Journal of Natural Products
Vol. 53, No. 1, pp. 87-93, Jan-Feb 1990

THE ROLE OF METHYLSULFONIUM COMPOUNDS IN THE BIOSYNTHESIS OF *N*-METHYLATED METABOLITES IN *CHONDRIA COERULESCENS*

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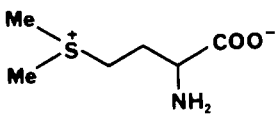
ABSTRACT.—The possible metabolic relationships between methylsulfonium and *N*-methylated compounds were investigated in the red alga *Chondria coerulescens* by means of radiotracer techniques. The alga, whose H_2O-soluble fraction contains *S*-methylmethionine [**1**], dimethyl-β-propiothetin [**2**], 4-hydroxy-*N*-methylproline [**3**], and γ-aminobutyric acid betaine [**4**], was fed with methionine, *S*-methylmethionine, dimethyl-β-propiothetin, and γ-aminobutyric acid betaine, all [$^{14}CH_3$]-labeled. Following administration of [$^{14}CH_3$]-dimethyl-β-propiothetin, methyl-labeled 4-hydroxy-*N*-methylproline was isolated, thus supporting the hypothesis that sulfonium salts may function as methyl donors in the biosynthesis of *N*-methylated compounds.

In a recent study on the distribution of Dragendorff-positive compounds in Mediterranean red algae (1) we found that only one out of 22 species examined lacked both sulfonium and quaternary ammonium (or tertiary amino) compounds; in all the other species members of the two classes co-occur. On the basis of this parallel distribution we supposed that sulfonium salts can act as methyl donors in the biosynthesis of *N*-methylated metabolites, and now we have tried to support this hypothesis by tracer experiments in the red alga *Chondria coerulescens* (Crouan) Falk. (Rhodomelaceae, Ceramiales). The H_2O extract of this species contains *S*-methylmethionine [**1**] (SMM; 4-dimethylsulfonio-2-aminobutyrate), dimethyl-β-propiothetin [**2**] (DMP; 3-dimethylsulfoniopropionate), 4-hydroxy-*N*-methylproline [**3**] (HMP), and γ-aminobutyric acid betaine [**4**] (GABAB; 4-trimethylammoniobutyrate) (1). [$^{14}CH_3$]Methionine ([$^{14}CH_3$]Met), [$^{14}CH_3$]SMM, [$^{14}CH_3$]DMP, and [$^{14}CH_3$]GABAB were fed to the alga, and the present paper describes the results of this investigation.

In order to reproduce as closely as possible the natural environment of the alga, in all the feeding experiments a home-made incubation apparatus was used which provided the most important extrinsic requirements of the plant: light (both intensity and day-night cycle), temperature, and turbulence. Continuous monitoring of the O_2 concentration in the incubation solution gave an indication of the photosynthetic activity



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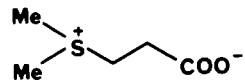

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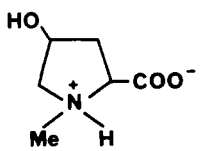

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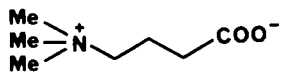

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of the plant and provided an indicator as to its well-being. A more detailed description of the apparatus is given in the Experimental section. At the end of the incubation period, SMM, DMP, GABAB, and HMP were isolated, purified by chromatography, and checked for radiochemical purity by 2D autoradiography. After they were quantified by nmr spectroscopy, their radioactivity was determined by liquid scintillation counting. To determine whether randomization of the label had occurred, each metabolite was subjected to appropriate chemical degradation. In particular, heating [^{14}C]SMM and [^{14}C]DMP with alkali produced [^{14}C]Me_2S which incorporated the total label. Similarly, Hofmann's elimination of [^{14}C]Me_3N from [^{14}C]GABAB showed that all the label was located at the methyls. [^{14}C]HMP was converted into the corresponding betaine, and this was decomposed by alkali fusion to give [^{14}C]Me_2NH of the same specific activity as the starting material.

