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Biogenesis of Sulfonium Compounds in a Dinoflagellate; Methionine Cascade.†

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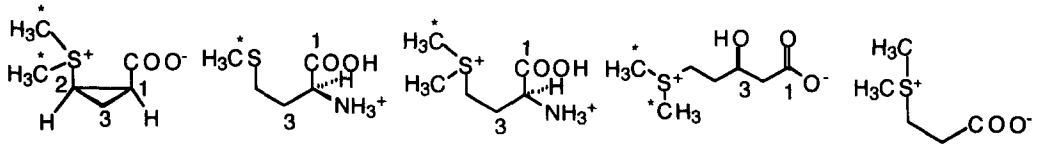
Abstract: Feeding experiments of ^{13}C-labeled compounds to dinoflagellates have unveiled existence of methionine cascade pathway from methionine to sulfonium compounds such as 3-dimethylsulfoniopropionate, gonyauline and gonyol in a dinoflagellate. © 1997 Elsevier Science Ltd.

INTRODUCTION

Gonyauline (**1**) was isolated from extracts of the bioluminescent dinoflagellate *Gonyaulax polyedra* based on its effect in shortening the circadian rhythm.[1] Due to their structural similarity between **1** and methionine, it was proposed that **1** originates biogenetically from L-methionine by a sequence of reactions including methylation and deamination-cyclopropanation accompanied by inversion of the configuration at the C2 carbon. As expected, feeding experiments of |methyl-$^{13}C_1$|methionine (Met) or |methyl-$^{13}C_1$|-methylmethionine (SMM) revealed that methyl groups in **1** originated from methionine.[2] However, no carbon in **1** was labeled with |1-$^{13}C_1$|methionine (Table 1, entries 1 and 2) even at a high concentration.



Gonylauline (1) L-Methionine S-Methylmethionine Gonyol (2) 3-Dimethylsulfonio-propionate

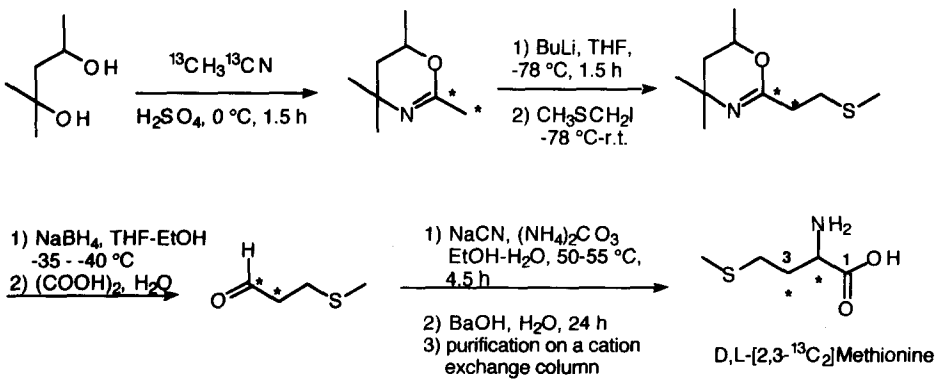
†Dedicated to Professor Yoshito Kishi on the occasion of his 60th birthday.

During the feeding experiments, it was shown that the cells accumulate a new sulfonium compound gonyol (**2**) when the cells were cultured in methionine-enriched medium and we found that the C1 and C2 carbons of gonyol originate from acetate (Table 1, entry 9),[2] suggesting that 3-methylthio- or 3-dimethylsulfoniopropionate is formed from methionine. Two metabolic pathways from methionine to 3-methylthio- or 3-dimethylsulfoniopropionate were known, i.e., 1) direct formation from methionine[3] by deamination and decarboxylation reactions and 2) successive transformation via 5-methylthioadenosine from *S*-adenosylmethionine (methionine salvage pathway)[4] by which only SMe group originates from methionine. Although it has been established that dimethylsulfoniopropionate is directly formed from methionine in higher plants, algae, and heterotrophic dinoflagellate,[3,5-7] it has not been reported that the detailed metabolic pathway of methionine in photosynthetic dinoflagellates. To establish the biogenesis of **1** and **2**, feeding studies were conducted with double labeled methionine (C2 and C3). Here we would like to report the detailed results of the feeding experiments.

