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Biosynthetic Pathway and Origin of the Chlorinated Methyl Group in Barbamide and Dechlorobarbamide, Metabolites from the Marine Cyanobacterium *Lyngbya majuscula*

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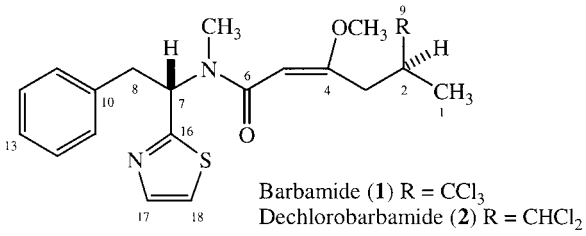
Abstract—Structural and biosynthetic studies have been conducted on the barbamide class of molluscicidal agent. Dechlorobarbamide was isolated from a Curaçao collection of the marine cyanobacterium *Lyngbya majuscula* and its structure determined through spectroscopic analysis and comparisons with barbamide. The absolute stereochemistry of the dolaphenine moiety of barbamide was determined to be *S*, defining the absolute configuration of barbamide as 2*S*,7*S*. Stable isotope feeding experiments conducted with cultured *L. majuscula* have provided clear evidence that barbamide biosynthesis involves chlorination of the unactivated *pro-R* methyl group of leucine. Experiments with L-[$^{2}H_{10}$]leucine demonstrated that chlorination of the pro-*R* methyl occurs without detectable activation via the leucine-catabolic pathway. Moreover, an extremely high level of incorporation of fed [2-^{13}C]-5,5,5-trichloroleucine into barbamide indicates that leucine is the probable substrate for the chlorination reaction. Incorporations of [1,2-$^{13}C_2$]acetate and [1-^{13}C, 1-^{18}O]acetate confirmed the origins of C-5 and C-6 whereas incorporation of L-[3-^{13}C]phenylalanine supported the hypothesis that the phenyl group and its three carbon side-chain in barbamide (C-7, C-8 and C-10–C-16) arise from phenylalanine. The thiazole ring (C-17–C-18) of **1** was shown to likely arise from cysteine through a [2-^{13}C, ^{15}N]glycine feeding experiment. Detection of intact ^{13}C–^{15}N bond was observed by application of a new GHNMBC NMR experiment. Results from this latter feeding experiment also indicated that the N–CH_3 and O–CH_3 groups of **1** originate from the C_1 pool; this was supported by enrichment in these methyl groups when cultures were provided with L-[methyl-^{13}C]methionine. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Marine organisms are prolific sources of halogenated secondary metabolites.[1] A majority of these halogen atoms are incorporated into positions which are suggestive of their biochemical reaction as electrophilic species. Haloperoxidase enzymes responsible for the formation of the X^{+}-halogenating species have been found in many classes of marine organisms and their study has been an area of intense interest.[2] In contrast, a number of sponge-cyanobacterial and cyanobacterial metabolites possess halogenated functional groups wherein the electronic nature of the halogenating species is uncertain.[1] Such an example is the unusual trichloromethyl group of barbamide (**1**), a molluscicidal metabolite our group isolated from the marine cyanobacterium *Lyngbya majuscula.*[3]



Barbamide is an intriguing natural product for several reasons. First, it possesses the rare trichloromethyl group, a feature which has previously only been found in a series of sponge-derived metabolites, such as dysidin (**4**)[4] and diketopiperazine derivative **5**.[5] However, it should be noted that these latter chlorinated metabolites have recently been localized to the sponge-associated cyanobacterium *Oscillatoria spongeliae*.[6] Second, barbamide contains a dolaphenine moiety, which is a structural feature found in several biologically active natural products such as dolastatin 10[7] and symplostatin 1.[8] Last, the discovery of these metabolites (barbamide and dechlorobarbamide) provides clarification of the metabolic origin of related compounds isolated

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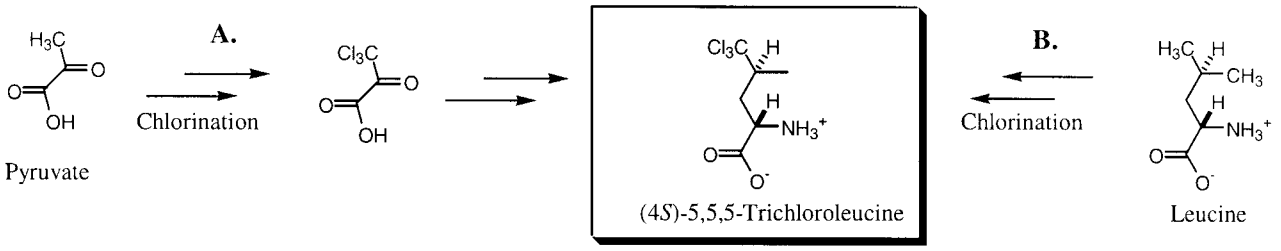
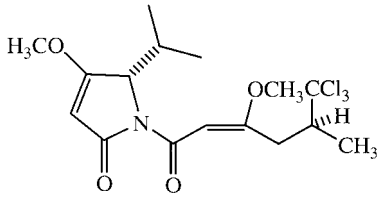


Figure 1. Biosynthetic hypotheses for the formation of barbamide: pathway A, chlorination predicted to occur during biosynthesis of leucine from pyruvate; pathway B, chlorination is believed to occur by novel mechanisms acting directly on leucine.

from sponge-cyanobacteria assemblages. Because barbamide is produced in laboratory cultures of *L. majuscula* originally collected off the coast of Curaçao (ca. 2.4% of the extract), we have been able to experimentally determine the biosynthetic precursors of barbamide using stable-isotope labeling methods.[9,10] We have found that the trichloromethyl group of barbamide derives from the *pro-R* methyl group of leucine,[11] and that this chlorination occurs without detectable activation of the methyl group to facilitate a potential nucleophilic or electrophilic process.[9] Hence, we have proposed that chlorination of this leucine methyl group occurs through novel biochemical processes, possibly involving radical chemistry.[9]



