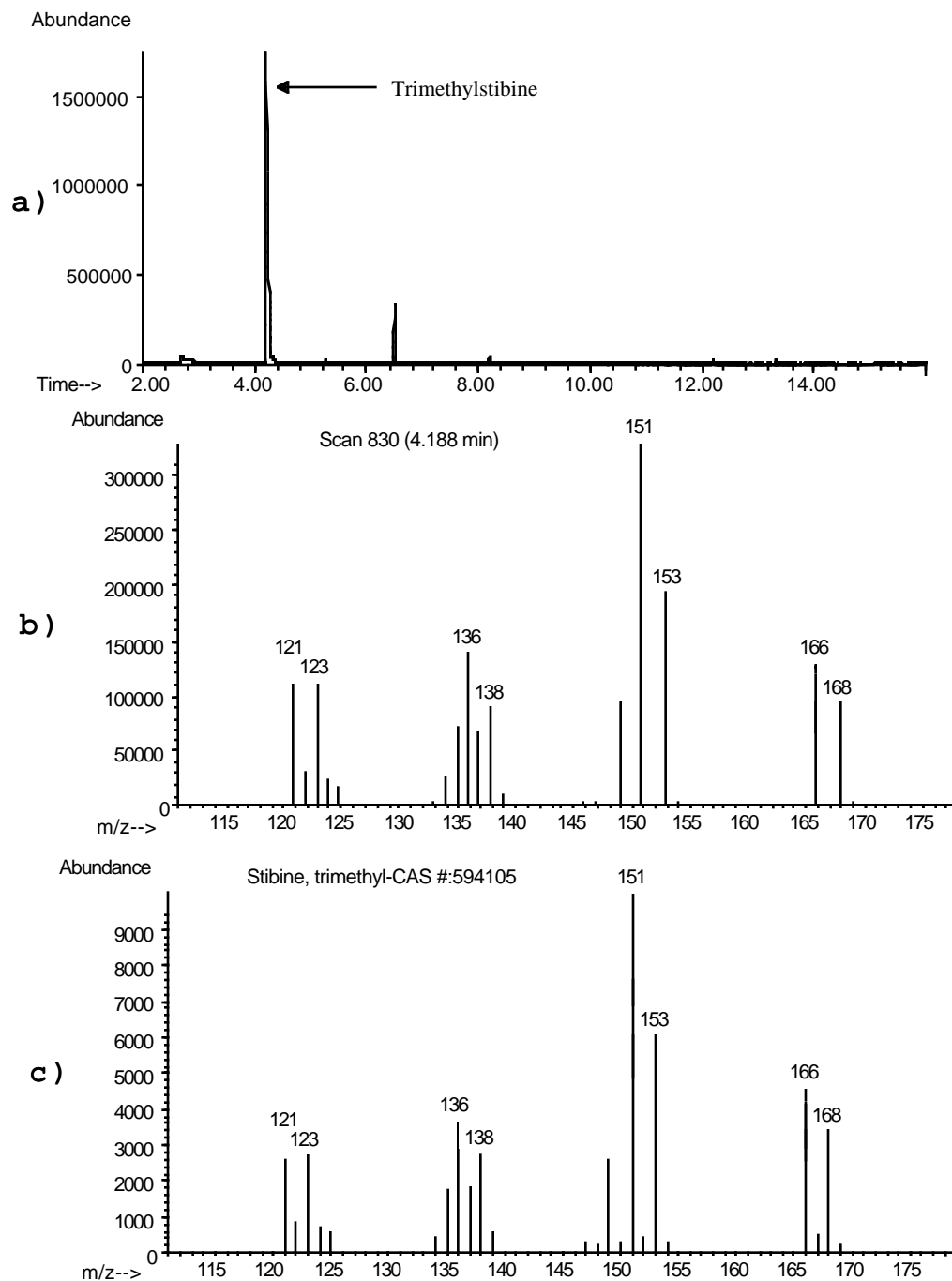
**Figure 17.** The chromatogram of the headspace of BSS soil in DM2 amended with 0.01 mM potassium antimonyl tartrate. The headspace was analyzed 1 month after amendment.