RESULTS AND DISCUSSIONS

Double labeled [2,3-$^{13}C_2$]methionine (99 atom % ^{13}C) was synthesized from [1,2-$^{13}C_2$]acetonitrile via [1,2-$^{13}C_2$]methylthiopropionaldehyde in a total yield of 4% as described in Scheme 1[8] and used as a racemate in feeding experiments. [1,Methyl-$^{13}C_2$]methylmethionine was prepared from [1-$^{13}C_1$]methionine. Methionine and methylmethionine showed toxicity to dinoflagellates and were used at a concentration below 100 and 20 μM, respectively. Since sodium cyanide and ethionine were highly toxic, these compounds were not able to use as either carbon sources or metabolic inhibitors. Feeding experiments were conducted to three dinoflagellates which are taxonomically close but accumulate different type of sulfonium compounds.[9]

Scheme 1. Synthesis of double labeled [1,methly-$^{13}C_2$]methionine.



Preliminary results of feeding experiments with [methyl-$^{13}C_1$]methionine revealed that efficient incorporation of ^{13}C was observed by using two or three weeks cultures. Cells were grown for one to several weeks after feeding labeled compounds, additives and antibiotics and were harvested by filtration. The crude water-soluble extracts gave a ^{13}C NMR spectrum consisting mainly with sulfonium compounds, however, some of carbon signals (especially carboxylate signals) were not clearly detected because of serious broadening.

Quantitative analyses were performed after purification on a cation exchange column and a proton-decoupled without NOE mode (NNE) was used for quantification of carbonyl carbons. Signal enhancement of incorporated carbons were expressed as relative intensity by using a value of 1% to non-labeled carbon signals. In case of double labeled compounds, the amount of incorporated ^{13}C was calculated by comparing with ^{13}C signal intensity of natural abundance. The results are summarized in Table 1.

In entry 3, labeled gonyauline was obtained from the *Gonyaulax* cells that were incubated for one week with the double labeled methionine after two weeks of pre-culturing. The ^{13}C signals of gonyauline from [2,3-$^{13}C_2$]methionine were clearly seen as one set of two doublet signals (δ 14.4, C3 and 20.4, C1) with a coupling constant of 12 Hz (Fig. 1). The content of incorporated ^{13}C was calculated based on the signal height ratio of the doublet signals to the natural abundant signal (~1.5:1). The biogenesis of gonyol from methionine and acetate was confirmed by entry 4 in which two sets of doublet signals (δ 29.0, C4 and 66.0, C3, J=38 Hz, and 41.0, C2 and 174.9, C1, J=55 Hz) were shown in the ^{13}C NMR spectrum.

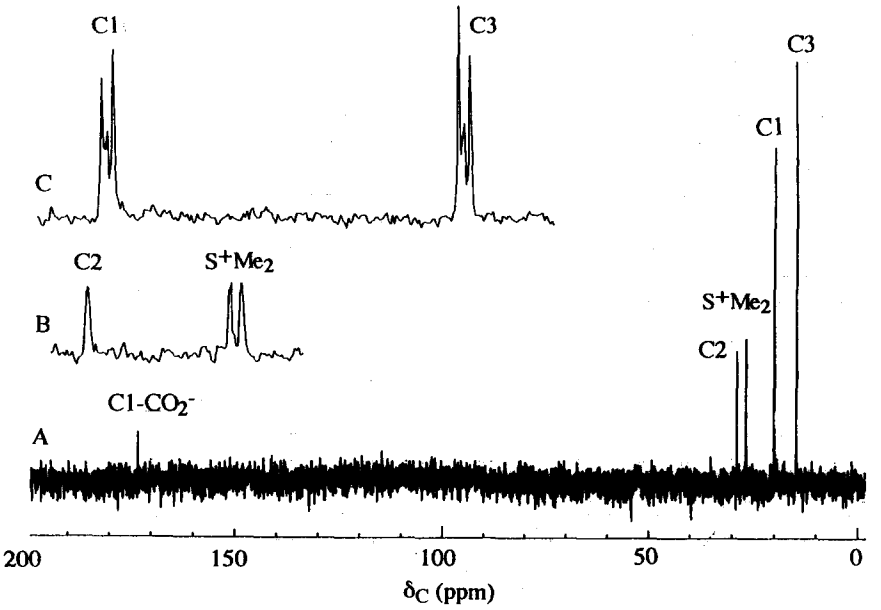


Fig. 1. 67.5 MHz Proton-decoupled ^{13}C NMR spectrum of gonyauline in D_2O isolated from *Gonyaulax polyedra* cultured in the presence of [2,3-$^{13}C_2$]methionine (entry 3): (A) all carbons; (B) expansion of C2 and S^+Me_2; and (C) expansion of C1 and C3.