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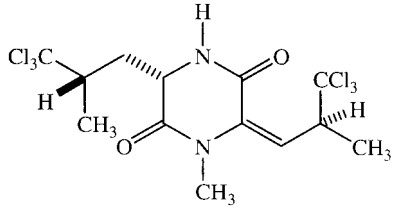

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In this paper, we report the isolation and structure determination of a new barbamide derivative, dechlorobarbamide (**2**), as well as the determination of the absolute stereochemistry at C-7 of barbamide (**1**). Moreover, we summarize our previously communicated stable isotope feeding experiments,[9,10] and describe several new ones which define the nature of the biosynthetic subunits composing these metabolites and which confirm that leucine is the substrate for the metabolic chlorination reaction. Additionally, the dichloromethyl group of dechlorobarbamide provides further insights into the unique halogenation process utilized in the biosynthesis of these metabolites.

We envision two possibilities in the biosynthesis of barbamide, both of which predict 5,5,5-trichloroleucine as the metabolic precursor to C-1–C-4 and C-9 of barbamide, but which differ in the timing and biochemical mechanism of the chlorination reaction (Fig. 1). In the first, we propose that chlorination occurs during the biosynthesis of leucine, perhaps at the pyruvate stage, during which point this methyl group is activated to electrophillic mechanisms of chlorination. In the second, we and others propose that chlorination occurs via a novel mechanism on the unactivated methyl group of intact leucine.[9,12] In either case, the intermediacy of 5,5,5-trichloroleucine (**3**) is envisioned; additional support for the existence of **3** is provided by the isolation of diketopiperazine derivatives, such as **5**, from the marine sponge-cyanobacterial assemblage *Dysidea herbacea/Oscillatoria spongeliae*.[5,13] Transamination and decarboxylation of **3** could give rise to **6**, which could then be ketide extended by malonyl CoA to provide intermediate **7** (Fig. 2). It is reasonable to predict that phenylalanine and cysteine serve as precursors to the phenyl and thiazole rings, respectively. In agreement with precedents from the biosynthesis of other nonribosomal polypeptides, *N*-methylation by *S*-adenosylmethionine (SAM) is predicted to occur prior to amide bond formation between the activated acyl group **7** and the phenylalanine residue.[14] Following amide bond formation with an activated cysteine residue, heterocyclization of the cysteine side chain with the carbonyl carbon of phenylalanine followed by oxidative decarboxylation is predicted to complete formation of the thiazole ring, although the timing of these reactions relative to other steps in the pathway is uncertain. Finally, at some point in the pathway, *O*-methylation of the enol hydroxy group at C-4 also occurs with involvement of SAM.

Results and Discussion

Isolation and structure of dechlorobarbamide (2)

The lipid extract from a 1996 Curaçao collection of *L. majuscula* was subjected to silica gel vacuum liquid chromatography (100% hexanes to 100% ethyl acetate, v/v). A relatively non-polar fraction (50% ethyl acetate/hexanes) was further fractionated using ODS vacuum liquid chromatography. A final purification utilizing ODS-HPLC yielded barbamide (**1**, ca. 2.4% of ext.) and dechlorobarbamide (**2**, ca. 0.1% of ext.).

Comparison of the ^{1}H and ^{13}C NMR data for dechlorobarbamide (**2**) with those of barbamide (**1**) clearly indicated

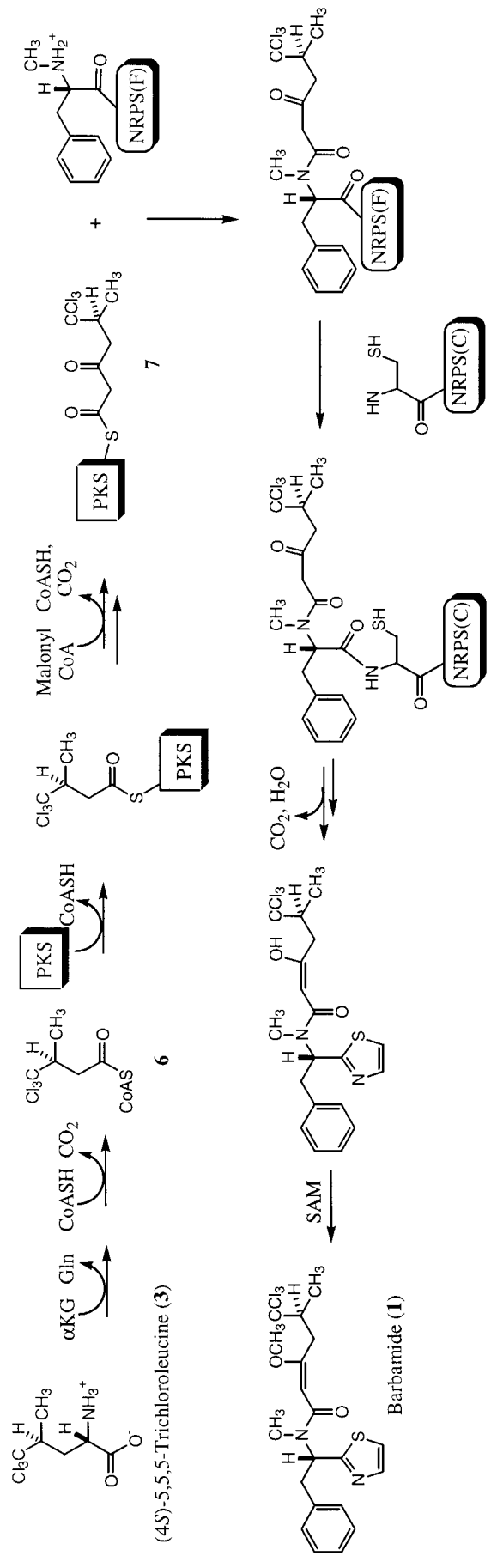

Figure 2. Proposed biogenesis of barbamide (**1**) from 5,5,5-trichloroleucine (**3**), acetate, *S*-adenosylmethionine, cysteine, and L-phenylalanine (PKS=polyketide synthase; NRPS(F)=non-ribosomal peptide synthetase for phenylalanine; NRPS(C)=non-ribosomal peptide synthetase for cysteine).

