

Synthesis of Dimethyl Selenone and Studies on the Biomethylation
Intermediates of Selenium Reduction and Methylation

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Synthesis of Dimethyl Selenone and Studies on the Biomethylation
Intermediates of Selenium Reduction and Methylation

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Abstract

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Purpose

This investigation was undertaken for the purpose of determining whether or not dimethyl selenone could be an intermediate of biomethylation and transformation of inorganic selenium in bacterial cultures. The possible pathways for the synthesis of dimethyl selenone were also investigated.

Method

The reactions of the synthesis of dimethyl selenone were carried out in a methylene chloride solution of 3-chloroperoxybenzoic acid and dimethyl selenide. Fourier transform infrared spectroscopy, nuclear magnetic resonance spectroscopy, elemental analysis and melting point analysis were used to characterize the dimethyl selenone formed.

Pseudomonas fluorescens K27 was grown in tryptic soy broth containing 0.1% potassium nitrate, TSN medium. All test tubes and filters were autoclaved before using. Both medium and aqueous

solutions of selenium containing salts were sterilized by using sterilized filters. A Hewlett Packard 5890 Series II gas chromatograph coupled with a Sievers Research Model 300 Sulfur Chemiluminescence Detector was used as the principal tool for this research.

Findings

3-Chloroperoxybenzoic acid (m-CPBA) (65%) was found to readily oxidize dimethyl selenide (DMSe) at 20°C in 2 hours. The optimum ratio of m-CPBA to DMSe was 3:1 mole equivalents.

Pseudomonas fluorescens K27 grew well in sterilized TSN medium. Dimethyl selenone was tentatively identified as one of the intermediates of biomethylation of inorganic selenium because very large amount of volatile selenium compounds such as dimethyl selenide, dimethyl diselenide (DMDS₂) and probably dimethyl selenenyl sulfide (DMSeS) were observed after the K27 cultures were dosed with dimethyl selenone. Gas phase concentrations of volatile DMSe and DMDS₂ formed above selenone dosed cultures were about 4000 times and 1000 times more (respectively) than those observed above sodium selenate dosed cultures poisoned with the same concentration. If dimethyl selenone is a biological intermediate of the reduction and methylation of selenium oxyanions, then the final reduction step to dimethyl selenide and dimethyl diselenide may occur by chemical means via reduction by dimethyl sulfide and dimethyl disulfide.

Thomas G. Chasteen
Thesis Director

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Mr. Steve L. McCarty in Chasteen's research group has also provided great support to my work. I would also like to thank him.

A special thanks go to my husband Fang Deng who has given me the assistance and constant support I needed to complete this thesis.

My thesis is dedicated to my parents who have always loved me, supported me and encouraged me.

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Chapter I

Introduction

Selenium and its compounds have been of considerable biological interest for a long time because they are both required by and highly toxic to human beings and animals. From the public health standpoint, trace amounts of selenium are important but they are harmful to organisms in the environment at higher concentrations.

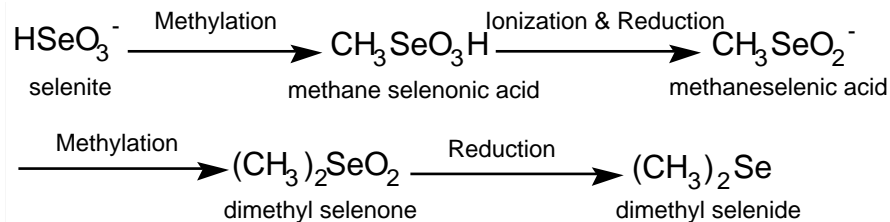
Movement of toxic inorganic selenium through the geocycle and its biological methylation in the environment to volatile products have also attracted attention. Methylation often leads to a change in both mobility and toxicity of the element. The conversion of oxyanionic compounds containing inorganic selenium such as selenate(VI) and selenite(IV) to volatile methylated species such as dimethyl selenide and dimethyl diselenide by plants, microorganisms and mammals has been well documented in the literature (Challenger and North, 1934; Lewis *et al.*, 1966; Jiang *et al.*, 1983).

The biological methylation of inorganic selenium by microorganisms has been investigated since the early 1930s beginning with Challenger and co-workers. In 1934, Challenger and North reported that fungi in culture are able to methylate inorganic selenium (Challenger and North, 1934). In the 1970s, information on microorganisms' ability to methylate some heavy metals such as Hg (Wood, 1974), As (Braman and Foreback, 1973; Wood, 1974), and Pb (Wong *et al.*, 1975) was published. For heavy metals such as Hg and Pb, microorganisms have the capacity to metabolize certain compounds containing these elements and the resulting transformations could

thus yield more toxic products (Wood, 1974; Wong, *et al.* 1975). In 1974, Francis and co-workers noted that dimethyl selenide was evolved from samples of soil (Francis *et al.*, 1974). Hence, methylation reactions may be of importance to the transformations of selenium in natural ecosystems.

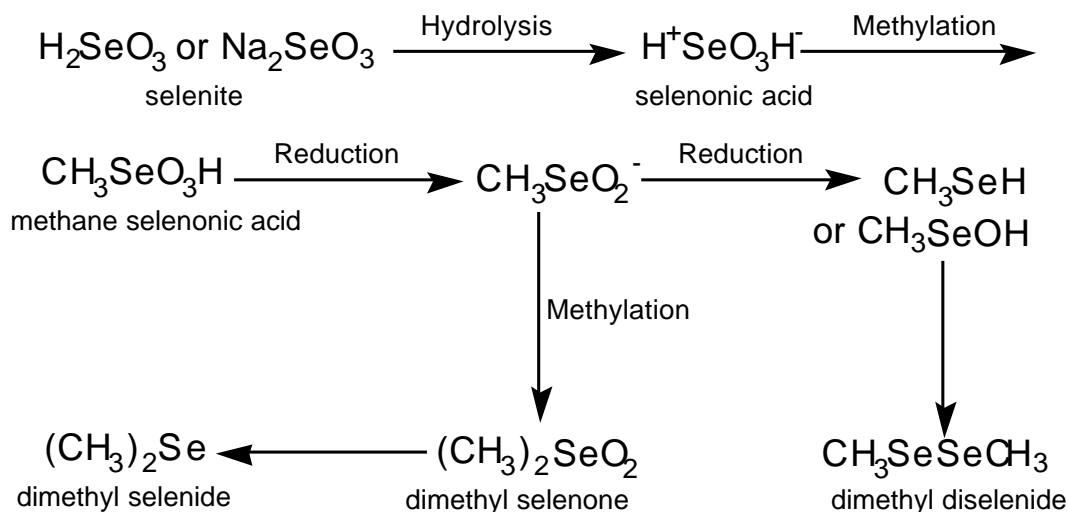
With inorganic selenium, biomethylation processes by microorganisms can produce volatile organic metabolic compounds that are less toxic than the original forms (McConnell and Portman, 1952; Sigel and Sigel, 1993). As selenium is of interest as a potential environmental toxicant, attentions have been focused on the biodegradation and transformation processes of selenium in the environment.

Some investigations have been done to develop a detailed understanding of the mechanism of biomethylation and transformation of selenium. In 1945, Challenger suggested a mechanism (Scheme 1) that involves four steps in which the selenium atom is methylated and reduced to form volatile dimethyl selenide (Challenger, 1945). But this mechanism did not involve dimethyl diselenide. Many workers have observed dimethyl diselenide in the headspace above selenium resistant cultures (Reamer and Zoller, 1980; Chasteen *et al.*, 1990; Chasteen, 1993; McCarty *et al.*, 1993). None of the postulated, intermediate selenium compounds was detected in culture solutions; however, dimethyl selenone is the only postulated intermediate which may be stable enough to be isolated by normal means. Dimethyl selenone has never been synthesized and purposely introduced into bacterial cultures to probe the mechanism.



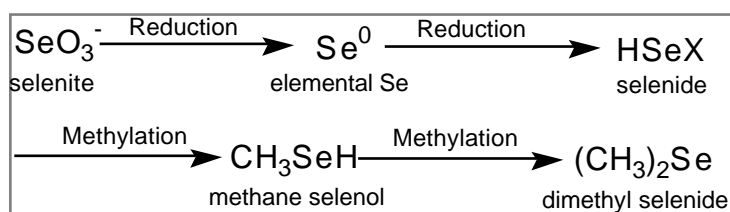
Scheme 1

More recently, Reamer and Zoller (1980) proposed an expanded mechanism (Scheme 2) based on their identification of dimethyl selenide, dimethyl diselenide, and dimethyl selenone as products from soil and sewage amended with selenite or elemental selenium. Recent evidence suggests, however that their detection of dimethyl selenone was probably in error and that they confused dimethyl selenone for dimethyl selenenyl sulfide instead (Chasteen, 1993).



Scheme 2

Doran (1982) suggested that the methylation of inorganic Se may first involve reduction of selenite to the elemental Se and then reduction to the selenide form, which is subsequently methylated to form dimethyl selenide (Scheme 3). Many workers have found elemental selenium in bacterial cultures doped with oxidized selenium salts (Francis *et al.*, 1974; Doran and Alexander, 1977; Steinberg and Oremland, 1990). Our bacterial cultures doped with oxyanions of selenium often yielded a red precipitate that is probably selenium.