A similar result was obtained in another dinoflagellate, *Amphidinium* sp (Y-5) (entry 8) which contains gonyol as a major sulfonium compound under the normal culture conditions. 3-Dimethylsulfoniopropionate (DMSP), the most widely distributed sulfonium compound in dinoflagellates,[9] was also shown to originate from methionine (entry 11, δ 28.4, C2 and 173.6, C1, J=55 Hz) in *Pyrocystis lunula* as well as other DMSP containing higher plants.

Labeling experiments with $NaH^{13}CO_3$ in the absence of additives showed no specific incorporation of ^{13}C into DMSP, gonyol and gonyauline either under light or dark conditions. As a result of photosynthesis

Table 1. Feeding experiments of labeled compounds to the dinoflagellates *Gonyaulax polyedra*, *Amphidinium* sp., and *Pyrocystis lunula*. .[a]

entry	labeled compound (μM)	additive (μM)	feeding schedule (day)	content of incorporated ^{13}C[b] gonyauline (**1**) (position)	gonyol (**2**) (position)
Gonyaulax polyedra					
1	[Me-$^{13}C_1$]Met (5+5)[c]	none	14-7-7	2% (Me)	N. D.
2	[1,Me-$^{13}C_2$]SMM (5+5)[c]	none	14-7-7	2% (Me)	N. D.
3	[2,3-$^{13}C_2$]Met (10)	none	14-7	3% (C1, C3)	-
4	[2,3-$^{13}C_2$]Met (10) and [1,2-$^{13}C_2$]AcOH (50)	Met (90)	14-7	1% (C1, C3)	2% (C1, C2) and 2% (C3, C4)
5	DMSP (54% $^{13}C_5$, 10)	none	21-1	5% (C1-C3, Me)	50% (C3-C5, Me)
6	$NaH^{13}CO_3$ (5000)	none	14-7	20% (all C)	-
7	$NaH^{13}CO_3$ (500)	DMSP (10)	14-1	2% (C1-CO_2^-)	4% (C1, C2)
***Amphidinium* sp. (Y-5)**					
8	[2,3-$^{13}C_2$]Met (10)	AcOH (50)	14-7	-	0.5% (C3,C4)
9	[1,2-$^{13}C_2$]AcOH (50)[c]	Met (10)	14-7	-	0.2% (C1, C2)
10	$NaH^{13}CO_3$ (500)	none	14-1	-	1% (all C)
Pyrocystis lunula				DMSP (position)	
11	[2,3-$^{13}C_2$]Met (10)	none	14-7	2% (C1, C2)	
12	$NaH^{13}CO_3$ (2000, 1000x2)	none	14-7-7	54% (all C)	

a) Dinoflagellates (*Gonyaulax polyedra, Amphidinium* sp. *and Pyrocystis lunula*) in the stationary phase were inoculated (about 1/10 volume) in fresh f/2 culture medium and fed along the feeding schedule under 12 h light-12 h dark cycles at 20 °C. Feeding schedule 14-7-7 means 14 days pre-culture, 7 days culture after first feeding of the labeled compound, the additive, and antibiotics (streptomycin 5 mg/L and chloramphenicol 2.5 mg/L), 7 days culture after second feeding of the labeled compound, and harvest by either filtration or centrifugation. Most of labeled compounds were used as 99 atom % ^{13}C. 3-Dimethylsulfoniopropionate (DMSP) was enriched with ^{13}C at a level of 54% (entry 12) and used for feeding experiments with *G. polyedra* (entry 5). b) Isolation yields of each compounds in the entries were as follows [purified amount (mg, compounds) / wet weight of harvested cells (g) / culture medium (L)]: entry 1 [10 (**1**) / 2.1 / 6]; entry 2 [5.2 (**1**) / 0.44 / 6]; entry 3 [5.2 (**1**) / 2.0 / 4]; entry 4 [7.5 (**1**) and 6.8 (**2**) / 2.1 / 4]; entry 5 [4.2 (**1**) and 2.2 (**2**) / 0.56 / 2]; entry 6 [10 (**1**) / 1.0 / 2]; entry 7 [3.3 (a mixture of **1** and **2**) / 0.90 / 2]; entry 8 [16 (**2**) / 3.3 / 4]; entry 9 [15 (**2**) / 4.4 / 4]; entry 10 [50 (crude extract) / 1.5 / 2]; entry 11 [10 (**DMSP**) / 2.6 / 4]; entry 12 [27 (**DMSP**) / 2 / 4]. c) Preliminary results were reported in lit. 2.