Table 1. ^{1}H NMR (600 MHz, DMSO) and ^{13}C NMR (150 MHz, DMSO) data for the major conformer of dechlorobarbamide (**2**) (the major conformer was shown to be of *Z* geometry by observation of NOE between H-5 and the NCH_3 resonance)

Position	^{1}H δ (mult, *J* in Hz)	^{13}C δ (mult[a])	HMBC correlations
1	0.93 (d, 6.6)	13.64 (q)	33.58, 41.58, 79.64
2	2.38 (m)	41.58 (d)	13.64, 33.58
3a	2.81 (dd, 12.1, 8.7)	33.58 (t)	13.64, 41.58, 79.64, 167.83,
3b	2.54 (dd, 12.1, 4.2)		13.64, 41.58, 79.64, 167.83
4		167.83 (s)	
5	5.30 (s)	93.15 (d)	33.58, 167.83
6		167.17 (s)	
7	6.31 (obscured)	54.04 (s)	35.72, 167.17, 169.52
8a	3.51 (dd, 14.8, 9.2)		54.04, 129.02, 137.76
8b	3.25 (dd, 14.8, 10.5)	35.72 (t)	54.04, 129.02, 137.76, 169.52
9	6.29 (obscured)	79.64 (d)	13.64, 33.58
10		137.76 (s)	
11	7.35 (br. d)	129.02 (d)	128.07, 126.19
12	7.27 (m)	128.07 (d)	126.19, 128.69, 137.76
13	7.19 (br. d)	126.19 (d)	128.69
14	7.27 (m)	128.69 (d)	126.19, 128.69,137.76
15	7.35 (br. d)	129.02 (d)	126.19, 128.69
16		169.52 (s)	
17	7.79 (d, 3.2)	141.85 (d)	
18	7.70 (d, 3.2)	120.55 (d)	141.85
NCH_3	2.86 (s)	30.64 (q)	54.04, 167.17
OCH_3	3.57 (s)	55.23 (q)	167.83

[a] Multiplicity was determined using the DEPT 135 pulse sequence.

that the two metabolites were closely related. However, HR-FABMS of **2** gave an $[M+H]^+$ at 427.1014 analyzing for $C_{20}H_{25}Cl_2N_2SO_2$, indicating that **2** contained one less chlorine atom than barbamide (**1**). Examination of ^{13}C NMR data for compounds **1** and **2** revealed that their only significant difference was the assigned chemical shift at C-9 (δ 105.6 for **1** and δ 79.64 for **2**). Moreover, an HSQC correlation showed that this new methine carbon in compound **2** had an associated proton at δ 6.29. ^{1}H–^{1}H COSY showed that this proton was adjacent to the H-2 methine (δ 2.38). Hence, it was clear that dechlorobarbamide (**2**) contained a dichloromethyl moiety at C-9 in contrast to the trichloromethyl group found at this position in barbamide (**1**). The remaining structural features of **2** were confirmed as being identical to those found in **1** by ^{1}H–^{1}H COSY,[15] HSQC,[16] and HMBC[17] data (Table 1). The $\Delta^{4,5}$ double bond geometry of **2** was established as *E* by observation of strong NOE between the $-OCH_3$ resonance (δ3.57) and H-5 (δ 5.30) upon selective irradiation of the latter signal using the DPFGSE 1D NOE pulse sequence.[18] At 50 ppm, dechlorobarbamide (**2**) was inactive in the *Biomphaleria glabrata* molluscicidal assay.

Determination of the C-2 and C-7 stereochemistry in barbamide (1)

The stereochemistry of C-2 in barbamide (**1**) was determined through feeding (2*S*,4*R*)-[5-^{13}C]leucine and (2*S*,4*S*)-[5-^{13}C]leucine (see below).[9] Selective incorporation of (2*S*,4*R*)-[5-^{13}C]leucine into C-9 of barbamide indicates that the stereochemistry of C-2 is *S*. In order to determine the absolute stereochemistry at C-7 of barbamide (**1**), *N*-methylphenylalanine was liberated through ozonolysis[19] and acid hydrolysis for subsequent derivatization and Marfey's analysis. Of interest, the acid hydrolysis (2 mL, 6 N HCl) was performed in 1 min in an ordinary microwave oven (550 W). This fast and simple procedure should find wide application in the hydrolysis of amides and esters for microanalysis procedures.[20] The released *N*-methylphenylalanine was derivatized with *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine (FDAA) for Marfey's analysis,[21] and determined to be *S* by comparison of retention times and co-injections with derivatized (*S*-), (*R*-), and (*S*,*R*)-*N*-methylphenylalanine standards. The stereochemistry of dechlorobarbamide (**2**) is proposed to be the same as that in barbamide (**1**-2*S*,7*S*) by virtue of (a) its co-occurrence in this cyanobacterium and likely similar biogenesis, and (b) its comparable optical rotation {lit. for **1** $[\alpha]_D^{25}=-89°$ (MeOH, *c* 1.3),[3] re-measurement of **1** $[\alpha]_D^{25}=-82°$ (MeOH, *c* 0.046); for **2** $[[\alpha]_D^{25}=-67°$ (MeOH, *c* 0.046)}.