Scheme 3

The present investigation was initiated to study one possible pathway of the biomethylation process of selenium salts by *Pseudomonas fluorescens* K27, a selenium resistant bacterial strain which was isolated from Kesterson Reservoir in the San Joaquin valley of central California (Burton *et al.*, 1987). Our studies were focused on identifying whether or not dimethyl selenone may act as an intermediate of the biomethylation process.

We have noticed that both of the mechanisms of Challenger and of Reamer and Zoller involved dimethyl selenone as an intermediate of the biomethylation process. This compound was never tested by Challenger but it was reported by Reamer & Zoller in soil and sew-

age sludge samples inoculated with sodium selenite or elemental selenium. Because dimethyl selenone has a *melting point* at 147°C-148°C (Paetzold and Bochmann, 1968), it is probably not possible to detect directly by gas chromatography as a volatile metabolic compound as proposed by Reamer & Zoller; we proposed another way to identify it as an intermediate.

The method that we proposed is to synthesize dimethyl selenone, dose cultures with it and analyze the headspace released from the cultures by gas chromatography coupled with sulfur chemiluminescence detection. If the methylation process involves this compound, we should observe volatile selenium compounds as final products in headspace above the cultures. If dimethyl selenone were not involved, no volatile selenium compounds should be observed (assuming no other sink for reduced selenium exists). This process was carried out in an anaerobic and facultative bacterial culture.

Little information on the synthesis of dimethyl selenone was available in the literature; however, Paetzold and Bochmann successfully synthesized dimethyl selenone by using ozone as oxidant in 1968 (Paetzold and Bochmann, 1968). More recently, workers reported the synthesis of dimethyl selenone by the oxidation of the corresponding dimethyl selenide by 3-chloroperoxybenzoic acid and potassium permanganate (Krief *et al.*, 1985). Rebane reported the mass spectrum of this compound in 1974 (Rebane, 1974).

As we did not have an ozone generator in our lab, we had to choose another appropriate oxidant for this synthesis.

3-Chloroperoxybenzoic acid was found to efficiently oxidize dimethyl selenide to the corresponding selenone, and the yield was

higher than when using potassium permanganate as oxidant (Krief *et al.*, 1985). So, the method that we followed was to use 3-chloroperoxybenzoic acid and dimethyl selenide as starting materials and methylene chloride as solvent to perform this synthesis. In order to achieve purer dimethyl selenone, a modification of the Krief *et al.* method was used in our lab.

Gas chromatography is the most widely used technique to analyze volatile compounds in all fields of science. A large number of detectors have been developed for gas chromatography. Most of them are specialized detectors which are selective for a certain kind of compounds. Three major sulfur/selenium selective detectors are discussed below.

The flame photometric detector (FPD) is the most popular detector for sulfur compounds (Brody and Chaney, 1966; Farwell and Barinaga, 1986), but is also used for volatile compounds containing selenium (Flinn and Aue, 1978). The most frequently cited impediments to its broader use is that the FPD produces an exponential response which is *close* to two for sulfur and selenium compounds. But it has been shown to have a number of inherent problems, such as non-linear response above two orders of magnitude (Farwell *et al.*, 1981), variation in the response factor with molecular structure, and quenching by hydrocarbons and other species which are eluted together with the analyzed compounds (Driscoll and Berger, 1989). The detection limits of FPD for sulfur and selenium compounds are in the nanogram range which is not very satisfactory for trace analysis. So, more recently, two other sulfur and selenium selective detection methods which are more sensitive and selective and re-

spond on a relatively constant mass basis, independent of the source of the sulfur or selenium, have become commercially available.

Sulfur chemiluminescence detection (SCD) is specific for alkylated sulfur/selenium compounds; atomic emission spectrometric detection (AED) is a multi-element method capable of detecting elements with atomic emission at vacuum-UV, UV, visible and near-IR portions of the electromagnetic spectrum. Both detectors have detection limits for sulfur or selenium in the low picogram range (Chasteen, 1990; Eckert-Tilotta *et al.*, 1992). The linear dynamic range with AED and SCD are approximately five orders of magnitude and four orders of magnitude respectively. However, atomic emission detector instrumentation is much more expensive and requires more laboratory space than sulfur chemiluminescence detector instrumentation. On account of its availability, high sensitivity, wide linearity, and good selectivity to alkylated selenium, the sulfur chemiluminescence detector was found well suited for our research.

Chapter II

Experimental Methods

Part I. Synthesis of Dimethyl Selenone

1. Apparatus and Reagents

All chemicals used in our synthesis were of analytical reagent grade and used without further purification. Dimethyl selenide was purchased from Strem Chemicals, Inc. (Newburyport, MA USA). 3-Chloroperoxybenzoic acid (65%) was acquired from the Spectrum Chemical Mfg. Corp. (Gardena, CA USA). Methylene chloride was obtained from the Aldrich Chemical Company, Inc. (St. Louis, MO).

2. Methods

Synthesis of dimethyl selenone

Dimethyl selenone was synthesized by oxidizing dimethyl selenide with an excess of 3-chloroperoxybenzoic acid in methylene chloride solution. One mL dimethyl selenide (0.013 moles) was dissolved in 5 mL methylene chloride. Three mol equiv. of 3-chloroperoxybenzoic acid (65%, 10.35 g), dissolved in 25 mL methylene chloride solution, was added to the selenide solution dropwise. The reaction was stirred for two hours at 20°C and the white cloudy solution was dried using an evaporator and extracted three times with 20 mL ethyl ether. The by-product, 3-chlorobenzoic acid, which dissolved in ether solution, was separated from the crude solid dimethyl selenone by vacuum filtration. The crude selenone was recrystallized with methanol two times to give white odorless crystals.

Elemental analysis of dimethyl selenone, $(\text{CH}_3)_2\text{SeO}_2$

Elemental analysis of dimethyl selenone was performed by Galbraith Laboratories, Inc. Knoxville, TN USA. A fifty-five mg sample was sent to this lab and the results returned by mail.

Fourier transfer infrared spectroscopy of $(\text{CH}_3)_2\text{SeO}_2$

A Bomem DA3 fourier transfer infrared spectrometer was used to identify dimethyl selenone. The spectrometer was controlled by a DEC PDP/11 computer. The data were exported to a 486sx personal computer for storage and display.

The sample preparation was performed by Mr. D. Ellinwood by mixing 2 mg dimethyl selenone and 200 mg potassium bromide in a small sample cell. The sample was pressed at 10,000 psi pressure for 2 minutes to achieve a thin transparent sample sheet in order for infrared radiation to pass through or be absorbed by the sample. The sample cell was kept in a desiccator after pellet preparation until FTIR analysis was performed.

Nuclear magnetic resonance measurement of $(\text{CH}_3)_2\text{SeO}_2$

The proton NMR spectrum of dimethyl selenone was carried out on a Varian EM 360 NMR spectrometer. This instrument was a 60 MHz system that provided an accurate and non-destructive method of determining structure in liquids and soluble chemical compounds.

Deuterium oxide was the solvent for dimethyl selenone in this NMR measurement. It was obtained from Aldrich Chemical Com-

pany, Inc. (St. Louis, MO USA) and NMR test tubes were purchased from Alltech (Deerfield, IL). About 10 mg of sample was dissolved in 6 drops of solvent and the spectrum was recorded at room temperature. The stability of dimethyl selenone was checked by NMR; the spectrum was taken one month after dimethyl selenone was synthesized.

Melting point analysis of dimethyl selenone

The measurement of melting point of dimethyl selenone was performed on a Fisher-John melting point apparatus in our lab. The thermometer for this measurement was not calibrated.

Part. II Studies on the Biomethylation Intermediates of Selenium Reduction and Methylation

1. Apparatus and Reagents

Dimethyl sulfide, dimethyl disulfide, and dimethyl diselenide were obtained as pure solutions from Aldrich Chemical Company, Inc. (St. Louis, MO USA). Dimethyl selenide and sodium selenate were purchased from Strem Chemicals, Inc. (Newburyport, MA USA). Tryptic soy broth was obtained from Difco Laboratories (Detroit, MI USA). One mL gas syringes were acquired from Alltech (Deerfield, IL USA), and sterilized filters were purchased from MSI (Westboro, MA USA).

2. Gas Chromatography-Chemiluminescence Detection

A gas chromatograph-sulfur chemiluminescence detector system was used for all the headspace experiments. The chromatograph was a Hewlett Packard Model 5890 Series II. The detector was a Sievers Research Model 300. In this instrument, organo-sulfur, -selenium and -tellurium compounds react with molecular fluorine (F_2) which is produced in-line by a high frequency electrical discharge of a stream of SF_6 (99.99%). This reaction is carried out in a steel cell in which the pressure is below one torr, maintained by a vacuum pump; the temperature of the cell is room temperature. A red sensitive photomultiplier tube using photon counting electronics is used to detect chemiluminescence which is produced by the reaction of the analytes with F_2 . The resulting analog signal from the detector is recorded by a Hewlett Packard Model 3396 Series II integrator. There is a heated nickel transfer line between the gas chromatograph and the detector. The capillary column from the GC is inserted through this transfer line into the reaction cell of the detector and the temperature of this transfer line is thermostatically maintained at approximately 213°C.