**Figure 18.** The chromatogram of the headspace of BSS soil in DM2 amended with 0.01 mM potassium hexahydroxo antimonate(V). The headspace was analyzed 1 month after amendment.



**Figure 19.** The chromatograms of the headspace analyses of sterilized BSS in DM2 amended with a) 0.01 mM potassium hexahydroxo antimonate(V); b) 0.01 mM potassium antimonyl tartrate; c) no antimony compounds.



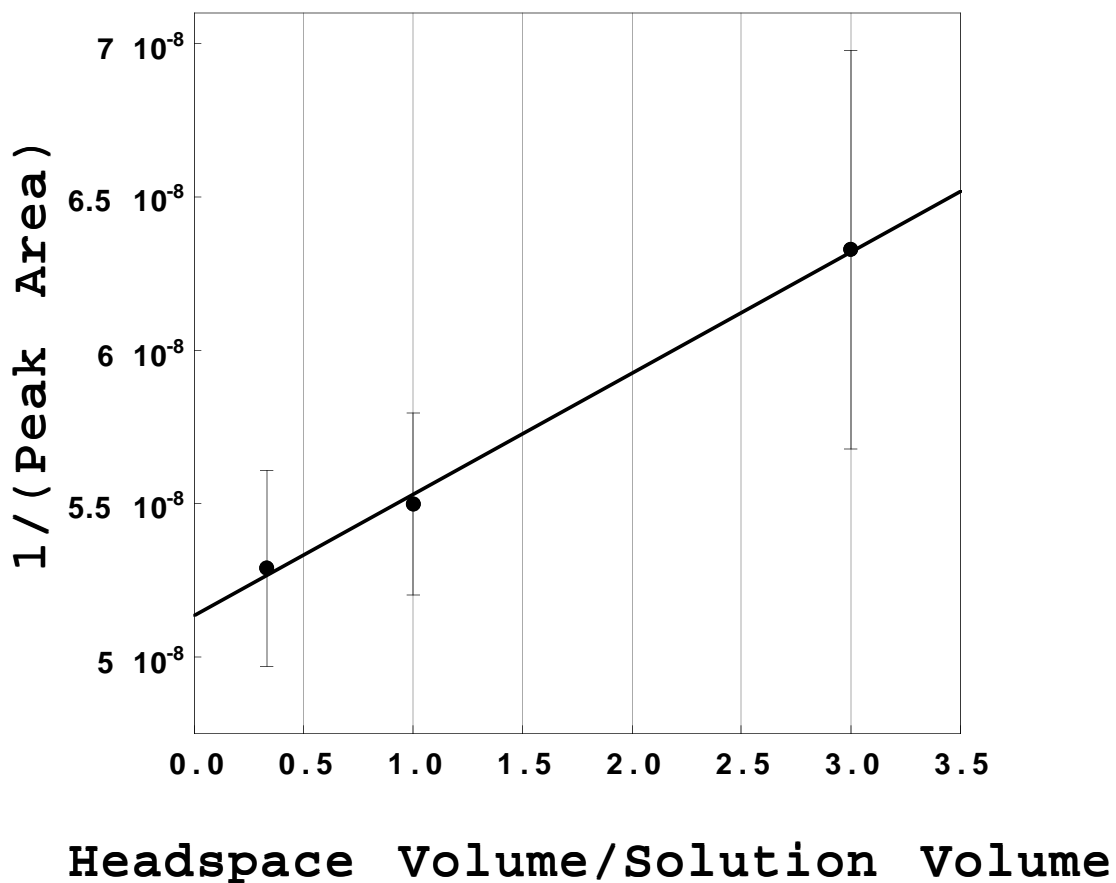
**Figure 20.** (a) Total ion chromatogram of the headspace above BSS soil sample amended with 0.01 mM potassium hexahydroxo antimonate(V) 1 month after amendment. (b) Mass spectrum of trimethylstibine found in the headspace (Peak at RT=4.188 min). (c) Reference mass spectrum of trimethylstibine from NIST Mass Spectra Library.