Isolation of barbamide (1) for biosynthetic studies

The marine cyanobacterium *L. majuscula* was cultured in our laboratory as described previously.[22] For the precursor incorporation experiments, 50–75 mL of wet-packed cells of *L. majuscula* (strain 19L) were inoculated into fresh medium (SWBG11).[22] After 3 days of acclimation, isotope-labeled precursors were administered to the cultured cyanobacterium, incubated for an additional 6–7 days and then harvested. The crude organic extract was subjected to normal phase vacuum liquid chromatography (NP-VLC), C_{18} solid phase extraction cartridge (SPE), and ODS-HPLC (C_{18}, 80% MeOH/H_2O), respectively, to provide the variously labeled barbamides (**1**).

^{2}H- and ^{13}C-Leucine feeding experiments

If chlorination occurs during the biosynthesis of leucine (hypothesis A, Fig. 1), then no incorporation of L-[2-^{13}C]leucine into barbamide should occur. On the other hand, if chlorination occurs on leucine or a leucine-derived product, ^{13}C enrichment in barbamide should be observed upon feeding L-[2-^{13}C]leucine (hypothesis B).

When normalized to unlabeled carbons, a 450% increase in signal integral at δ 166.80 (C-4) was observed from providing *L. majuscula* cultures L-[2-^{13}C]leucine, clearly indicating that leucine or a catabolite of leucine is the substrate for metabolic chlorination.[9] It should be noted that this carbon resonance was originally misassigned to the amide carbonyl (C-6).[3] However, re-examination of the HMBC spectrum of **1** showed correlations from the O–CH_3 protons at δ 3.60 to δ 166.80 and from the N–CH_3 protons at δ 2.88 to δ 167.04, thus leading to a reversal in the original assignments.

Having established that C-2 to C-6 of leucine contributed to C-1–C-4 plus C-9 of **1**, it remained in question whether the carbonyl carbon of leucine contributed to C-5 of **1** or if it was lost during the biosynthetic process. To probe this and to be able to draw firm conclusions from a negative incorporation result, similar amounts of both L-[1-^{13}C]leucine and L-[2-^{13}C]leucine were simultaneously provided to cultures of the cyanobacterium. Analysis of the ^{13}C NMR spectrum of barbamide produced under these conditions showed the expected enhancement at C-4 (230% when normalized to the unenriched signals; deriving from L-[2-^{13}C]leucine) whereas C-5 of **1** showed no enhancement (100%). Hence, C-1 of leucine is lost in the biosynthetic pathway leading to barbamide (**1**).

To examine the chirality of the chlorination of the prochiral methyl group of leucine (the C-2 chirality of **1**), synthetically prepared (2*S*,4*R*)-[5-^{13}C]leucine and (2*S*,4*S*)-[5-^{13}C]leucine were separately provided to cultures.[23–25] Routes for the synthesis of L-leucine selectively labeled with ^{13}C in either diastereotopic methyl group have been described recently.[26] For both (4*R*)- and (4*S*)-L-[5-^{13}C]leucine, the source of isotopic label was $^{13}CH_3I$ and the stereogenic center at C-2 was created with complete stereocontrol via a reductive amination of the analogous α-keto acid catalyzed by leucine dehydrogenase. In each case, the stereogenic center at C-4 was established using a chiral auxiliary; for the (4*R*)-diastereomer a valine derived oxazolidinone was used giving 85% *de* whereas for the (4*S*)-isomer, a camphor sultam led to 70% *de* at C-4.

Analyses of the ^{13}C NMR spectra of barbamide from these two experiments demonstrated that (2*S*,4*R*)-[5-^{13}C]leucine selectively enhanced the signal for the C-9 trichloromethyl group of **1** by 530% whereas (2*S*,4*S*)-[5-^{13}C]leucine selectively enhanced the signal for the C-1 methyl group by 610%. In this latter feeding experiment, the slight enrichment observed for the C-9 signal (270%) was likely due to the 70% *de* at C-4 in this synthetic leucine preparation. Results from the incorporation of the two chirally ^{13}C-labeled leucines into **1** demonstrated that: (1) the chlorination reaction occurs at the *pro-R* methyl group of leucine, and (2) the stereochemistry at C-2 in barbamide is *S*. Trichloromethyl moieties possessing the same stereochemistry at comparable centers have been observed in several 'sponge-cyanobacterial' metabolites, such as **4** and **5**.[5,27]

While the above experiments conclusively showed that chlorination occurs post-leucine biosynthesis, they did not distinguish between chlorination of leucine directly or of a leucine-derived catabolite. During the degradation of leucine, carboxylation of the C-4 methyl group occurs,[28] an intermediate which conceivably could be channeled through successive electrophillic additions by $C1^+$ and decarboxylation to yield 4,4,4-trichloroisovaleroyl CoA. To examine this possibility, L-[$^2H_{10}$]leucine was fed to cultures of *L. majuscula* and the resulting barbamide (**1**) was analyzed by ^{2}H NMR. This ^{2}H NMR spectrum showed two bands, one centered at δ 3.13 (for H-2 and/or H_2-3) and the other at δ 1.22 (for H_3-1) [the assignments of these signals were confirmed by 2D NMR analysis of **1** in toluene-d_8]. Integration of these two peaks gave a ratio for ($^2H_3$-1):(^{2}H-2+$^2H_2$-3) of 3.00:2.77, indicating that there was no loss of deuterium from C-3 or C-4 of leucine during its incorporation into barbamide. Therefore, the trichloromethyl group of barbamide (**1**) is not activated to electrophilic chlorine addition via the leucine catabolic pathway.

[2-^{13}C]-5,5,5-Trichloroleucine feeding experiment

To directly evaluate the possible intermediacy of L-5,5,5-trichloroleucine in the biosynthetic pathway of barbamide, synthetic [2-^{13}C]-5,5,5-trichloroleucine was prepared. Routes for the synthesis of trichloromethylbutanoic acid have been previously reported.[29] [1-^{13}C]Trichloromethylbutanoic acid was converted to [2-^{13}C]trichloroleucine via a Strecker reaction. A mixture of diastereomers was formed, the major isomer possessing the required (4*S*)-stereochemistry. This was fed to cultures of *L. majuscula*(2×1 *L*, 80 mg each), and after 10 days total, the cells were harvested and barbamide isolated. ^{13}C NMR analysis of this sample in toluene-d_8 showed specific and very high (ca. 30-fold over natural abundance) incorporation of ^{13}C into C-4 (Fig. 3).