The gas chromatograph was equipped with a cryogenic oven which was used 1) to trap samples at the head of the column upon injection at the very beginning of temperature program and 2) to cool down the column in a short time when the temperature program ran out. A thirty meters long capillary column (Alltech, Deerfield, IL USA) was used. The chromatographic phase of the column was SE-54 (5% phenyl methyl silicone), which was an almost completely non-polar phase so that the elution order of analytes

were generally based on the boiling point of compounds. Helium was used as the carrier gas at a flow rate of about 1 mL/min in our experiments.

3. Microbial Incubations

Bacterial strains used in this study were *Pseudomonas fluorescens* K27, isolated from Kesterson Reservoir in central California (Burton *et al.*, 1987). The K27 cultures were supplied by Ray Fall at University of Colorado, Boulder. This selenium resistant bacterial strain was grown at 30°C in TSN medium containing 10 g/L tryptic soy broth and 1 g/L potassium nitrate as the electron sink (terminal electron acceptor). The medium was filter sterilized before inoculation and the inoculated cultures were incubated at 30°C overnight in a thermostatic water bath. After 24 hours of growth, the cultures were diluted 1:1 with fresh sterilized medium and dosed with a desired amount of poison (selenate or selenone) using the stock solution described below. The cultures were then incubated for 24 hours at 30°C before headspace sampling.

The TSN medium is a complex medium, for which the exact chemical composition can not be defined. It is made of nutrients such as digests of proteins from soy broth so that the energy, carbon, nitrogen, and sulfur requirements of the growing microorganisms are met largely by protein. In order to avoid complex medium, two chemically defined media in which the exact chemical composition are known were also attempted for the growth of K27. The minimal succinate medium consisted of 40 mM $\text{Na}_2\text{C}_4\text{H}_4\text{O}_4$, 20 mM NH_4Cl , 2.5 mM K_2HPO_4 , 2.5 mM KH_2PO_4 , 0.8 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in

100 mL distilled water with 0.1% (vol/vol) trace mineral solution (250 mg of EDTA, 500 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 154 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 10 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 24.5 mg of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 17.7 mg of $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, water to 100 mL), and adjust to pH 6.8 (Bryan *et al*, 1985). Another minimal medium consisted of 0.544 g KH_2PO_4 , pH 7.4 and 0.6 mL of a salt solution consisting of (L^{-1}) 10 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.4 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.1 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ in 100 mL distilled water with the addition of 0.2 g glucose and 0.38 g NH_4Cl (McEldowney and Fletcher, 1986). Both minimal media did not grow the K27 bacteria under the same incubation conditions that was successful using TSN medium. So, the TSN medium was the only medium used for growing the K27 bacteria in this research.

4. Sample Preparation

Sterilization was accomplished by autoclaving all test tubes, caps and septa before preparing samples. Solutions were sterilized via sterile filtering. One mole/L aqueous solutions of sodium selenate, sodium selenite and dimethyl selenone were prepared as stock solutions. The dimethyl selenone was recrystallized the same day that the stock solution was prepared. The diluted solutions were prepared by taking different amount of filter sterilized stock solutions, diluting into 2 mL and 10 mL cultures to get either 1 mM or 10 mM final concentration in the media.

5. Standard solutions preparation

The volatile sulfur and selenium compounds released from our

samples including methanethiol, dimethyl sulfide, dimethyl selenide, dimethyl disulfide and dimethyl diselenide were determined by comparing their retention times with those of standard solutions prepared by serial dilutions. The standard solutions of DMS, DMSe, DMDS, DMDS₂Se were prepared by diluting 50 μ L pure standards into 1 mL acetonitrile solution in a 2 mL glass vial, then diluting 50 μ L solutions from the first vial into 1 mL acetonitrile in another 2 mL glass vial. This process was repeated five to six times to get different concentration diluted standard solutions.

The standard solution of methanethiol was prepared in our lab by mixing 25 μ L dimethyl disulfide and 0.2 gram powder zinc with 1 mL concentrated HCl into 2 mL water in a 6 mL glass vial sealed with a Teflon[®] lined septum (Chasteen, 1990; Chasteen, 1993). Methanethiol was produced after the mixture was allowed to react for 15 min at room temperature.

One mL gas of headspace above these standard solutions were removed from the vials by a gas syringe and analyzed by GC. The retention times of these standards were applied to identify headspace species in our samples.

6. Experimental Procedure

In our experiments, two media blanks and two culture blanks (with microorganisms) were prepared as controls. Also, we grew several 2 mL and 10 mL cultures in 15 mL glass vials sealed with Teflon lined septa and poisoned them with different concentrations of sodium selenate and dimethyl selenone. On the other hand, we poisoned three media blanks (without microorganisms) with 10 mM

sodium selenate, sodium selenite and dimethyl selenone respectively, with the addition of DMS and DMDS to see if the reduction of dimethyl selenone was a chemical reaction only. Known volumes of headspace gas above samples were taken from the vials by a gas tight syringe for GC analysis after 24 hours incubation at 30°C. A splitless injection technique was used for sodium selenate or selenite samples and a split injection technique was used for dimethyl selenone samples to avoid column overloading. The split ratio was 1/65, which means only 1/65 of the sample introduced into the injector was injected into the column and the other was thrown away to waste.

The peak areas that we reported were normalized to a one mL gas sample, 10 mL culture volume, and splitless injection. For example, a 0.5 mL gas sample taken from above a 2 mL culture sample and injected in the split injection mode had the chromatogram's peak areas multiplied by 2 (to normalize to 1 mL gas samples), by 5 (to normalize to 10 mL culture samples) and by 65 to normalize to a splitless injection.

In order to get high resolution chromatograms, we set up a two-step temperature program. In the first ramp, the headspace samples were cryogenically trapped in the capillary column at -20°C for one minute, then the temperature was increased at 8°C/min until the temperature arrived at 20°C. In the second ramp which began immediately, the temperature was increased at 15°C/min to a final temperature of 200°C for three minutes. We used the same temperature program for all the standards and headspace samples analysis.

Media blanks and culture blanks (sample vials inoculated with the K27 bacteria without selenate salts or dimethyl selenone) were run along with poisoned samples as controls. By this method, we routinely identified which gases detected in the headspace were the result of the addition of the selenate salts or dimethyl selenone and which were not.

A syringe cleaning device was used to clean the gas syringes before each headspace analysis; one mL of lab air in the gas tight syringe was analyzed by GC to make sure the syringe was clean before each sample run.

The stability of sodium selenate and sodium selenite containing TSN solution with regard to added DMS and DMDS was also measured by GC-SCD. Sodium selenate and selenite amended TSN solutions with DMS and DMDS were prepared by Mr. Steven L. McCarty in our research group. First, sodium selenate and sodium selenite were dissolved in two 100 mL TSN media blanks at a final concentration of 10 mM. Nanogram range concentrations of DMS and DMDS solutions were prepared by serial dilutions of concentrated DMS and DMDS solutions. Then 1 ng DMS and 1 ng DMDS in water solutions were pipetted into the TSN media with selenate and selenite salts by Eppendorf pipet. The final solutions were sterilized by sterilized filters before sampling.

Chapter III

Data

1. Elemental analysis of synthesized dimethyl selenone.

Element	Theoretical	Determined	Difference
Carbon	17%	16.86%	0.14%
Hydrogen	4.25%	4.09%	0.16%
Selenium	56%	57.96%	1.96%
Oxygen	22.75%	21.1%	1.65%

2. FTIR experimental and literature spectra of dimethyl selenone. (Figure 1)

3. FTIR spectrum of dimethyl selenone from 800 to 1100 wavenumbers. (Figure 2)

4. NMR spectrums of dimethyl selenone and dimethyl selenone as it decayed. (Figure 3)

5. Melting point of dimethyl selenone.

The melting point of dimethyl selenone was 147⁰C-148⁰C. Before it was recrystallized, the melting point was 137-138⁰C. Ten days after synthesis, the melting point of dimethyl selenone dropped to 140-141⁰C.

6. The chromatograms of autoclaved medium and filter sterilized medium. (Figure 4)

7. The chromatogram of 1 mM sodium selenate dosed K27 culture. (Figure 5)

8. The chromatogram of 1 mM dimethyl selenone dosed K27 culture. (Figure 6)

9. The chromatogram of 10 mM dimethyl selenone in medium blank with DMS and DMDS. (Figure 7)

10. The chromatogram of 10 mM sodium selenate in medium blank with DMS and DMDS. (Figure 8)

11. The chromatogram of 10 mM sodium selenite in medium blank with DMS and DMDS. (Figure 9)

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Figure 1. FTIR spectra of dimethyl selenone. Upper spectrum is experimental spectrum. Lower spectrum is from Paetzold and Bochmann, 1968.