**Table VI**  
Results of the ICP analysis of the soil sample BSS

	<b>Sb</b>	<b>As</b>	<b>Se</b>	<b>Pb</b>	<b>Sn</b>
<b>Blank 1</b>	0	0	0	0	0
<b>Blank 2</b>	0	0	0	0	0
<b>Blank 3</b>	0	0	0	0	0
<b>Sample 1</b>	0	1411.3	265.7	920.3	0
<b>Sample 2</b>	408.6	2052.3	780.9	1867.9	2896.5
<b>Sample 3</b>	17.5	1470.5	313.3	1230.2	448.9
<b>Sample 4</b>	0	1392.6	278.3	831.2	323.6
<b>Average</b>	106.5	1581.7	409.6	1212.4	917.3

The concentrations reported are in ppb in solid sample.

$$y = 5.6459e-09 + 8.1963e-10x \quad R = 0.999$$



**Figure 21.** The graph that was used to calculate the Henry's Law constant for dimethyl sulfide. The points are the averages of triplicate analysis and the error bars represent the deviation of the triplicates from the average.

**Table VII.** Results of the Henry's Law constant calculations for dimethyl sulfide (DMS), dimethyl disulfide (DMDS), dimethyl selenide (DMSe), and dimethyl diselenide (DMDS<sub>2</sub>) in SMM medium at 25 °C.

	DMS	DMDS	DMSe	DMDS <sub>2</sub>
Dimensionless				
Henry's Law	0.1441	0.0538	0.0879	0.0879
constants				

## CHAPTER IV

### Results and Discussions

Trimethyldibromoantimony was successfully synthesized in our lab with minor modifications of the method described by Doak *et al.* (1967). The glove box purged with nitrogen helped prevent the air oxidation of the intermediate, trimethylstibine. After 2 low yield syntheses, an excess of magnesium was used to increase the yield in the Grignard step. The yield of the crude product was 11.41 g [55.7% of the theoretical based upon antimony(III) chloride]. After crystallization from 95% ethanol, the yield was 7.53 g (36.8%).

The product was identified using elemental analysis, Fourier transform infrared spectroscopy and nuclear magnetic resonance spectroscopy. The elemental analysis of the product showed acceptable match to the theoretical values and it was better than the values reported in the reference used (Doak *et al.*, 1967) (Table II).

Goel *et al.* (1971) calculated that the asymmetric stretching of the carbon—antimony bonds would appear at  $563\text{ cm}^{-1}$  and in their experiments they found it to be at  $570\text{ cm}^{-1}$ . In our experiments, this asymmetric stretching peak showed up at  $568.34\text{ cm}^{-1}$  which is closer to the calculated value (Figure 2a and b).

The NMR spectrum of trimethyldibromoantimony (Figure 3) gave a singlet at  $\delta$  2.61 since the position (chemical environment) of all the hydrogens are the same in trimethyldibromoantimony. Parris and Brinckman (1976) reported a singlet at  $\delta$  2.62 for the trimethyldibromoantimony NMR spectrum.

Calibrations were run using a  $\text{F}_2$ -induced chemiluminescence detector in order to quantitate the bacterial productions of dimethyl sulfide, dimethyl

disulfide and trimethylstibine (Figures 4, 5, and 8 respectively). Calibrations were also carried out for dimethyl selenide and dimethyl diselenide using F<sub>2</sub>-induced chemiluminescence detection and were used in a different project (Figures 6 and 7 respectively). The detector responded linearly around 4 orders of magnitude and therefore, the linearity couldn't be observed well using an x-y plot in the software used. As a result, the calibrations were plotted in log-log fashion that presented the linearity in a better way (Fowles and Scott, 1963). The detection limits for these compounds were calculated using a signal-to-noise ratio of 3 and the limits are presented in Table III.