The results of the administration experiments are summarized in Table 1. [$^{14}CH_3$]Met acted as a methyl source for both *S*- and *N*-methylated compounds, though more efficiently for DMP. [$^{14}CH_3$]SMM was absorbed by the plant, but no incorporation of the label was observed in any of the compounds considered. After administration of [$^{14}CH_3$]DMP, HMP was found labeled at the methyl, but no radioactivity was detected in either SMM or GABAB. Because ammonium compounds, e.g., choline or glycine betaine, have been claimed to be involved in transmethylation reactions in plants (2,3), an additional feeding experiment was carried out with [$^{14}CH_3$]GABAB, but no radioactivity was found in any of the co-occurring *N*- and *S*-methyl compounds.

Finally, in order to provide further proof for the validity of the observed incorporations, two separate control experiments were carried out in which appropriate amounts of either [$^{14}CH_3$]Met or [$^{14}CH_3$]DMP were added to aqueous-EtOH extracts of the alga; the extracts were subsequently subjected to the same purification procedure as in the feeding experiments. The former experiment revealed no radioactivity in any of the metabolites **1–4**, while the latter, as expected, showed that only metabolite **2** was radiolabeled.

As a result of the present study it seems that sulfonium salts other than *S*-adenosylmethionine can actually act in plants as methyl donors in transmethylation reactions leading to *N*-methyl compounds, and the fact that DMP is very frequently found in algae can be due to this physiological function. Moreover, the observation that in *C. coerulescens* the methyl groups are transferred to HMP and not to GABAB is a good indication of the specificity of the acceptor in the transmethylation process. As yet, in this alga no evidence has been obtained as to whether DMP (or even SMM) acts as methyl donor in the biosynthesis of compounds other than the ones considered in the present study.

Finally, it must be recalled that Greene (4) showed that in the green alga *Ulva lactuca* both the carbon skeleton and the methyls of DMP originate from methionine, while SMM is not a biosynthetic intermediate. Considering the results here reported on *C. coerulescens*, it appears likely that the biosynthesis of DMP follows the same route in both green and red algae.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H-nmr spectra were recorded at 250 MHz on a Bruker AC-250 instrument. Unless otherwise specified, D_2O was used as solvent and 3-(trimethylsilyl)-2,2,3,3-d_4-propanoic acid sodium salt (TSP) as internal reference. Hptlc were run on glass-precoated HPTLC Si gel-F_{254} plates (Merck) using the following solvent systems: (1) *n*-BuOH–HOAc–H_2O (12:3:5) and (2) EtOH-30% NH_4OH (7:3). Ninhydrin, iodine vapors, or Dragendorff's reagent (5) was used as chromogenic reagent. The location of radioactivity on tlc plates (Si gel-F_{254}; Merck) was determined by autoradiography on Kodak X-OMAT AR-2 X-ray film. Radioactivity measurements were carried out with a Beckmann LS-1801 liquid scintillation counter in Ready Safe scintillation cocktail (Beckmann).

TABLE 1. Distribution of Radioactivity in Different Metabolites from *Chondria coerulescens* Supplied with [$^{14}CH_3$]-Labeled Methionine ([$^{14}CH_3$]Met), *S*-Methylmethionine ([$^{14}CH_3$]SMM), Dimethyl-β-propiothetin ([$^{14}CH_3$]DMP), or γ-Aminobutyric Acid Betaine ([$^{14}CH_3$]GABAB).[a]