uniformly labeled compounds were obtained (entries 6, 10, and 12). Uniformly labeled DMSP at a high level of 54% prepared with *Pyrocystis lunula* allowed to establish that DMSP is the common precursor for gonyauline and gonyol (entry 5) whose carbons were found as broad or multiplet signals due to direct and long-range ^{13}C-^{13}C couplings. But the origin of carboxylate carbon in gonyauline is still not known.

After several trials using C1 carbon sources or inhibitors such as NaCN and ethionine, we fount that a short duration of feeding of $NaH^{13}CO_3$ in the presence of either DMSP or methionine with *G. polyedra* resulted in specific increment of two carboxylate carbon signals (Fig. 2). The enriched carbons with a combination $NaH^{13}CO_3$ and DMSP were assigned as the carboxylate carbons of gonyauline and gonyol on the basis of the chemical shifts of δ 173.7 and 175.5 and analyses of ^1H-^{13}C long-range couplings by HMBC experiments; the protons on the cyclopropane ring of gonyauline were correlated to the carboxyl carbon signal at δ 173.7 and the oxymethine proton of gonyol was correlated to the carbon signal at δ 175.5. Incorporation of $^{13}CO_2$ was observed in day time (light) cultures but not in night time (dark) cultures. These data may suggest that CO_2 is the direct origin of the carboxylate carbon of gonyauline. However, uniformly labeling of an acetate unit of gonyol (C1' and C2' in Fig. 2) with ^{13}C suggested that rapid scrambling among photosynthetic products was achieved under the culturing conditions. Therefore, it is difficult to specify one compound as a carboxylate source of gonyauline under the culturing conditions among possible chemicals including inorganic CO_2 and the products at an early stage of photosynthesis. In the reaction, carboxylase or transcarboxylase might be involved.

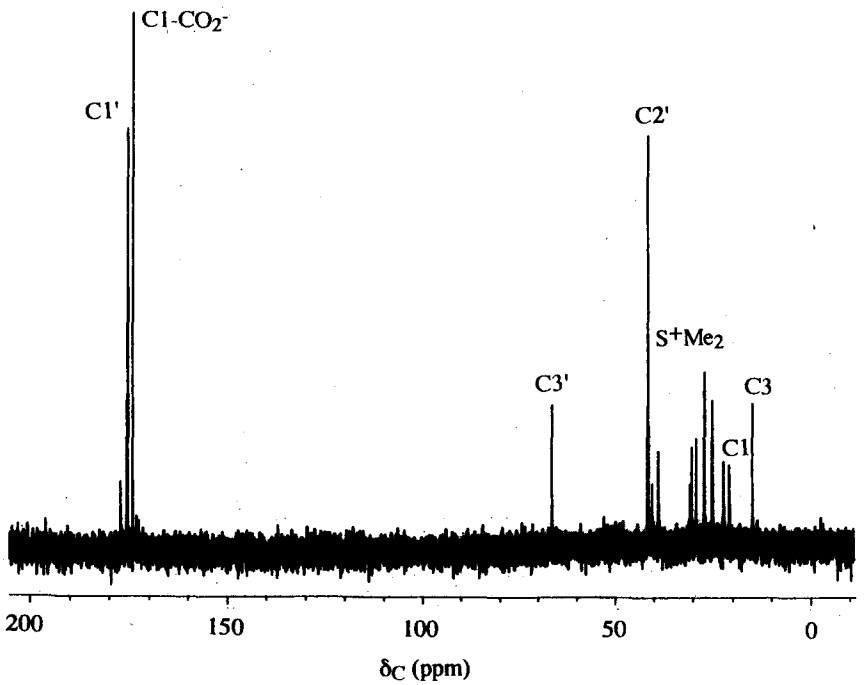
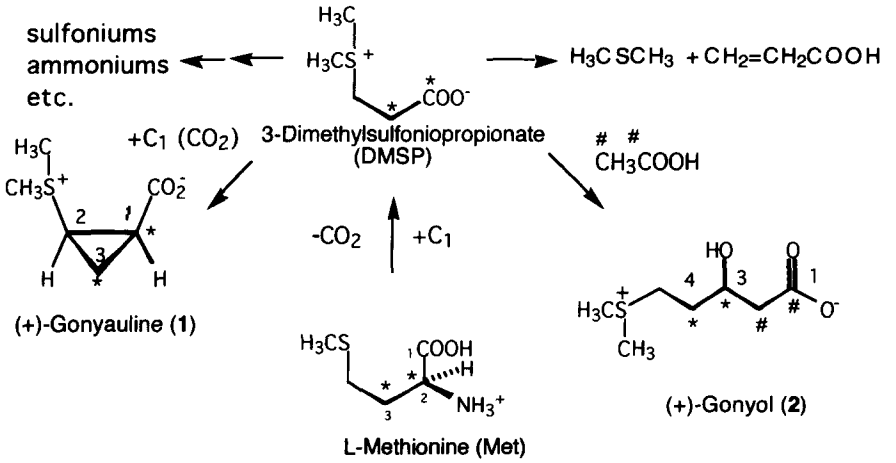