Since the antimony content of the soil sample is an important aspect of our experiments, elemental analysis of the soil sample was necessary. For this purpose, an inductively coupled plasma spectrometer was calibrated for antimony, arsenic, selenium, lead, and tin. The spectrometer was extremely linear for the above elements for 3 orders of magnitude as can be seen in Figures 9, 10, 11, 12, and 13.

When *P. fluorescens* K27 was inoculated in DM2 medium separately with 0.01 mM of trimethyldibromoantimony, potassium antimonyl tartrate and potassium hexahydroxo antimonate(V), trimethylstibine was found after 24 hours only in the headspace of samples that contained trimethyldibromoantimony. The blanks for trimethyldibromoantimony amendment (described in Chapter 2, part 2) **did not** produce any trimethylstibine. This shows that the reduction of trimethyldibromoantimony to trimethylstibine in these samples is the result of a biological activity.

In the headspace of samples that contained potassium antimonyl tartrate and potassium hexahydroxo antimonate(V), no trimethylstibine was found after 24 hours. The experiment was carried out over a month period with analysis of a sample every other 4 or 5 days, but still no trimethylstibine was found.

The bacterial growth in these liquid cultures was measured by optical density and Figure 14 and 15 shows that the reduction of trimethyldibromoantimony is significant 8 hours after amendment. The production of trimethylstibine by K27 starts to increase exponentially as the culture reaches the stationary phase of growth. In the tubes that were amended with PAT and PHA no bacterial growth was observed even 1 month after of amendment. The lack of growth in these cultures could be due to the toxicity of these two compounds to *P. fluorescens* K27 and the reason for the absence of production of trimethylstibine from PAT and PHA.

Although the above experiment shows the reduction of an antimony compound by an organism, it still lacks conclusive evidence for biomethylation since trimethyldibromoantimony already has three methyl groups on it. Therefore, we collected soil samples and postulated that they would contain an organism that could show methylation activity. The three different soil samples (SS, SPS, and BSS) were inoculated into different biological media (DM2, DMVTE, and DMAATE) to increase the chances for that specific organism to grow and then produce trimethylstibine. The tubes were kept in a water bath until their headspaces were analyzed two weeks later. The presence of the soil in the tube made it impossible for us to measure the bacterial growth correctly. Therefore, we examined the growth by shaking the tubes gently without disturbing the soil and looking for a turbulence in the solution which would suggest bacterial growth. There was bacterial growth in all soil samples amended with PAT and PHA but when the headspaces in the tubes that contained SS and antimony compounds were analyzed, no trimethylstibine was found. On the other hand, trimethylstibine was found in the headspaces of most of the tubes that contained the other two soil samples with antimony compounds (Table IV). In addition to trimethylstibine,

dimethyl sulfide and dimethyl disulfide were also found in the headspace (Table V).

In order to narrow the scope of our experiments, we chose BSS as our soil sample and the simplest DM2 medium as our biological medium. This combination produced significant amounts of trimethylstibine. The samples with BSS were prepared including all the necessary blanks. All these samples were left in a water bath at 30 °C for a month and then their headspaces were analyzed for trimethylstibine (Figures 16, 17, 18, and 19). Out of 24 samples of each set, 15 tubes that contained potassium antimonyl tartrate, 9 tubes that contained potassium hexahydroxo antimonate(V) and only 1 tube that contained no antimony compounds were found to have trimethylstibine in their headspaces. The trimethylstibine was identified by its retention time in GC/chemiluminescence detection compared to a standard and by analyzing the sample using a mass selective detector. The retention time window for trimethylstibine in the chemiluminescence detector was  $\pm 0.04$  minutes which corresponds to less than 2.5 seconds of deviation. The mass spectrum of trimethylstibine was obtained from the chromatogram (Figure 20a) acquired by the mass selective detector (Figure 20b). This spectrum was then compared to the spectrum of trimethylstibine in National Institute of Standards mass spectra library (Figure 20c). The Hewlett Packard ChemStation software was asked to match the two spectra and the resulting match quality was 99%. An old dimethyl disulfide standard gave peaks at the same retention times as the first eluting peaks in Figures 17 and 18 which suggest that those peaks are sulfur containing compounds such as hydrogen sulfide and methanethiol.