Metabolite	Labeled precursor											
	[$^{14}CH_3$]Met[b]			[$^{14}CH_3$]SMM[c]			[$^{14}CH_3$]DMP[d]			[$^{14}CH_3$]GABAB[e]		
	Inc. %	Sp. Inc. %	Total wt (mg)	Inc. %	Sp. Inc. %	Total wt (mg)	Inc. %	Sp. Inc. %	Total wt (mg)	Inc. %	Sp. Inc. %	Total wt (mg)
S-Methylmethionine	0.07	0.308	0.51	2.55	81.909	5.23	n. i.[f]	n. i.	0.94	n. i.	n. i.	1.01
Dimethyl-β-propiothetin	1.90	2.616	1.31	n. i.	n. i.	0.94	2.60	54.814	4.73	n. i.	n. i.	1.63
4-Hydroxy-*N*-methyl-proline	0.13	0.0024	104.72	n. i.	n. i.	107.26	0.39	0.378	111.42	n. i.	n. i.	105.82
γ-Aminobutyric acid betaine	0.44	0.038	22.78	n. i.	n. i.	33.43	n. i.	n. i.	27.96	5.58	6.380	28.40

[a]Inc. % = % absolute incorporation, Sp. Inc. % = % specific incorporation. Total wt is the total amount of metabolite isolated from 50 g (fresh wt) of alga after the feeding experiment.

[b]Total activity 50 μCi, specific activity 3.7 mCi/mM.

[c]Supplied as hydroiodide. Total activity 48.46 μCi, specific activity 46.94 μCi/mM.

[d]Supplied as hydroiodide. Total activity 16.01 μCi, specific activity 21.5 μCi/mM.

[e]Total activity 6.36 μCi, specific activity 28.4 μCi/mM.

[f]n. i. = no incorporation.

[$^{14}CH_3$]Methionine and [$^{14}CH_3$]MeI, purchased from NEN Radiochemicals, had specific activity of 54.2 mCi/mM and 13.16 mCi/mM, respectively. When required, they were diluted with cold material to the desired specific activity. The reported specific activity for [^{14}C]MeI is as given by the supplier, and the purity of this compound was not checked prior to use.

PLANT MATERIAL.—Thalli of *C. coerulescens* were collected near Catania, Sicily. They were put in sea-water thermostated at 16° and immediately transferred to the laboratory. After the removal of macroscopic epibionts, the alga was rinsed with sea-water (filtered through 0.22 μm membrane filter) and used for tracer experiments. Voucher specimens were deposited in the University Herbarium, Institute of Botany, Catania.

INCUBATION APPARATUS.—The apparatus for tracer experiments was composed of the following principal components (Figure 1): an incubation vessel, an alga holder, a dc motor with its power supply and control unit, a light source, a cool H_2O circulating system, an oxygen sensor, and an appropriate recorder.

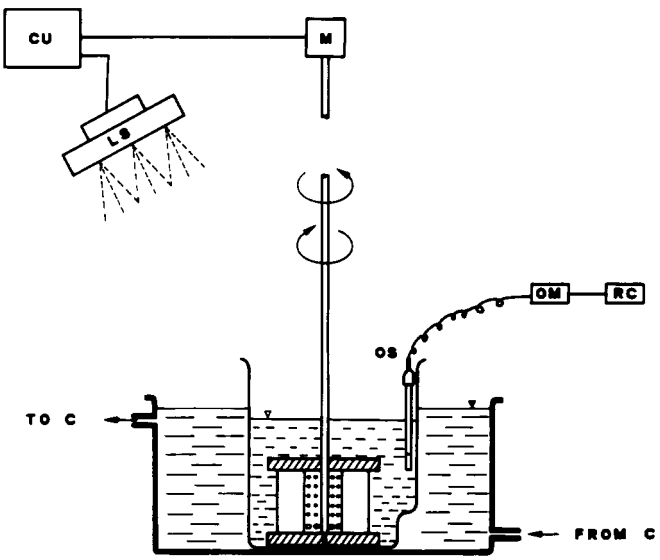

FIGURE 1. Schematic diagram of incubation apparatus. C, cryostat; CU, control unit; LS, light source; M, motor; OM, oxygen meter; OS, oxygen sensor; RC, recorder.

The incubation chamber was a cylindrical Pyrex beaker (diameter 14 cm, 2 liters) on one side of which a semicylindrical (diameter 2 cm) niche had been heat-molded to accommodate an O_2 sensor.