Fig. 2. 125 MHz Proton-decoupled without NOE ^{13}C NMR spectrum of a mixture of gonyauline (**1**) and gonyol (**2**) in D_2O isolated from *Gonyaulax polyedra* cultured in the presence of 3-dimethylsulfoniopropionate and $NaH^{13}CO_3$ (entry 7). C1-CO_2^-, C1, C3 and S^+Me_2 for **1** and C1'-C3' for **2**.

Feeding experiments of ^{13}C labeled compounds showed that the rapid catabolic pathway of methionine to sulfonium compounds occurs in dinoflagellates, which transform to various compounds including acrylic acid and quaternary ammonium (methionine cascade, Scheme 2). In *G. polyedra*, the rates of biosynthesis and catabolism of gonyauline are slower than those of methionine, DMSP and gonyol since methyl carbon signals of methionine and gonyol were seen in the *G. polyedra* extracts only by short period feeding of [methyl-$_{13}C_1$]Met (20 μM). After the origin of DMSP was established as methionine in the green alga *Ulva lactuca*,[3] methionine was shown to be a DMSP precursor in a wide range of plants, including a heterotrophic dinoflagellate *Crypthecodinium cohnii*,[5] a red alga *Chondria coerulescens*[6] as well as lower and higher plants.[7] Recently methylmethionine and 3-dimethylsulfoniopropionaldehyde were reported to be the biosynthetic intermediates and the enzymatic activities of methyltransferase and dehydrogenate involved in the biosynthesis were found in cytosol and chloroplast, respectively, in a flowering plant *Wollastonia biflora*.[10]

Gonyauline was found only in *Gonyaulax polyedra* so far and gonyol distributed in several dinoflagellates[9] These compounds may act as an osmoprotectant and a methyl donor like DMSP.[11] It is important to know the detailed catabolic pathways in marine organisms, since sulfoniums contribute to the biogenic production of atmospheric dimethylsulfide, which has a central role in the global sulfur cycle.[12] The unusual biogenesis of gonyauline from methionine in *G. polyedra* might have some relation to the structural characteristics of dinoflagellate metabolites.[13,14] Physiological and chemical studies on sulfonium compounds and unusual metabolic pathway in dinoflagellates are in progress in our laboratory.

Scheme 2. Methionine cascade in a dinoflagellate.



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EXPERIMENTAL

General: ^1H and ^{13}C NMR spectra were measured by JEOL FX-270, GX-400, EX-400, Alpha-400 and Alpha-500 instruments. t-BuOH was used for an internal standard in D_2O (δ_H 1.23, δ_C 30.3). L-[1-$^{13}C_1$]Methionine (99 atom % ^{13}C), sodium [$^{13}C_1$]bicarbonate (99 atom % ^{13}C), and [1,2-$^{13}C_2$]acetonitrile (99 atom % ^{13}C) were purchased from ISOTEC Inc. L-[Methyl-^{13}C]methionine, [$^{13}C_1$]MeOH, and sodium [1,2-$^{13}C_2$]acetate (99 atom % ^{13}C) were obtained from Aldrich Inc.

Dinoflagellates: *G. polyedra* and *P. Lunula* were isolated from sea water and the cultures were kept at Harvard University. *Amphidinium* sp. (Y5) was isolated from a flat warm *Amphiscolops* sp. by Prof. Yamasu (Ryukyu University). These specimens were cultured in a 3 L flask containing 2 L sea water medium f/2 using soil extracts instead of silicate under 12 h dark/12 h ligh cycle at 22 °C. Dinoflagellates in the stationary phase were inoculated (about 1/10 volume) in fresh culture medium and used for the feeding experiments.