The presence of trimethylstibine in the headspace of soil samples amended with potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) was due to a biological activity since none of the headspace analyses of blanks that

contained sterilized soil samples produced trimethylstibine. Only one of the blanks that contained the soil sample and no added antimony compounds produced a little amount (20.4 ppbv) of trimethylstibine. This could be due to the high antimony content of that specific soil sample some of which could have been dissolved in the biological medium. In order to confirm this hypothesis, elemental analysis on BSS soil was performed to see its antimony content. Four samples were analyzed for antimony and 4 other elements (As, Se, Pb, and Sn) using inductively coupled plasma spectroscopy (Table VI). Two out of four samples were found to contain no antimony at all whereas the other two contained some. As it can be seen with the results from the antimony analysis of the soil sample, one of the major problems in using soil samples is the homogeneity of the soil sample. Although the samples were collected from the same place, environmental soil samples are seldom homogeneous. In fact, the only blank that produced trimethylantimony can still be considered as an evidence for the biomethylation of antimony since it is still methylation of an antimony compound although this antimony compound was not added to the medium.

In conclusion, we can say that we have strong evidence that an organic and an inorganic salt of antimony have been methylated biologically by living organisms producing trimethylstibine.

Different antimony compounds such as salts of stibonic and stibinic acids and any water soluble antimony compound can be used to amend the cultures. More important than this is the isolation of the culture from the soil samples that performs methylation and once a pure culture is obtained, a better understanding of this process could be achieved.

Henry's Law constants were calculated for dimethyl sulfide, dimethyl disulfide, dimethyl selenide, and dimethyl diselenide (Table VI). The headspaces of the tubes with different headspace to liquid volume ratios were analyzed and

the reciprocal of the resulting peak areas were plotted versus the headspace to liquid volume ratios (Figure 20). The headspace analysis of each tube in each phase ratio generally produced high standard deviation but the plot of averages of the results for specific headspace to liquid volume ratios versus the phase ratios produced a line with high linearity. Our results for dimethyl sulfide and dimethyl selenide is comparable to the ones in the literature (Dacey *et al.*, 1984; Karlson *et al.*, 1994). For dimethyl sulfide, Dacey *et al.* (1984) reported dimensionless Henry's Law constants of 0.0693 in distilled water at 24.2 °C and 0.0821 in sea water at 23.5 °C. For dimethyl selenide, Karlson *et al.* (1994) reported a Henry's Law constant of 0.058 in deionized water at 25 °C. Although the value reported for dimethyl sulfide is lower than our value (0.1441), one should consider that these values were not measured in 25 °C and Henry's Law constant increases exponentially with increasing temperature. On the other hand, our value for dimethyl selenide (0.0879) also seems acceptable since the literature value was measured in deionized water whereas ours was in a biological medium with high ionic strength. These constants were calculated in an effort to calculate the total extracellular productions of these compounds. Until now, headspace analysis has been a way of quantitating this bacterial production but these constants help us find out the amounts in the solution from the amounts in the headspace, basically improving our estimations for the real amount.