The alga holder, which can be disassembled for easy cleaning (Figure 2), was composed of two rectangular containers sandwiched corner to corner between two circular discs, the upper of Plexiglas, the lower of Teflon. The rectangle walls of each container were also of Plexiglass, two being bored (Figure 2, a). The two remaining significant spaces helped the incubation medium (sterilized sea-water, 0.22 μm membrane filter, 650 ml) to circulate; the medium was maintained by cryostat at 16°. An alternating flow of H_2O through the bored walls was provided by simply inversely rotating the holder on a central vertical stainless steel rod.

The rotation speed and cycle duration were set by a timeable polarity-inverting potentiometer.

Additional lighting was provided by 2 × 25 W cool-white fluorescent tubes (Philips SL25) and 2 × 25 W tungsten bulbs. The lights were orientable for desired light intensity and were connected in series to a photoperiod timer (photoperiod 12:12).

The O_2 sensor was a PTI-401 (Kent) and was connected to a Leeds-Northrup recorder.

LABELED PRECURSORS.—L-[$^{14}CH_3$]Methionine used for feeding experiments had specific activity 3.7 mCi/mM.

For L-[$^{14}CH_3$]*S*-methylmethionine hydroiodide ([methyl-^{14}C](*S*)-(3-amino-3-carboxypropyl)dimethylsulfonium iodide) ([$^{14}CH_3$]SMM·HI), the original procedure (6) was adapted as follows. A mixture of [$^{14}CH_3$]Met (250 mg, specific activity 59.6 μCi/mM), HCOOH (2.75 ml), MeCOOH (0.9 ml), and MeI (0.45 ml) was kept at room temperature for 3 days. Distillation in vacuo left a syrupy residue which

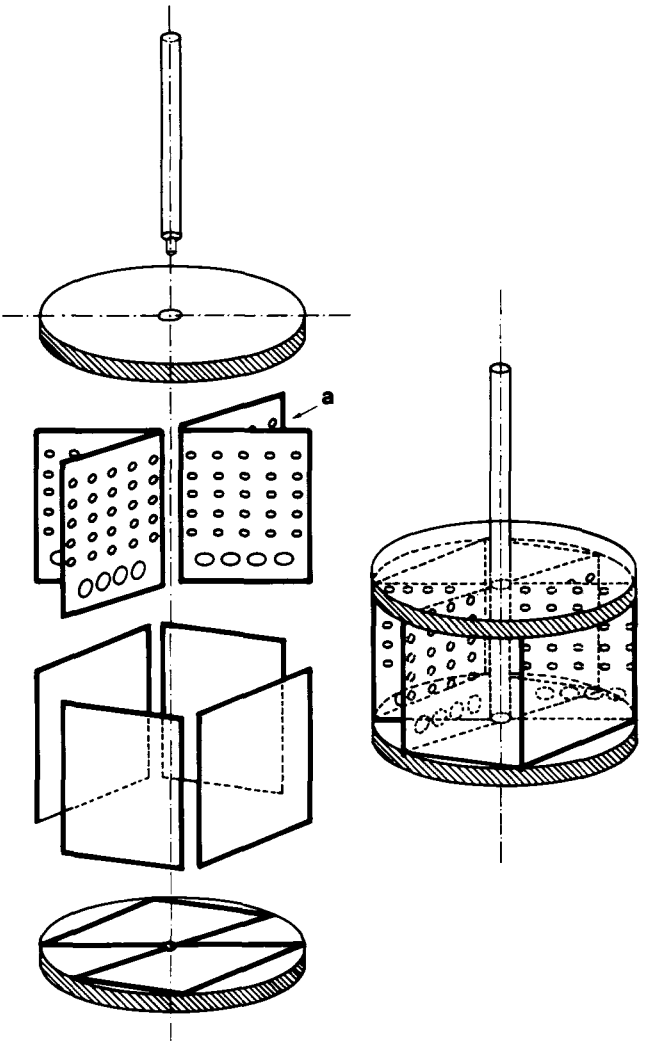

FIGURE 2. Alga holder and its exploded view.

was digested with MeOH to give a precipitate of crude [$^{14}CH_3$]SMM·HI. Subsequent recrystallization from EtOH gave a product showing constant specific activity (46.9 μCi/mM) after the third crystallization.