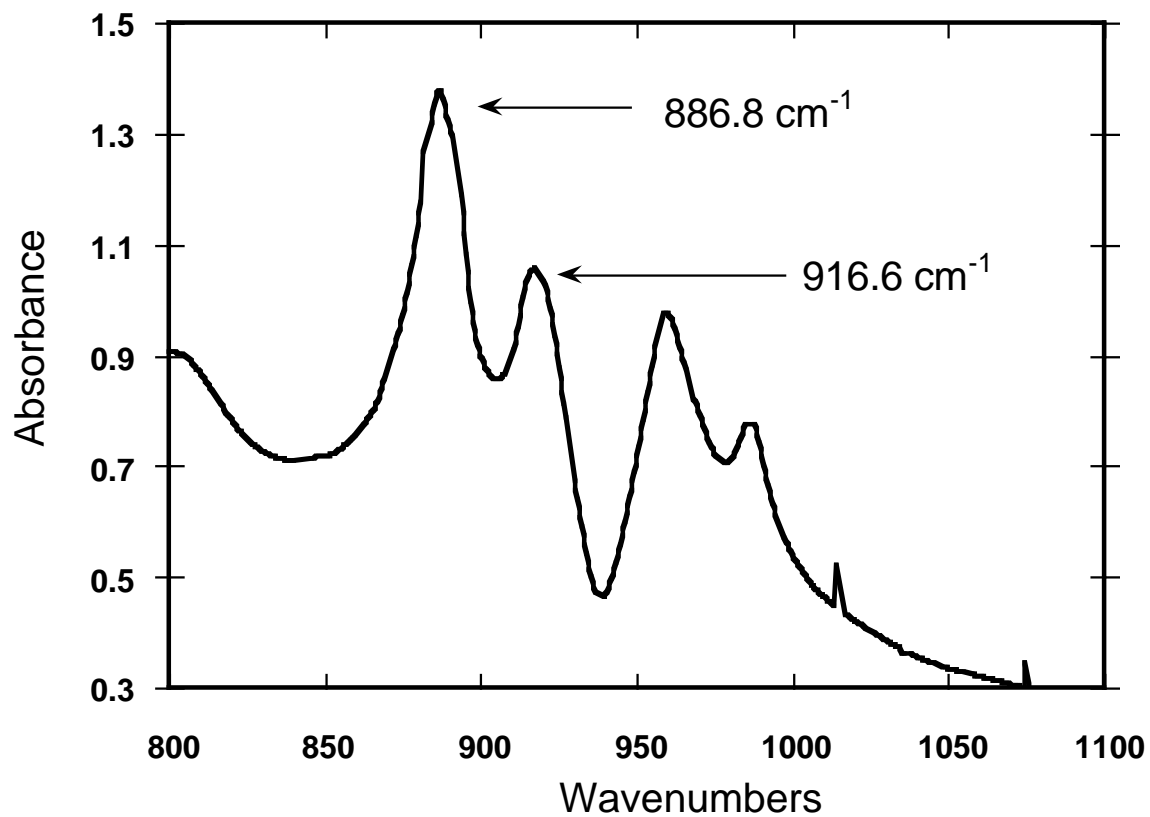


Figure 2. The two characteristic absorptions of dimethyl selenone in our experimental FTIR spectrum. These absorptions are due to SeO₂ stretching.

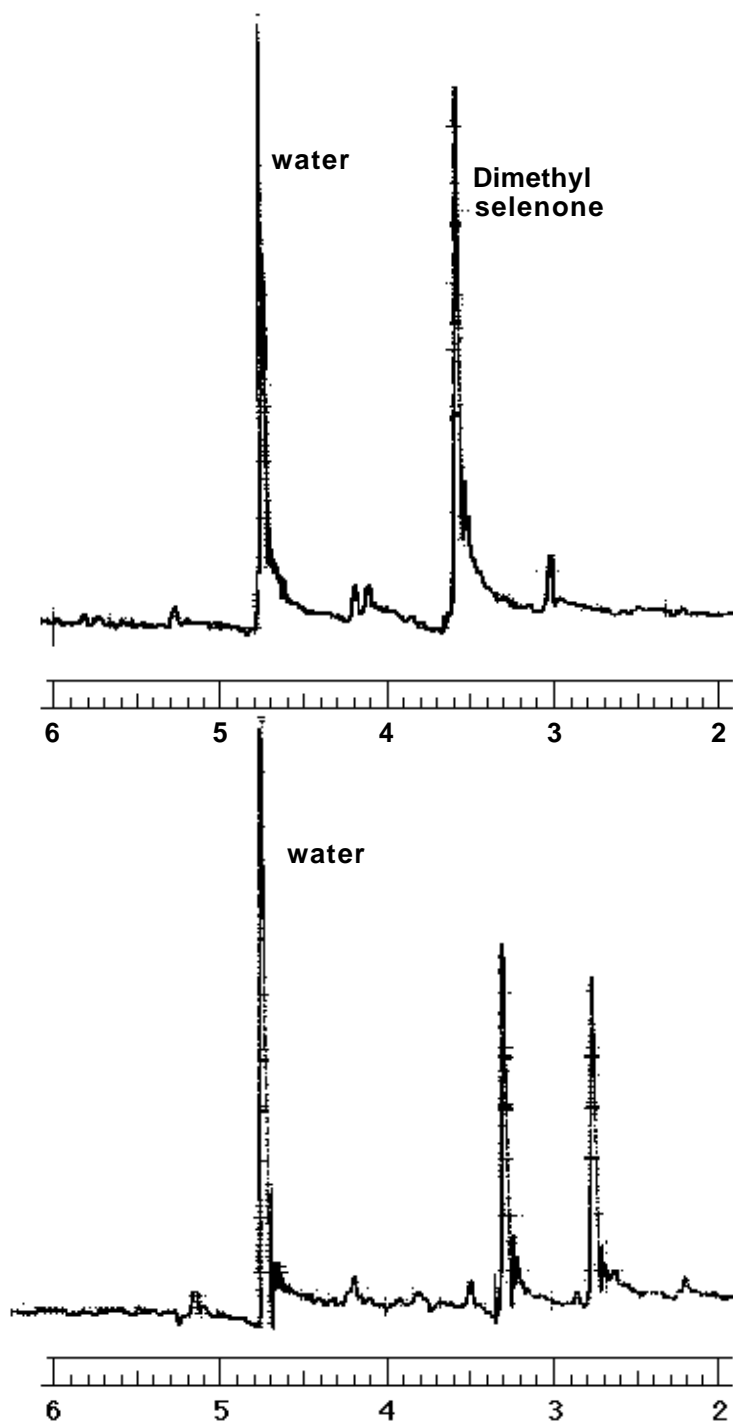


Figure 3. Nuclear magnetic resonance spectra of dimethyl selenone in deuterium oxide. Spectrum A was performed soon after it was synthesized. Spectrum B was performed one month after the compound was synthesized.