# CHAPTER V

## Conclusions

- Trimethyldibromoantimony was successfully synthesized and used in amending bacteria to investigate the possible bioreduction of this compound.
- *P. fluorescens* K27 reduced trimethyldibromoantimony to trimethylstibine in anaerobic cultures at 30 °C grown in a minimal medium. This reduction was confirmed to be a biological process because the sterile blanks showed no detectable trimethylstibine production. The culture amended with trimethyldibromoantimony reached its stationary growth phase after around 30 hours where it started to produce high amounts of trimethylstibine in the headspace. On the other hand, the same culture failed to reduce and methylate potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) as measured by trimethylstibine production in the headspace. No growth was observed with the cultures amended with these compounds at a concentration of 0.01 mM even after 1 month of amendment.
- Out of three soil samples examined, two of them methylated potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) in various biological media after 2 weeks of amendment. The product, trimethylstibine, was identified using its retention time in GC/chemiluminescence detection compared to a standard and by analyzing the sample using a mass selective detector.

- In all the blanks prepared (96) and analyzed, no trimethylstibine was found except for one which contained a soil sample but no added antimony compounds. This could be due to a high concentration of environmental antimony present in that specific soil sample used ( $\approx 408.6$  ppb).
- We can say we have strong evidence that the trimethylstibine found in the headspace above soil samples amended with potassium antimonyl tartrate and potassium hexahydroxo antimonate(V) is a result of biological methylation of these compounds.
- As expected, the fluorine-induced chemiluminescence detector responded to trimethylstibine as well as it responded to methyl sulfides and selenides. The detection limit for trimethylstibine was comparable to these compounds and the detector response was linear for over 3 orders of magnitude.
- The Henry's Law constants for dimethyl sulfide, dimethyl disulfide, dimethyl selenide, and dimethyl diselenide were measured in a defined medium at 25 °C using the static headspace method.

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# APPENDIX

## Chemical Abstract Service Registry Numbers

<b>Compound Name</b>	<b>CAS Registry Number</b>
Acetonitrile	75-05-8
Ammonium sulfate	7783-20-2
Antimony(III) chloride	10025-91-9
Aspartic acid	56-84-8
Biotin	58-85-5
Boric acid	10043-35-3
Bromine	7726-95-6
Calcium chloride dihydrate	10035-04-8
Carbon tetrachloride	56-23-5
Dimethyl diselenide	75-09-2
Dimethyl disulfide	7101-31-7
Dimethyl selenide	624-92-0
Dimethyl sulfide	75-18-3
(ethylenedinitrilo)-tetraacetic acid disodium salt	60-00-4
Ethanol	64-17-5
Ether (diethyl ether)	60-29-7
Iron(II) sulfate heptahydrate	7782-63-0
Glutamic acid	56-86-0
Glycerol	56-81-5

Magnesium sulfate heptahydrate	10034-99-8
Magnesium turnings	7439-95-4
Manganese sulfate	7785-87-7
Methyl iodide	74-88-4
Nicotinamide	98-92-0
Nitric acid	7697-37-2
Nitrilotriacetic acid	139-13-9
Potassium antimonyl tartrate	28300-74-5
Potassium hexahydroxo antimonate(V)	12208-13-8
Potassium hydroxide	1310-58-3
Potassium phosphate dibasic	7758-11-4
Potassium phosphate monobasic	7778-77-0
Sodium chloride	7647-14-5
Sodium citrate	6132-04-3
Sodium molybdate dihydrate	10102-40-6
Succinic acid	110-15-6
Thiamine	67-03-8
Trimethylstibine	594-10-5
Zinc sulfate	7446-20-0

## VITA

Hakan Gürleyük was born in Istanbul, Turkey, on March 03, 1971. He graduated from Kadıköy Anatolian High School in 1989 and entered Bogaziçi University where he received a Bachelor of Science in Chemistry in 1994. Upon completion of his B.S., he attended Sam Houston State University as a graduate student, and graduated with a Master of Science degree in Chemistry in May 1996. He was accepted by the doctoral program in chemistry at the University of Massachusetts, Amherst in February 1996.