Acetate feeding experiments

To determine if C-5 and C-6 of barbamide (**1**) originate from an intact acetate unit, a [1,2-$^{13}C_2$]acetate feeding experiment was conducted. The ^{13}C NMR spectrum of **1** produced under these conditions showed an additional doublet structure for the C-5 and C-6 signals ($^1J_{cc}$=71.8 Hz) compared to a natural abundance spectrum, indicating that C-5 and C-6 of **1** are derived from an intact acetate unit.

To determine the orientation of the acetate unit in barbamide, as well as the origin of the C-6 oxygen atom, a feeding experiment using [1-^{13}C,$^{18}O_2$]acetate was conducted. The ^{13}C NMR spectrum of **1** produced from this feeding experiment revealed an ^{18}O-shifted ^{13}C resonance (0.03 ppm upfield) for C-6 at δ 167.04 (38% of the ^{13}C at C-6).[30] The results from this experiment demonstrate that C-6 originates from C-1 of acetate and that the carboxyl oxygen atom is not lost during barbamide biosynthesis.

L-[3-^{13}C]Phenylalanine feeding experiment

To examine the biosynthetic origin of the phenyl moiety and adjacent carbon atoms of barbamide (C-7–C-8 and C-10–C-16), L-[3-^{13}C]phenylalanine was provided to *L. majuscula* cultures. Analysis of the ^{13}C NMR spectrum of **1** isolated from this feeding experiment showed a 290% enhancement

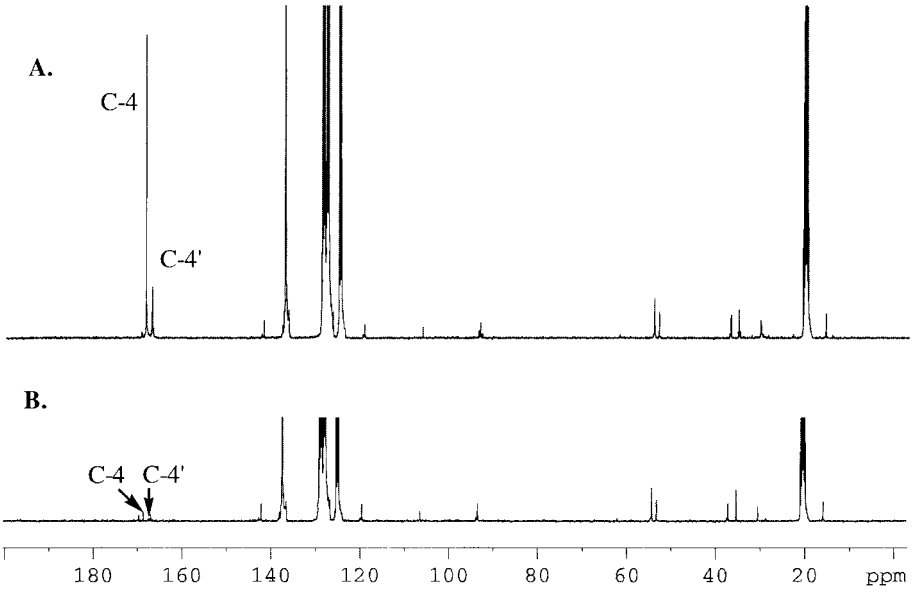

Figure 3. ^{13}C NMR spectra of barbamide (**1**) produced by *L. majuscula* culture 19 L (a) supplemented with [2-^{13}C]-5,5,5-trichloroleucine, and (b) natural abundance control [C-4 of barbamide is indicated (deriving from C-2 of [2-^{13}C]-5,5,5-trichloroleucine] (C-4=major amide isomer; C-4′=minor amide isomer). Both 100 MHz ^{13}C NMR spectra were acquired in toluene-d_8 with 24K data points and 3.0 Hz line broadening.

in signal integral for C-8, supporting our hypothesis that C-7–C-8 and C-10–C-16 of **1** arise from L-phenylalanine.

[2-^{13}C,^{15}N]Glycine feeding experiment

The biosynthetic proposal (hypothesis B) presented in Figs. 1 and 2 requires cysteine to be a direct precursor to the thiazole ring of **1** (except for C-16). However, a straightforward examination of this hypothesis is problematic, as isotopically labeled cysteine is very expensive, metabolically labile, and toxic to the organism when more than 20 mg/L of D,L-cysteine was supplied to the cultures.[10] These concerns prompted our exploration of other approaches to provide information on the origin of the thiazole moiety in **1**.

Because cysteine metabolically derives from serine, a feeding experiment utilizing this latter amino acid was attempted. Unfortunately, serine was also toxic to the organism when more than 25 mg/L of L-serine was supplied to the cultures. As an alternative to feeding cysteine or serine, we reasoned that glycine, the metabolic precursor of serine and cysteine, might be better tolerated by the cyanobacterium.[31] In the conversion of glycine into cysteine, the C-2 carbon and nitrogen atom remain intact. Hence, if an intact incorporation of the ^{13}C–^{15}N doubly labeled glycine fragment is observed in **1** from a [2-^{13}C,^{15}N]glycine feeding experiment, this would provide supporting evidence that the thiazole ring of **1** arises from cysteine.