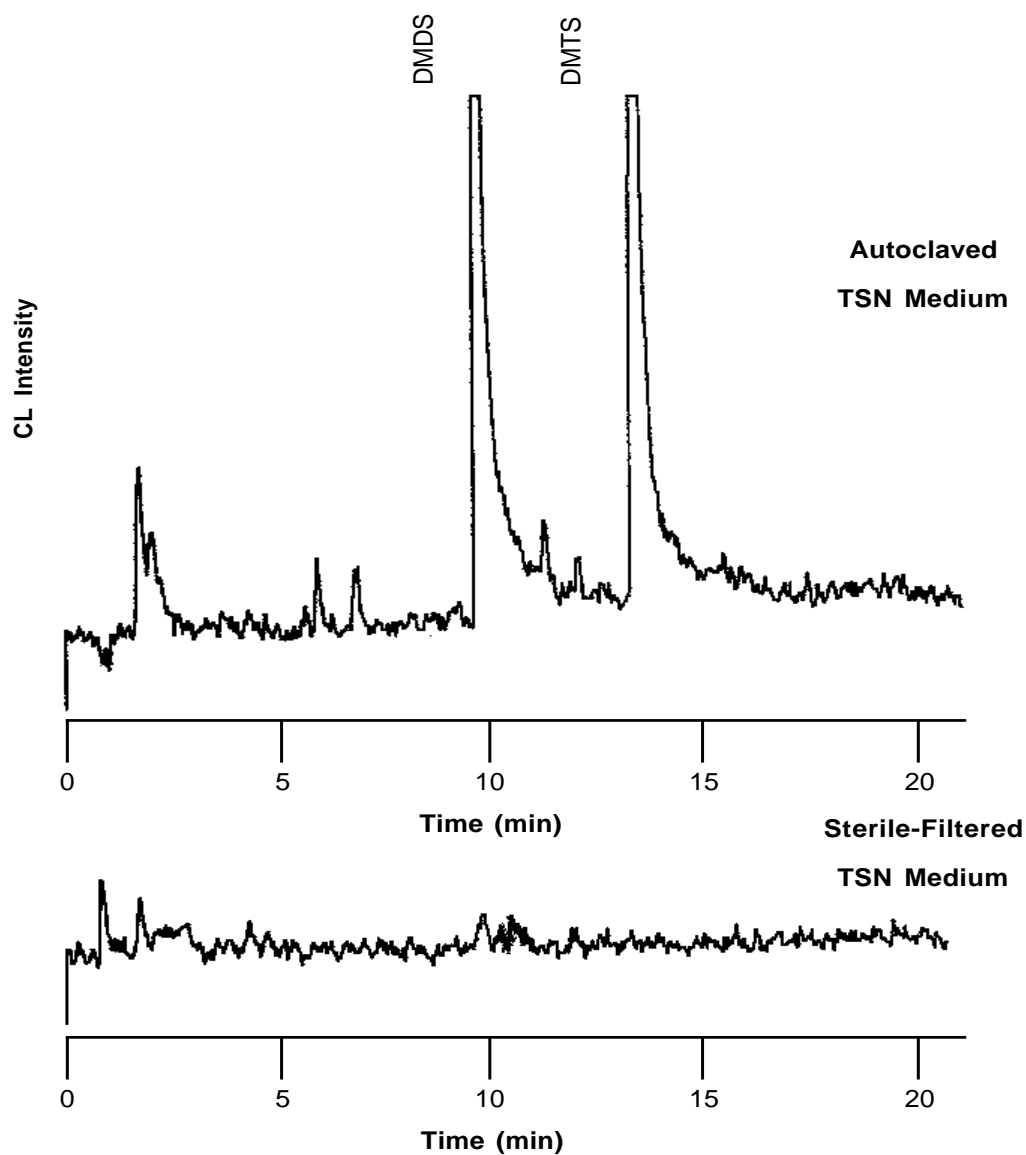


Figure 4. Headspace analysis of media blanks. Chromatogram A is from autoclaved medium. Chromatogram B is from filter sterilized medium.

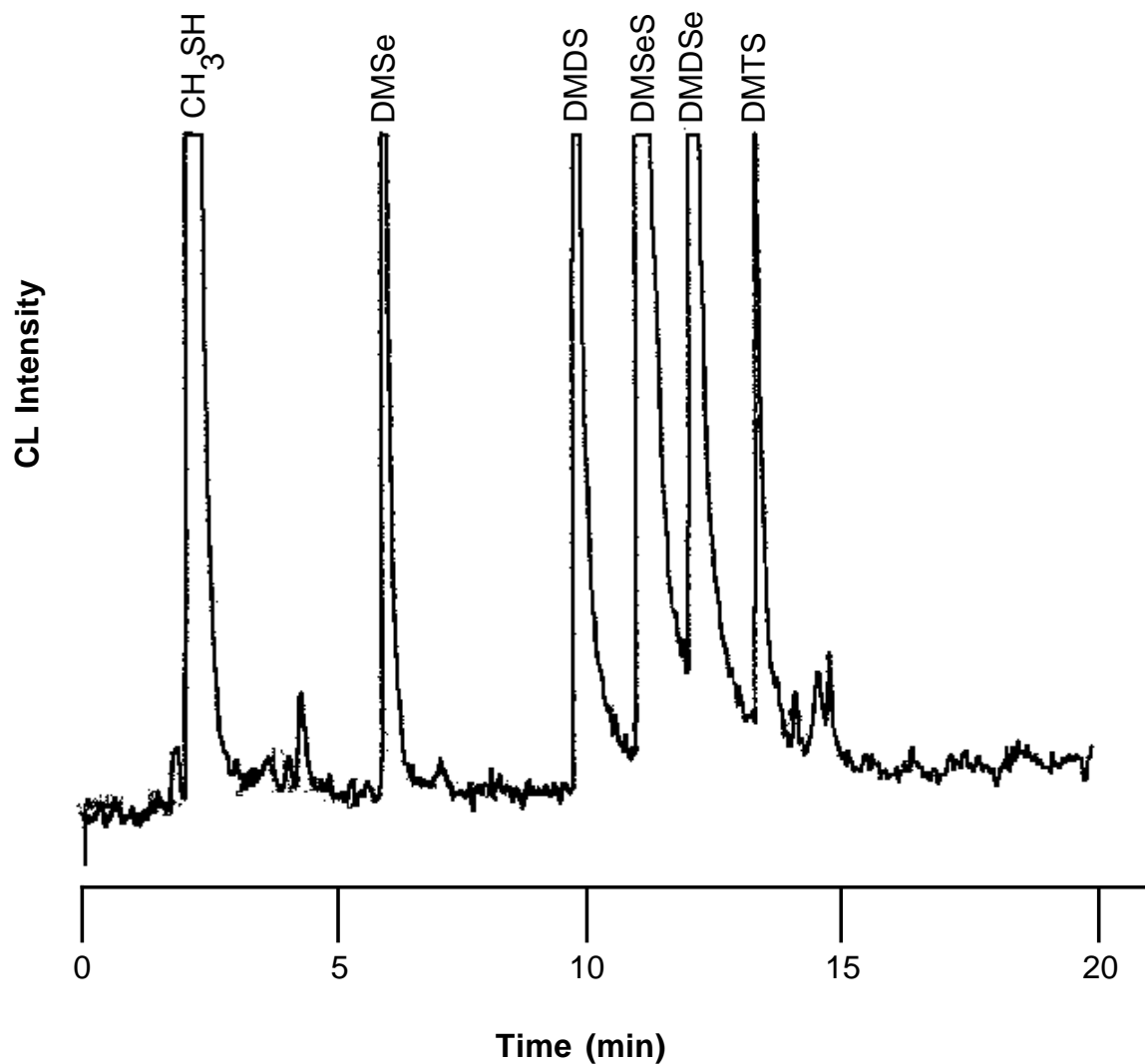


Figure 5. Headspace analysis of 1 mM sodium selenate in K27 culture. Culture was sampled 24 hours after being poisoned.

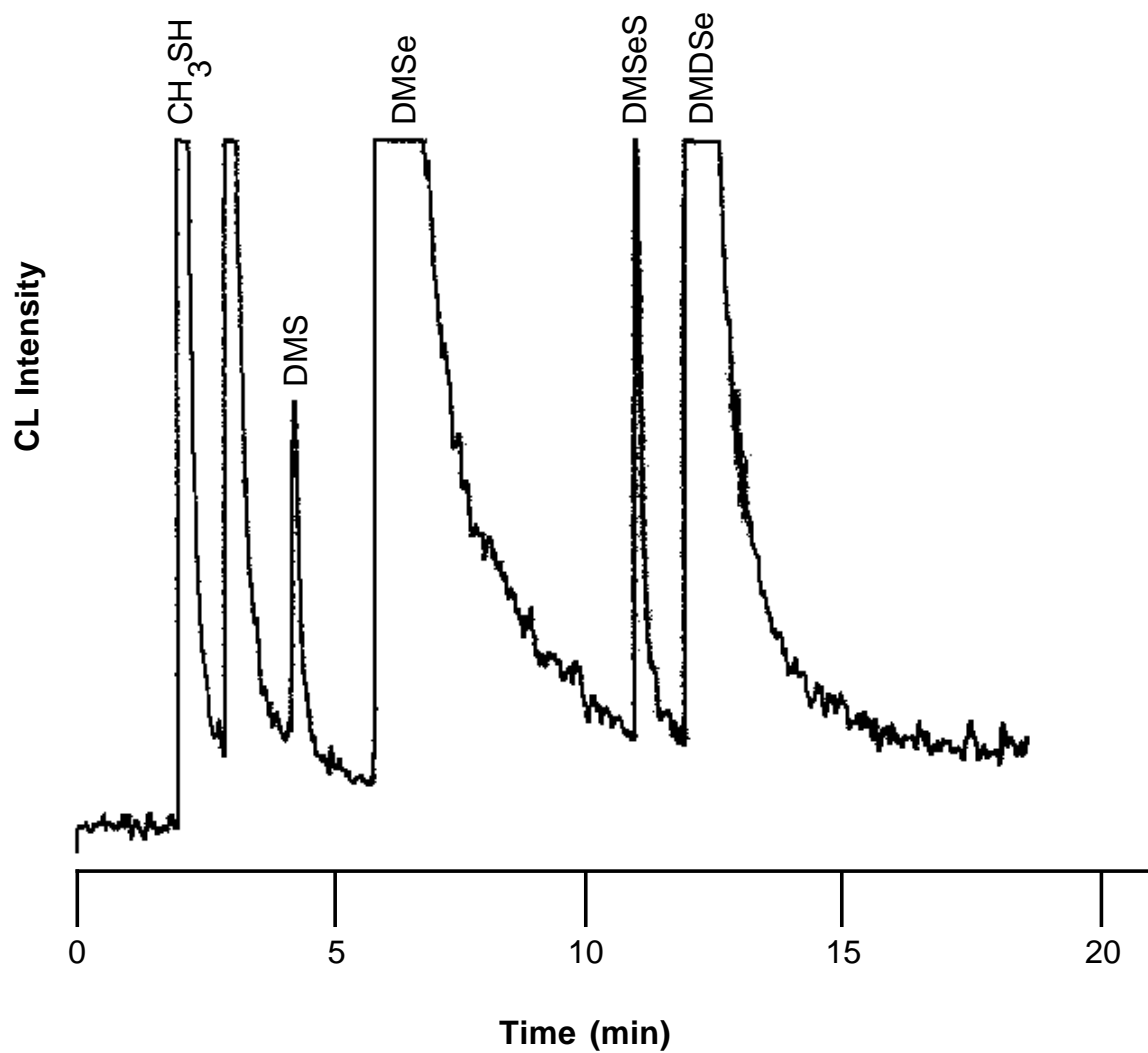


Figure 6. Headspace analysis of 1 mM dimethyl selenone in K27 culture. Culture was sampled 24 hours after being poisoned.