For [$^{14}CH_3$]dimethyl-β-propiothetin hydroiodide ([methyl-^{14}C](2-carboxyethyl)dimethylsulfonium iodide) ([$^{14}CH_3$]-DMP·HI), 3-mercaptopropanoic acid (2 ml) was dissolved in 10 N NaOH (9 ml), and after addition of Me_2SO_4 (2.4 ml), the mixture was stirred at room temperature for 1 h. After acidification to pH 1 with 6 N HCl, the solution was partitioned with EtOAc and the organic layer dried over Na_2SO_4. Evaporation of the solvent gave 3-methylthiopropanoic acid, whose identity was confirmed by ^{1}H nmr (δ 2.11, s, 3H,-SMe; δ 2.74, m, 4H). 3-Methylthiopropanoic acid (320 mg) was exhaustively methylated with [^{14}C]MeI (0.66 ml, specific activity 93.7 μCi/mM) in the conditions described above for SMM to give [$^{14}CH_3$]DMP·HI. This was diluted with cold DMP·HI and recrystallized from EtOH to constant specific activity (21.5 μCi/mM).

Methylation of γ-aminobutyric acid (GABA) (150 mg) with [^{14}C]MeI (0.19 ml, specific activity 326.8 μCi/mM) according to the procedure of Patchett and Witkop (7) yielded a mixture of [$^{14}CH_3$]GABAB hydroiodide and [$^{14}CH_3$]GABAB methylester iodide along with a small amount of unreacted GABA. This mixture was then treated with 22% HCl at 110° for 3 h. Evaporation to dryness in vacuo gave a residue which was dissolved in H_2O and applied to a column of Amberlite IRC-50 (H^+). The resin was washed with H_2O, and [$^{14}CH_3$]GABAB was recovered by elution with 2 N NH_4OH. The eluate

was added with cold GABAB and taken to dryness. Recrystallization from MeOH/Me_2CO (3 times) gave a product of constant specific activity (28.45 μCi/mM).

FEEDING EXPERIMENTS.—*C. coerulescens* (50 g) was put into the holder and immersed in filtered sea-water contained in the Pyrex beaker. Labeled precursor, dissolved in the minimum amount of H_2O, was administered by adding to the incubation liquid. After a day, the alga was carefully rinsed with sea-water and blotted with filter paper.

EXTRACTION AND ANALYSIS OF RADIOLABELED METABOLITES.—Extraction and isolation of the amino acid fraction from the aqueous-EtOH extract of the alga were carried out as reported earlier (8). An aqueous solution of this fraction was applied to a column of Dowex-1 ($^-$OAc), and both neutral and basic amino acids were eluted with H_2O. Subsequent elution with 2 N HOAc gave a fraction containing acidic amino acids, which were consistently unlabeled. This fraction, therefore, was not further investigated. The aqueous eluate, after concentration to a small volume, was further chromatographed on Amberlite IRC-50 (H^+). Elution with H_2O resulted in a partial resolution of the components. Fractions containing HMP (hptlc, solvent 1, R_f 0.23) were pooled and further purified by ion-exchange chromatography on Dowex-50W (H^+) eluting with a linear gradient of HCl from 0 to 1 N (9), while DMP (hptlc, solvent 1, R_f 0.15) was isolated from impure fractions by preparative liquid chromatography (plc) on LiChroprep Si-60 (25–40 μm) in solvent 1. The mixture of basic components, recovered from the Amberlite column by eluting with 2 N NH_4OH, was subjected to plc (LiChroprep Si-60, 25–40 μm, solvent 1) to give chromatographically pure GABAB and SMM (hptlc, solvent 1, GABAB R_f 0.19, SMM R_f 0.08). Radiochemical purity of the isolated metabolites was checked by 2D tlc (solvents 1 and 2) followed by autoradiography. For quantitative determination, a solution in D_2O of each metabolite was added with a known amount of TSP and the ^{1}H-nmr spectrum recorded. From the ratio of the area of the TSP peak to that of *S*- or *N*-Me resonance, the amount of the isolated compound was easily determined. An aliquot of the solution was then counted in liquid scintillation cocktail.