In the analysis of **1** from the [2-^{13}C,^{15}N]glycine feeding experiment, we encountered a number of challenges in the detection of an intact ^{13}C–^{15}N bond incorporation. First, C-2 of glycine is used in the biosynthetic conversion of glycine to serine, thereby enriching C-3 of serine/cysteine. As a result, ^{13}C–^{13}C coupling between C-2 and C-3 of cysteine (C-17 and C-18 in **1**) greatly reduces the sensitivity of detection in the one-dimensional ^{13}C NMR analysis. Second, barbamide exists in two different N–CH_3 amide conformations in DMSO-d_6, which further complicated this analysis.

Improvement in the detection of ^{13}C–^{15}N intact incorporation in barbamide was obtained in two ways. First, the *N*-methyl amide rotamer population in **1** was minimized in toluene-d_8 at 305 K. Second, we developed a new gradient-selected ^{1}H–^{15}N heteronuclear multiple bond correlation (GHNMBC) method for observing the coupling within intact ^{13}C–^{15}N units based on a modification of the HMBC experiment proposed by Seto, Watanabe, and Furihata for the detection of long-range ^{13}C–^{13}C couplings.[32,33] As in the Seto et al. experiment, our method relies on an HMBC pulse sequence[17] without a low-pass ^{1}J filter with the indirectly observed nucleus being ^{15}N.[10] Protons attached to ^{13}C are split by the $^{1}J_{CH}$ coupling of 160–210 Hz for an sp^2 hybridized carbon. In the case of the incorporation of [2-^{13}C–^{15}N]glycine, the proton that resides on the ^{13}C-labeled glycine-derived carbon will appear as a doublet of ca. 180 Hz. A two-bond coupling from this proton to the thiazole ^{15}N will result in ca. 180 Hz doublet in the GHNMBC spectrum at the nitrogen chemical shift. In contrast, if C-17 of the thiazole is unlabeled (^{12}C), the correlation of H-17 to nitrogen will give rise to an apparent singlet at the H-17 chemical shift. Both of these long-range correlations were observed in the spectrum of **1** from the [2-^{13}C,^{15}N]glycine feeding experiment, confirming the intact ^{13}C–^{15}N bond incorporation.

L-[Methyl-^{13}C]-methionine feeding experiment

To establish that the N–CH_3 and O–CH_3 of **1** derive from the C_1 pool via SAM, L-[methyl-^{13}C]methionine was administered to cultures of *L. majuscula*. Analysis of the isolated barbamide showed only slight enrichment when

compared to natural abundance (140% for N–CH_3 and 150% for O–CH_3). Unfortunately, L-[*methyl*-^{13}C]-methionine was found to be toxic to the organism when administered at a higher level (>45 mg/L). However, the ^{13}C NMR spectrum of **1** from the [2-^{13}C,^{15}N]glycine feeding experiment revealed increases in signal intensities of the N–CH_3 (200%) and O–CH_3 (190%) resonances. This apparently results from the contribution of C-2 of glycine to the C_1 pool, a carbon source for both N–CH_3 and O–CH_3 groups.

Conclusion

The incorporation studies described above have provided insight into the biosynthetic origin of all carbon atoms and most heteroatoms in barbamide (**1**). As depicted in Fig. 4, incorporations of L-[3-^{13}C]phenylalanine and [1,2-$^{13}C_2$]acetate into **1** provided insights into the origins of the phenyl moiety and C-5–C-6 of the molecule, respectively. The intact incorporation of [2-^{13}C,^{15}N]glycine into the thiazole ring of **1**, detected using a new modified GHNMBC NMR experiment, strongly supports cysteine as a direct precursor to this part of barbamide. Analysis of the ^{13}C NMR spectrum of **1** from this latter feeding experiment, as well as from experiments wherein exogenous [methyl-^{13}C]methionine was provided, supplied convincing evidence that the N–CH_3 and *O*-CH_3 groups both derive from the C_1 pool.

The leucine feeding experiments have shown that C-1–C-4 plus C-9 of **1** originate from L-leucine. Results from incorporation of the two chirally labeled leucines established the 2*S* stereochemistry of barbamide and that the chlorination reaction occurs at the *pro-R* methyl of leucine. An incorporation experiment using L-[$^{2}H_{10}$]leucine showed that the leucine *pro-R* methyl group is not activated via the leucine catabolic pathway. The very high level of incorporation from exogenously applied [2-^{13}C]-5,5,5-trichloroleucine strongly suggests that trichloroleucine (**3**) is an intermediate in the pathway. Taken together, these results indicate that L-leucine is the substrate for chlorination in this strain of *L. majuscula*, and that this reaction occurs without activation of the *pro-R* methyl group to electrophilic or nucleophilic mechanisms of chlorine addition. Therefore, we suspect that novel chlorination reactions, perhaps involving radicals, are involved.[9,12] Moreover, our isolation of dechlorobarbamide (**2**) as a minor natural product of *L. majuscula* suggests that this chlorination process does not occur by oxidation to a carboxylic acid equivalent followed by multiple additions of chlorine, but rather, occurs stepwise to form dichloro- and then trichloromethyl functionalities. Leucine-derived natural products from cyanobacteria living in association with sponges show a similar spectrum of di- and trichlorinated methyl groups.[5]

Experimental

General

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker AM400 and DPX 400 instruments operating at 400.13 MHz for ^{1}H NMR, 61.45 MHz for ^{2}H NMR, and at 100.61 MHz for ^{13}C NMR. Spectroscopic characterization of synthetic ^{13}C-labeled precursors utilized JEOL 400 MHz (^{1}H) and 270 MHz (^{13}C) instruments. The GHNMBC experiment performed on **1**, and various NMR experiments in support of the structure elucidation of dechlorobarbamide, were acquired on a Bruker DRX600 spectrometer operating at a ^{1}H frequency of 600.08 MHz and a ^{13}C frequency of 150.01 MHz. Proton spectra were referenced to 2.50 and 2.09 ppm for DMSO-d_6 and toluene-d_8, respectively. Carbon spectra were referenced to 39.51 ppm for DMSO-d_6 and 20.4 ppm for toluene-d_8. High-performance liquid chromatography (HPLC) utilized Waters M6000A or Waters 515 pumps, a Rheodyne 7125 injector, and a Waters Lambda-Max 480 LC spectrophotometer or Photodiode Array Detector model 996. Merck aluminum-backed thin layer chromatography (TLC) sheets (silica gel 60 F254) were used for TLC. Vacuum liquid chromatography (VLC) was performed with Merck Silica Gel G for TLC or with Baker Bonded Phase-octadecyl (C18). All solvents were either distilled from glass or of HPLC quality. All stable isotope labeled substrates, other than sodium