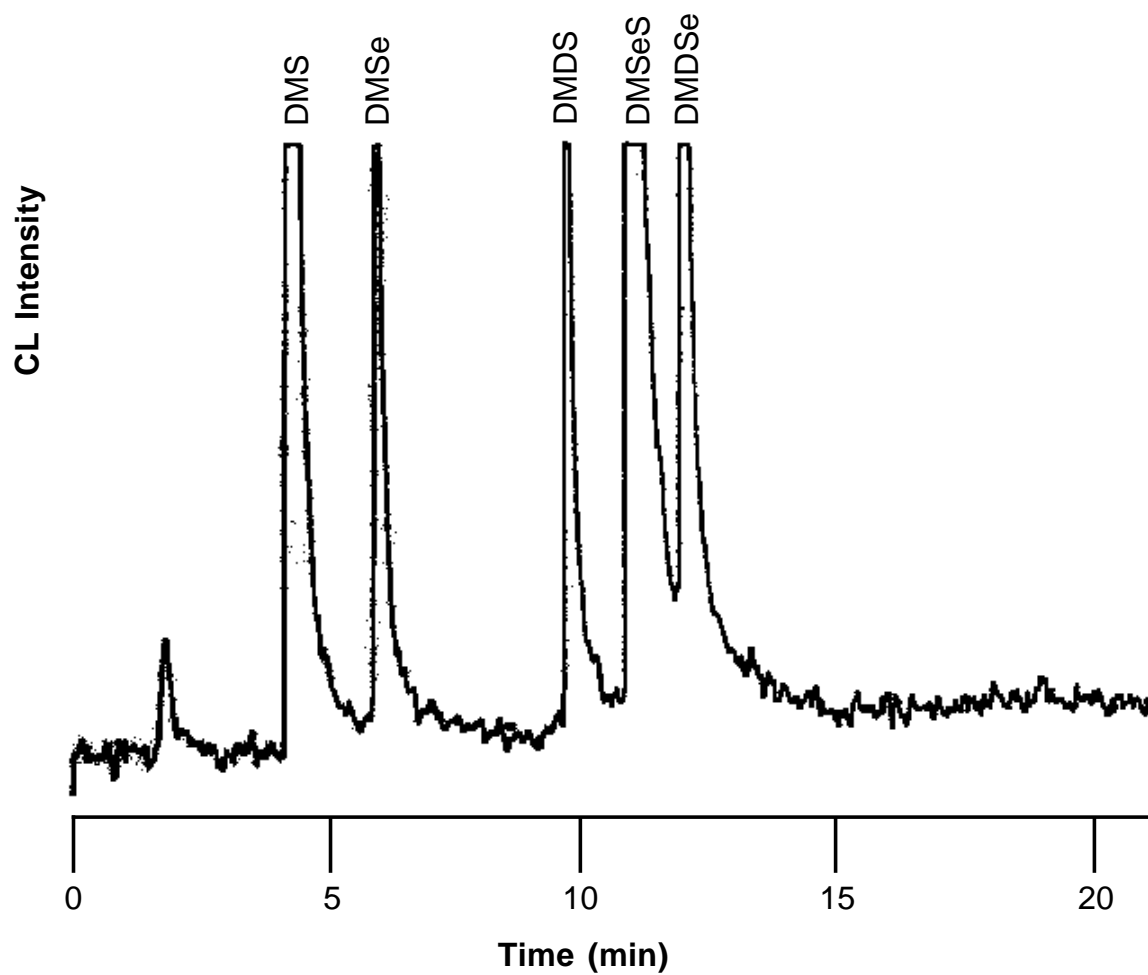


Figure 7. Headspace analysis of 10 mM dimethyl selenone in medium blank with DMS and DMDS. Solution was sampled 24 hours after being prepared.

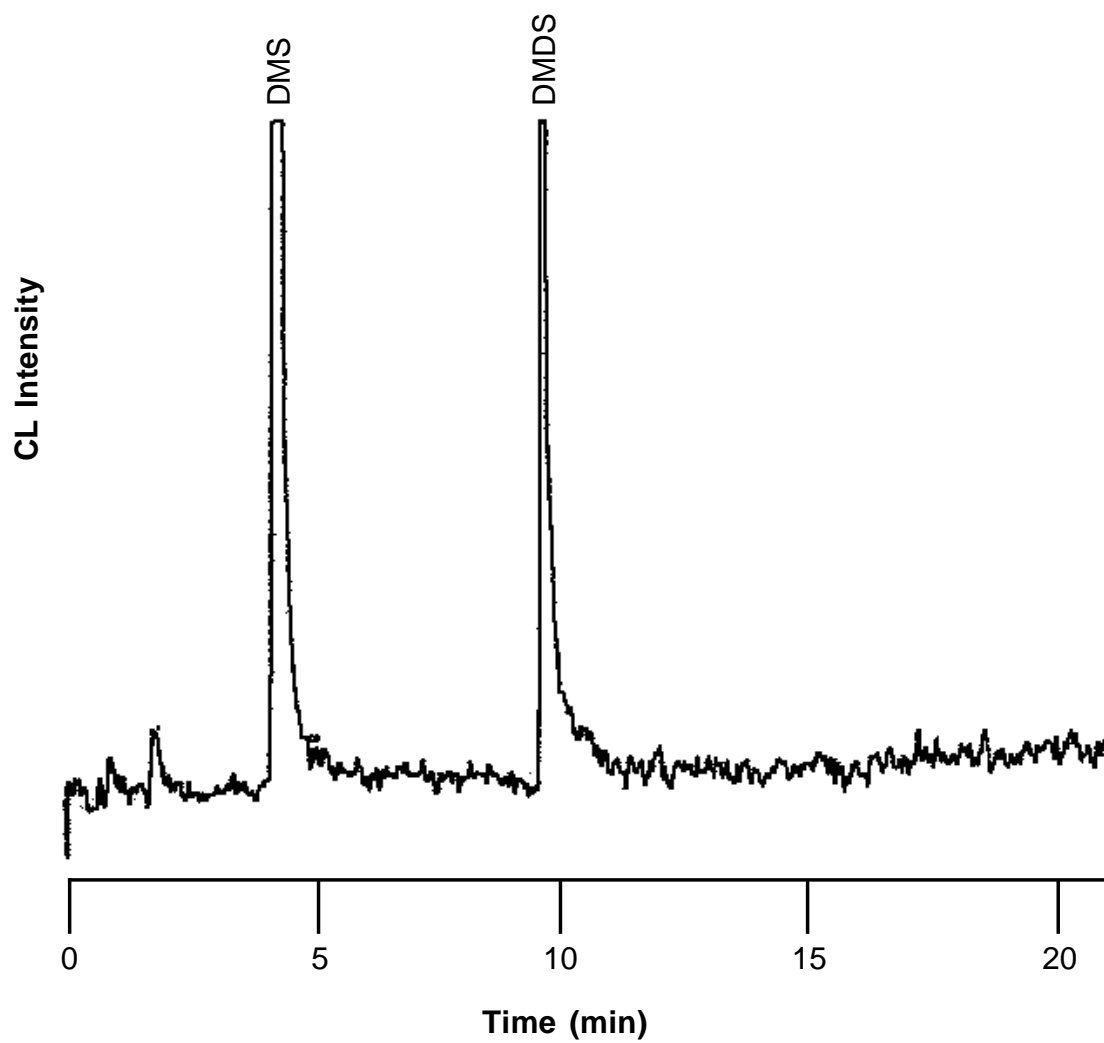


Figure 8. Headspace analysis of 10 mM sodium selenate in medium blank with DMS and DMDS. Solution was sampled 24 hours after being prepared.