CONTROL EXPERIMENTS.—To check the validity of the separation procedure described above, the following experiments were carried out. [$^{14}CH_3$]Met (0.24 mg, specific activity 1.35 mCi/mM) was added to the aqueous-EtOH extract from 50 g (fresh wt) of the alga and the resulting mixture processed by the above-described purification protocol. After the isolation of compounds **1–4**, not one of these was found to to be radioactive. A similar experiment was carried out by adding 5.865 mg of [$^{14}CH_3$]DMP·HI (specific activity 21.5 μCi/mM) to the aqueous-EtOH extract of the alga (50 g fresh wt); compounds **1**, **3** and **4**, subsequently isolated, were free of radioactivity.

CHEMICAL DEGRADATION OF LABELED METABOLITES.—Degradations of labeled compounds, isolated from the alga, were carried out in an air-tight glass apparatus which allowed for the collection directly into an nmr tube of volatile compounds evolved during the reactions.

Radioactive SMM (specific activity 11.41 μCi/mM) isolated after the feeding experiment with [$^{14}CH_3$]Met was treated with 1 N NaOH at 40°, and the evolved [^{14}C]Me_2S was collected in a cooled (liquid air) nmr tube containing CD_3OD and a known amount of TSP. The ^{1}H-nmr spectrum was recorded and Me_2S quantified from the area ratio of the peaks of TSP and Me_2S. Subsequently, the solution was transferred to a cooled vial and counted. The specific activity of the labeled Me_2S was 11.05 μCi/mM.

Labeled DMP (specific activity 96.8 μCi/mM) obtained following the feeding experiment with [$^{14}CH_3$]Met gave, after the alkali treatment as above, [^{14}C]Me_2S whose specific activity was 93.6 μCi/mM.

Radioactive HMP was converted into the corresponding betaine iodide (betonicine iodide) according to the method of Patchett and Witkop (7), and this was purified by recrystallization from EtOH. Alkali fusion of the betaine, using the method reported by Jahns (10) for stachydrine, yielded a volatile base which was absorbed in CF_3COOH contained in an nmr tube. ^{1}H-nmr spectrum of this solution allowed the identification of the volatile base as Me_2NH based on the observed multiplicity of the methyl resonance (δ 2.98, t, J = 5.7 Hz, $Me_2\overset{+}{N}H_2$). After the amine was quantified against a known amount of added TSP, the solution was taken to pH 7 and its radioactivity determined by liquid scintillation counting.

Labeled HMP (specific activity 0.090 μCi/mM) isolated from the alga after the feeding experiment with [$^{14}CH_3$]Met gave [^{14}C]Me_2NH whose specific activity was 0.087 μCi/mM. Degradation of radioactive HMP (specific activity 0.081 μCi/mM) isolated following the feeding experiment with [$^{14}CH_3$]DMP gave [^{14}C]Me_2H whose specific activity was 0.080 μCi/mM.

Hofmann's degradation of radioactive GABAB (specific activity 1.40 μCi/mM) isolated from the alga after administration of [$^{14}CH_3$]Met was carried out by treating the betaine with 10 N NaOH at 100° for several hours. This afforded [^{14}C]Me_3N which was collected in CF_3COOH (δ 3.04, d, J = 5.17 Hz, $Me_3\overset{+}{N}H$). Its specific activity was 1.38 μCi/mM.

ACKNOWLEDGMENTS

Our thanks are due to Mr. F. Coco for its invaluable technical assistance in the building of incubation apparatus. This work was supported in part by a grant from Ministero Pubblica Istruzione, Rome.

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Received 23 June 1989