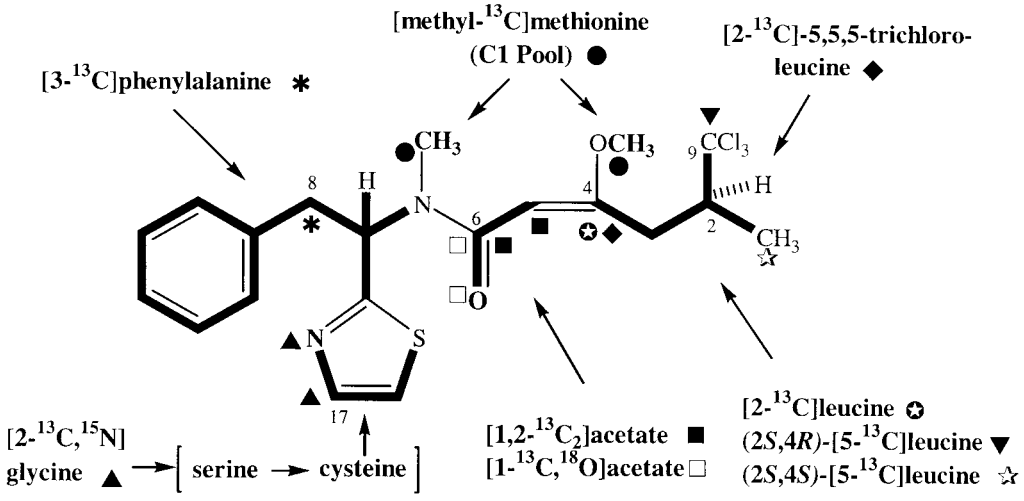

Figure 4. Summary of biosynthetic precursors of barbamide (**1**). Note, intermediates in parentheses (serine and cysteine) are hypotheses not yet demonstrated by direct precursor feeding-incorporation experiments (see text), and several carbon atoms (C-7 and C-10–C-16) hypothesized to derive from L-phenylalanine have not yet been confirmed through specific incorporation experiments.

[1-^{13}C,^{18}O]acetate which was a gift of S. J. Gould (Chemistry, OSU), were purchased from Cambridge Isotope, Inc.

Collection

The marine cyanobacterium *L. majuscula* (voucher specimen available from WHG as NSB-4 May 96-1) was collected from shallow water (0.1–1 m) on 4 May 1996, at Barbara Beach (Spanish Waters), Curaçao, Netherlands Antilles, and stored in 2-propanol at reduced temperature until workup.

Bioassay for molluscicidal activity

Evaluation of the molluscicidal activity of dechlorobarbamide (**2**) was performed as previously detailed using the test organism *Biomphalaria glabrata*.[3] Variable amounts of dechlorobarbamide (**2**) were dissolved in 20 μL of EtOH and added to 20 mL distilled H_2O. The snails (2 snails/assay vessel) were observed after 24 h and considered dead when no heartbeat could be detected upon microscopic investigation.

Extraction and isolation of dechlorobarbamide (2). A total of 83.2 g (dry wt) of the alga was extracted with CH_2Cl_2/MeOH (2:1) twice to give 2.29 g of crude extract. The extract was fractionated using vacuum liquid chromatography (VLC, 9.5 cm×4 cm) on TLC grade Si gel with a stepwise gradient of hexanes/EtOAc. Eluted material was collected, visualized by TLC, and similar fractions recombined. A fraction eluting with 50% EtOAc/hexanes was further fractionated by ODS VLC (3 cm×5 cm) using a MeOH/H_2O gradient (50% MeOH–100%MeOH). Final purification was achieved by ODS-HPLC (Phenomenex 250 mm×10 mm Sphereclone 5 μ, UV detection at 254 nm) using MeOH/H_2O (4:1) as eluent to give pure dechlorobarbamide (**2**, ca. 1.9 mg, 0.1% of extract) as an oil.

Dechlorobarbamide (2). Dechlorobarbamide (**2**) was isolated as a pale yellow oil showing the following: UV (MeOH) λ_{max} 238 nm (ϵ=16,000); $[\alpha]_D^{25}=-67°$ (MeOH, *c* 0.046); IR ν_{max} (film) 2927, 1643, 1603, 1453, 1441, 1244, 1167, 1114, 742 cm^{-1}; FABMS (3-NBA/2%TFA) obs. $[M+H]^+$ cluster at *m/z* 427/429/431 (100:67:17), 209/211/213 (100:67:17); HRFABMS (3-NBA/2% TFA) 427.1014 (−0.6 mmu dev. for $C_{20}H_{25}Cl_2N_2O_2S$); for ^{1}H and ^{13}C NMR see Table 1.

Ozonolysis and acid hydrolysis of barbamide

A slow stream of O_3 was bubbled into a 15 mL CH_2Cl_2 solution of barbamide (**1**, 0.73 mM) which was then sealed in a reaction flask for approximately 8 min. The solution was then dried under a stream of argon and subjected to acid hydrolysis. Hydrolysis of the barbamide ozonide (ca. 2.5 mg) was carried out in 2 mL of 6N constant boiling HCl under argon in a threaded Pyrex heavy wall tube sealed with a Teflon screw cap. The reaction vessel was then placed in a microwave oven (high power setting, 550 W) for 1 min.[20] The reaction mixture was dried under a stream of argon, and derivatized with Marfey's reagent.