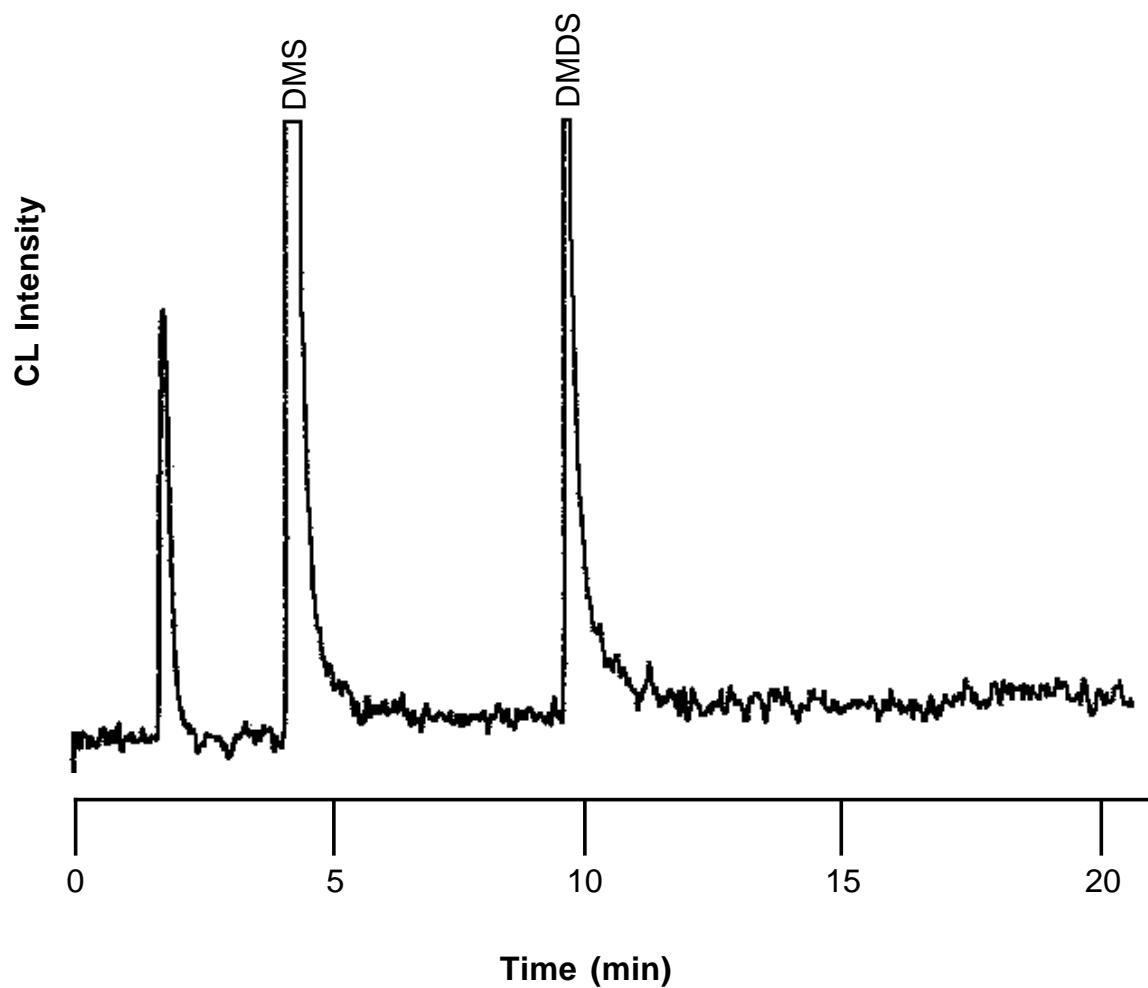


Figure 9. Headspace analysis of 10 mM sodium selenite in medium blank with DMS and DMDS. Solution was sampled 24 hours after being prepared. The early eluting peak is not methanethiol.

12. Table of integrated peak area of samples in 10 mL media volumes. All peak areas are normalized to 1 mL head-space gas and splitless injection. (Table 1)

Sample Name	CH ₃ OH	DMS	DMSe	DMDS	DMSeS	DMDSe	DMTS
TSN blank	0	0	0	0	0	0	0
K27 Blank	2.50e + 06	1.67e + 06	0	6.17e + 06	0	0	3.02e + 06
K27 + 1 mM SeO ₄ ²⁻	3.10e + 08	3.52e + 06	2.17e + 07	6.43e + 07	5.75e + 07	1.13e + 08	1.30e + 07
K27 + 10 mM SeO ₄ ²⁻	1.39e + 07	1.44e + 06	9.87e + 06	6.50e + 07	9.55e + 07	9.81e + 07	1.38e + 07
K27 + 1 mM DMSeO ₂	2.99e + 10	2.82e + 09	1.03e + 11	5.54e + 08	1.64e + 10	1.42e + 11	0
K27 + 10 mM DMSeO ₂	0	5.51e + 08	5.65e + 09	0	2.51e + 09	5.12e + 10	0
TSN + 10 mM DMSeO ₂ DMS & DMDS	0	6.48e + 07	1.43e + 07	1.89e + 07	4.83e + 07	1.02e + 07	0
TSN + 10 mM SeO ₄ ²⁻ + DMS & DMDS	1.29e + 06	5.42e + 07	0	2.08e + 07	0	0	1.58e + 06
TSN + 10 mM SeO ₃ ²⁻ + DMS & DMDS	0	7.38e + 07	0	2.37e + 07	0	0	0

13. Table of the retention times of volatile sulfur and selenium compounds using the chromatographic system described in this work. (Table 2)

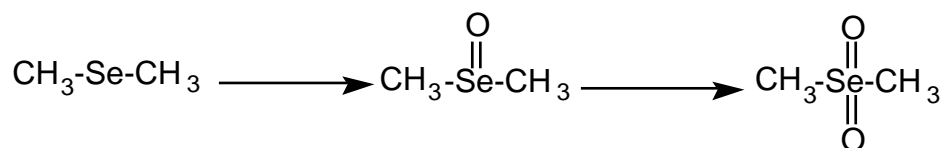
Compound Name	Retention Time
Methanethiol	2.05 min
Dimethyl sulfide	4.34 min
Dimethyl selenide	6.04 min
Dimethyl disulfide	9.72 min
Dimethyl selenenyl sulfide	10.93 min
Dimethyl diselenide	11.989 min
Dimethyl trisulfide	13.2 min

Chapter IV

Discussions and Results

Synthesis of dimethyl selenone

At the very beginning of this research, a literature search was done to find various oxidants able to oxidize dimethyl selenide to dimethyl selenone. Ozone was an effective oxidant for oxidizing dimethyl selenide to dimethyl selenone (Paetzold and Bochmann, 1968), but since it required an ozone generator, it was too expensive. Thirty percent hydrogen peroxide was also employed as oxidant to oxidize dimethyl selenide (Bird and Challenger, 1942). We found that it was unable to produce the corresponding selenone in our work. Then we attempted to approach this synthesis by employing the use of 3-chloroperoxybenzoic acid (m-CPBA) as oxidant with dimethyl selenide as the starting material (Krief *et al*, 1985).



Conditions:

3.0 mol. equiv. m-CPBA/CH₂Cl₂

20⁰, 2 hours

% Yield

50

Scheme IV

It was reported that m-CPBA was efficient for the oxidation of dimethyl selenide to dimethyl selenone (Krief *et al.*, 1985). But under the conditions that these workers used, the formation of

selenoxide was instantaneous whereas further oxidation to selenone occurred more slowly; therefore, our final product had both the selenone and selenoxide present. To minimize the problem caused by selenoxide, we modified the procedure by increasing the equivalents of m-CPBA and prolonging the reaction time. We achieved a 50% yield of pure dimethyl selenone by this means.

For purifying the crude product, we selected ethyl ether to separate dimethyl selenone from the by-product, m-chlorobenzoic acid. As m-chlorobenzoic acid was freely soluble in ether but dimethyl selenone was not, dimethyl selenone was removed as a precipitate by vacuum filter and collected.

Melting point consideration

The melting point of dimethyl selenone published in the literature (Paetzold and Bochmann, 1968) ranged from 147⁰C-148⁰C, which was higher than that of the crude product that we extracted from ethyl ether. Recrystallization was then performed using methanol as the solvent. Since methanol is a relatively polar solvent, it was found to work well for the recrystallization of dimethyl selenone. The melting point of our final product was 147⁰C-148⁰C, as reported by Paetzold and Bochmann. This was the first straight forward evidence for successful synthesis dimethyl selenone in our work.

Elemental analysis

The elemental analysis of dimethyl selenone showed some difference between the theoretical data and the determined data. The

largest difference was for selenium. The reason for this is possibly due to the degradation of dimethyl selenone to dimethyl selenoxide or other reduced selenium compounds, since this elemental analysis was performed ten days after it was synthesized.

Fourier transfer infrared spectrum of dimethyl selenone

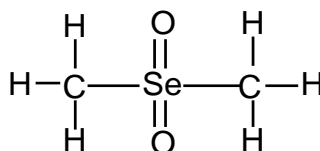
FTIR instruments have three major advantages. They can give greater signal to noise ratio; they have extremely high wavelength accuracy and precision and can detect all elements of the source reaching the detector simultaneously.

FTIR spectroscopy was carried out to further confirm the synthesis of dimethyl selenone in our experiment. Both published (Paetzold and Bochmann, 1968) and experimental infrared spectra of dimethyl selenone are shown in Figure 1. The two spectra agree well. The most characteristic absorptions of dimethyl selenone (Figure 2) were two strong bands in the 889 cm^{-1} and 917 cm^{-1} regions because of asymmetric and symmetric SeO_2 stretching, respectively (Paetzold and Bochmann, 1968). The stretching vibrations of C-S linkage occurred in the region of $700\text{-}600\text{ cm}^{-1}$. Two bending vibrations can occur within a methyl group. The symmetric bending vibration occurred near 1375 cm^{-1} and the asymmetric bending vibration near 1450 cm^{-1} . Absorption arising from C-H stretching of methyl group generally occurred in a high frequency region which was about $3000\text{-}2840\text{ cm}^{-1}$ (data not shown). Our experimental spectrum did confirm these peaks clearly. It was reported that dimethyl selenoxide had a characteristic absorption at 820 cm^{-1} region (Paetzold and Bochmann, 1968) which was not

observed in our experimental spectrum. This evidence suggested that there was little dimethyl selenoxide in our sample soon after recrystallization.