Feeding experiments: After pre-culturing, the labeled compound, the additive, and antibiotics (streptomycin 5 mg/L and chloramphenicol 2.5 mg/L) were fed and additional feeding was made after an interval. All the feeding materials were dissolved in water and sterilized by a membrane filter (Millex GV 0.22 μm). Cultured cells were harvested by either filtration or centrifugation and stored at -20 °C until use. The amounts of cultured cells (wet weight) were summarized in Table 1.

Analyses of incorporated ^{13}C: The harvested cells were extracted with 70% EtOH (3 times) by using Ultradisspensor. The extracts were dissolved in water and partitioned between water and EtOAc. The water layer was evaporated in vacuo. The preliminary analysis of ^1H and ^{13}C NMR was made by dissolving the residue in D_2O. Quantitative analyses was carried out after chromatographic purification on an ion exchange column (Bio RaD AG50W-X8, H^+ form). Gonyauline, DMSP and gonyol were eluted at 0.8, 0.9 and 1.0 M HCl, respectively and concentrated in vacuo. Incorporation of ^{13}C carbon was determined quantitatively by ^{13}C NMR spectrum in D_2O and expressed as relative intensity of labeled compound to natural abundance (1%). In case of double labeled compounds, the amount of incorporated ^{13}C was calculated by comparing with ^{13}C signal intensity of natural abundance. Enrichment of ^{13}C with $NaH^{13}CO_3$ was determined on the basis of ^1H satellite signals of the methyl groups. Carboxylate carbons were quantified as proton-decoupled signals without NOE (pulse delay=10 s). The isolation yields were depended on the harvest. and the typical amounts of the purified compounds were summarized in Table 1. These materials were pure enough for the analyses but may contain certain amount of salts.

Synthesis of [1,methyl-$^{13}C_2$]*S*-methylmethionine: L-[1-$^{13}C_1$]Methionine was methylated with $TsO^{13}CH_3$ (prepared from $^{13}CH_3OH$ and TsCl) in formic acid at 39 °C for 5 h. After evaporation of the solvent the residue was partitioned between water and EtOAc. The water layer was passed through a column of DEAE-Sephadex A-25 (Cl^- form) with water and concentrated to give an oil (yield 98%): ^1H NMR (D_2O) δ 3.88 (1H, m), 3.48-3.30 (2H, m), 2.85 (6H, dd, *J*=148, 4 Hz), and 2.35-2.25 (2H, m); HR-EI-MS *m/z* 165.0751 $(M)^+$, Calcd for $^{12}C_4{}^{13}C_2H_{13}O_2SN$ 165.0728.

Synthesis of [1,2-$^{13}C_2$]methionine: Double labeled methionine was prepared from [1,2-$^{13}C_2$]acetonitrile (ISOTEC, 99 atom % ^{13}C) via [1,2-$^{13}C_2$]methylthiopropionaldehyde by a sequence of reactions developed on the basis of literature methods[3] in total yield 4% and finally purified by ion chromatography (Dowex AG 50WX8, H^+ form, eluted by 2 M HCl). The purity was determined by amino acid analysis and characterized by NMR: ^1H NMR (D_2O, 270 MHz) δ 4.21 (1H, ddt, 148, 7, 5 Hz), 2.70 (2H, tt, *J*=13, 5 Hz), 2.225 (2H, dm, *J*=133 Hz), 2.12 (3H, s). HR-FAB-MS *m/z* 152.0664 $(M+1)^+$: Calcd for $^{12}C_3{}^{13}C_2H_{12}O_2SN$ 152.0716.

Synthesis of [$^{13}C_5$]3-dimethylsulfoniopropionate: 70% EtOH extracts of *Pyrocystis lunula* (2 g, wet weight) cultured for three weeks in the presence of $NaH^{13}CO_3$ (2 mM and 1 mMx2, entry 11) was purified on an ion exchange column to give DMSP (27 mg), 65% of which was labeled with ^{13}C (85% atom % ^{13}C). ^{13}C NMR (67.8 MHz, D_2O) δ 25.0 (s), 28.4 (m), 38.6 (s and d, *J*=35.6 Hz), 173.6 (s and d, *J*=55.4 Hz).

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