Nuclear magnetic resonance spectrum of dimethyl selenone

The experimental nuclear magnetic resonance spectrum for dimethyl selenone is shown in Figure 3. In a proton NMR spectrometry, protons in different chemical environments have different chemical shifts. If dimethyl selenone were present, only one proton NMR peak would be observed because the six protons in the two methyl groups are in the same chemical environment.



Structure of dimethyl selenone

We used deuterium oxide as the solvent because our sample did not dissolve well in deuterated chloroform. The TMS (reference) peak was set up at 0 Hz on the chart before the sample was run. Since the deuterium oxide was not completely deuterated or was contaminated by atmospheric moisture, a proton peak at 4.55 ppm was observed in our experimental spectrum. A water blank was run to identify this peak and we observed a proton peak appearing at the same place (4.55 ppm). In our product plus deuterium oxide sample, another singlet peak was observed at 3.45 ppm which was

tentatively identified as dimethyl selenone. No literature proton NMR spectrum of dimethyl selenone was found. NMR spectra of dimethyl selenide and dimethyl diselenide were run to identify the 3.45 ppm peak. Both of them had nothing to do with this peak. So, given the other characterization data, we feel confident that dimethyl selenone exhibits the 3.45 ppm singlet.

One month after dimethyl selenone was synthesized, we performed a proton NMR spectrum again (Figure 3). The spectrum showed two peaks appearing at 3.25 ppm and 2.75 ppm respectively and little or no peak at 3.45 ppm. This was probably due to the degradation or conversion of dimethyl selenone to another selenium compound. Methyl methaneseleninate $\text{CH}_3\text{Se}(\text{O})\text{OCH}_3$ is a good possibility because two methyl groups in this compound are in different environment. There should be two singlet peaks appearing in its NMR spectrum.

Dimethyl selenone as an intermediate of the biomethylation in selenium resistant bacterial cultures

Preparation work

Initially, we used only autoclaving of solutions and media as a means of sterilization for this work; however, headspace analysis of media blanks showed that some volatile sulfur compounds such as dimethyl disulfide and dimethyl trisulfide were produced after autoclaving TSN media (Figure 4). The reason for this may be due to high temperature and high pressure degrading the medium. The

sulfur containing amino acids in the complex TSN medium are presumably the source of the volatile sulfur compounds observed. This problem caused confusion because we could not differentiate which gases released from our samples were from methylation process by the microorganisms and which were from sterilization process. In order to overcome this problem, we sterilized the medium by employing the use of sterilized filters. With this technique, only trace amount of dimethyl diselenide were detected in medium blank in our chromatograms (Figure 4).

Chromatographic analysis

Table 1 summarizes the results of headspace analysis above sodium selenate dosed culture, dimethyl selenone dosed culture, solution of dimethyl selenone with DMS and DMDS (reduced sulfur compounds) in medium blank, and solution of sodium selenate or sodium selenite with DMS and DMDS in media blanks. We observed many volatile sulfur and selenium compounds such as methanethiol, dimethyl sulfide, dimethyl disulfide, dimethyl selenide, dimethyl diselenide and some unknown compounds in these chromatograms (Figure 5, 6, 7, 8, 9). We identified these volatile compounds by GC-SCD, using known compounds for comparison (Table 2). No selenium containing species were detected above selenium free cultures.

Since 1 mL gas of headspace above selenone dosed cultures extremely overloaded the column, we had to take small amounts of gas and used split injection technique for GC analysis. So, we also prepared 2 mL cultures dosed with sodium selenate and dimethyl

selenone in order to achieve more headspace, smaller cell populations, and lower concentration of volatile species. When we compared these results with 10 mL samples poisoned with the same concentration, the peak areas of 2 mL samples were multiplied by five to normalize to 10 mL cultures volume.

Almost all chromatograms of selenium amended cultures indicated that there was a peak eluted between dimethyl disulfide and dimethyl diselenide. Since the column that we used in our experiment was a nonpolar column, and the elution order of compounds should generally reflect the order of increasing boiling points, the compound eluted between DMDS and DMDS_e in our chromatograms was probably dimethyl selenenyl sulfide based on previous work (Chasteen, 1993); however, GC/MS confirmation using these cultures has not been performed. Another unknown peak was noted at 13.2 min, which was probably due to the dimethyl trisulfide. A dimethyl trisulfide standard has recently been synthesized in our laboratory and this 13.2 min peak has the same retention time as the major peak in that standard.

The most exciting aspect of this research was that we observed much larger amount of volatile selenium compounds (DMSe and DMDS_e) above selenone dosed cultures than those above selenate dosed cultures. (All comparisons are based on integrated peak areas). Gas phase concentrations of DMSe and DMDS_e above selenone dosed cultures were respectively about 4000 times and 1000 times more than those above selenate dosed cultures poisoned with the same concentration (Table 1). This suggests that dimethyl selenone could indeed be one of the biometabolic intermediates of the biom-

ethylation of selenium in the Se cultures. This lends some credence to the methylation mechanism proposed by Challenger (Challenger, 1945). If it were not true, we might not observe an increase of volatile selenium compounds released from the cultures if a stable intermediate such as dimethyl selenoxide, $(\text{CH}_3)_2\text{SeO}$, were created. These results demonstrate the ready and efficient bioreduction of dimethyl selenone; however, the mechanism of reduction may actually be a sulfur pathway which accommodates the structurally related and isoelectronic selenium species.

Meanwhile, we also noticed that gas phase concentrations of volatile sulfur compounds evolved from the 10 mM selenone dosed cultures were less than those evolved from the selenate dosed cultures poisoned with the same 10 mM concentration. Further experiments were undertaken to identify the correlation between selenone and volatile sulfur compounds. One mL of headspace gas of three media blanks separately dosed with 10 mM selenone, 10 mM selenate and 10 mM selenite in the presence of 1 ng DMS and 1 ng DMDS were analyzed by GC. The chromatograms showed that some volatile selenium compounds such as dimethyl selenide and dimethyl diselenide were formed in the selenone dosed medium in the presence of reduced sulfur compounds (Figure 7); however, no volatile selenium compounds were observed in the selenate or selenite dosed medium with DMS and DMDS (Figure 8, 9). This evidence proved that DMS and DMDS could reduce dimethyl selenone to DMSe and DMDS₂Se by a chemical reaction without microorganisms. But the reduced sulfur compounds could not methylate and reduce selenate or selenite salts to volatile selenium compounds directly.

On the basis of the present research, it seems plausible to suggest that the K27 bacteria can methylate nonvolatile sulfur compounds in TSN media to volatile sulfur compounds such as DMS and DMDS and thereby to provide a reducing environment. In the meantime, the K27 bacteria also reduce and methylate selenate or selenite salts to some intermediate, possibly dimethyl selenone, which may then be reduced by both chemical and biological means to volatile reduced selenium species simultaneously. Whether or not this final step is only a chemical reaction with no biological involvement can not be determined at this point.

Conclusions

The results of this research show that:

1) 3-Chloroperoxybenzoic acid was efficient at oxidizing dimethyl selenide to dimethyl selenone. This was confirmed by FTIR, NMR, elemental analysis and melting point.

2) Pure dimethyl selenone was found to be unstable in the presence of reduced sulfur species in trypticase soy broth medium with 0.1 % nitrate added.

3) TSN medium was better than two different minimal media for the growth of *Pseudomonas fluorescens* K27 bacteria.

4) Dimethyl selenone is possibly one of the intermediates of the biological reduction and methylation of inorganic selenium. This conclusion is based on the observation of an increase amount of volatile selenium species such as dimethyl selenide and dimethyl diselenide above selenone dosed cultures when compared with analogous selenate dosed cultures.

5) Chemical reduction and bioreduction by microorganisms may be simultaneously involved in the conversion of dimethyl selenone to volatile selenium compounds in *Pseudomonas fluorescens* K27 cultures.

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Appendix

Chemical Abstract Service Registry Numbers

Compound Name	CAS Registry Number
Dichloromethane	75-09-2
Dimethyl diselenide	7101-31-7
Dimethyl disulfide	624-92-0
Dimethyl selenide	593-79-3
Dimethyl selenenyl sulfide	41884-42-8
Dimethyl selenone	22089-69-6
Dimethyl sulfide	75-18-3
Ether (diethyl ether)	60-29-7
meta-Chloroperoxybenzoic acid	937-14-4
Sodium selenate	13410-01-0
Sodium selenite	10102-18-8

Vitae

Limin Zhang was born in Shanghai, P.R. China on June 13th, 1968. She graduated from the First High School of Wuhan in Wuhan, Hubei of China in 1986. Limin received a Bachelor of Science degree in Textile Chemical Engineering department from China Textile University in July, 1990. In January, 1992, she entered the chemistry graduate program at Sam Houston State University in Huntsville, Texas. She received her Master of Science degree in Chemistry department in December, 1993.