Amino acid analysis using Marfey's reagent

To a vial containing 50 μL of a 50 mM solution of pure amino acid standard in H_2O was added 100 μL of a 36 mM solution of *N*-α-(2,4-dinitro-5-fluorophenyl)-L-alanine (FDAA) in $(CH_3)_2CO$ followed by 20 μL of 1 M $NaHCO_3$. The reaction mixture was stirred at room temperature for 1 h, at which time 10 μL of 2N HCl was added and let stand for several minutes. The barbamide hydrolysate was derivatized by the addition of 100 μL of H_2O, followed by 500 μL of a 36 mM solution FDAA in $(CH_3)_2CO$ followed by 100 μL of 1 M $NaHCO_3$. The reaction mixture was stirred at room temperature for 1 h, at which time 50 μL of 2N HCl was added and let stand for several minutes. The dry reaction mixture was dissolved in 500 μL of MeOH and analyzed by ODS-HPLC (Phenomenex 250 mm×10 mm Sphereclone 5 μ, UV detection at 340 nm) with a linear gradient elution [9:1 triethylammonium phosphate (50 mM, pH 3.0):CH_3CN to 1:1 triethylammonium phosphate (50 mM, pH 3.0):CH_3CN over 60 min]. The derivative of standard *N*-methyl-D-phenylalanine showed t_R=37.31 min, standard *N*-methyl-L-phenylalanine showed t_R=36.75 min, and *N*-methyl-L-phenylalanine obtained from barbamide (**1**) showed a t_R=36.74 min.

Analytical data for (2*S*,4*R*)-leucine

90% incorporation of ^{13}C; mp 280–285°C (decomp) [lit. unlabelled leucine 293–295°C];[34] $[\alpha]_D=+11.3$ (*c* 1.56 in 6 M HCl) [lit. unlabelled leucine +15.2 (*c* 1.58 in 6 M HCl];[35] ^{1}H NMR (400 MHz, D_2O) δ 3.71 (1H, m, H-2), 1.63–1.77 (3H, m, H_2-3 and H-4), 0.95 (3H, dd, *J*=125.1, 5.7 Hz, $^{13}CH_3$-5), 0.94 (3H, br t, *J*=5.5 Hz, H_3-6); ^{13}C NMR (67.9 MHz, D_2O) δ176.3 (C-1), 54.2 (d, *J*=3 Hz, C-2), 40.6 (C-3), 25.0 (d, *J*=35 Hz, C-4), 22.8 (enriched CH_3), 21.7 (slightly enriched CH_3); CI MS (relative abundance) obs. *m/z* 133.1057 (28%) [(MH^+), $C_5{}^{13}CH_{14}NO_2$ requires 133.1058] and 87 (100).

Analytical data for (2*S*,4*S*)-leucine

98% incorporation of ^{13}C; mp 286°C (decomp); $[\alpha]_D=-10.5$ (*c* 2.0 in water) [lit. unlabeled leucine −10.7 (*c* 2.0 in water];[36] ^{1}H NMR (400 MHz, D_2O) δ 3.71 (1H, m, H-2), 1.63–1.78 (3H, m, H_2-3 and H-4), 0.96 (3H, br t, *J*=5.5 Hz, H_3-6), 0.95 (3H, dd, *J*=125.0, 6.1 Hz, $^{13}CH_3$-5); ^{13}C NMR (67.9 MHz, D_2O) δ175.4 (C-1), 53.4 (C-2), 40.7 (C-3), 23.7 (d, *J*=35 Hz, C-4), 22.0 (slightly enriched CH_3), 20.8 (enriched CH_3).

Analytical data for [2-^{13}C]-5,5,5-trichloroleucine

^{1}H NMR (400 MHz, D_2O, both diastereomers) δ 3.8 (1H, dm, *J*=125 Hz, H-2), 2.98 (1H, m, minor diastereomer, H-4), 2.87 (1H, m, major diastereomer, H-4), 1.97–2.55 (2H, m, H_2-3), 1.40 (3H, d, *J*=6.5 Hz, H_3-6); ^{13}C NMR (67.9 MHz, D_2O, major diastereomer) δ 174.1 (C-1), 104.6 (C-5, $-CCl_3$), 53.2 (C-2, enriched 99%), 51.1 (C-4), 34.8 (C-3), 15.9 (C-6, $-CH_3$); CI MS (relative abundance) obs. *m/z* 189.9835 (100%) [(MH^+-CO_2H) $C_4{}^{13}CH_9N^{35}Cl_3$ requires 189.9834], 153 (70), 82 (35), 75 (96).

General culture conditions and isolation procedure

Approximately 3 g of *L. majuscula* strain 19 L were inoculated into a 2.8 L Fernbach flask containing 1 L of SWBG11 medium. The culture was grown at 28°C under uniform illumination (4.67 μmol photon $S^{-1} m^{-2}$), aerated, and acclimated for 3 days prior to addition of isotopically labeled precursors. Cultures of *L. majuscula* were harvested 10 days after inoculation, blotted dry, weighed, and repetitively extracted with 2:1 CH_2C1_2/MeOH. The filtered organic extracts were dried in vacuo, weighed, and applied to silica gel columns (1.5 cm I.D.×15 cm) in 5% EtOAc/hexanes, and eluted with a stepped gradient elution of 5% EtOAc to 100% EtOAc. Fractions containing barbamide (eluted with 50% EtOAc/hexanes) were further fractionated by RP-VLC using a stepped gradient elution from 60% MeOH/H_2O to 100% MeOH. The fractions eluting with 80% MeOH (barbamide-containing fraction) were subjected to a final purification by ODS-HPLC [Phenomenex Spherisorb ODS (2), 4:1 MeOH/H_2O, flow rate 3 mL/min, detection at 254 nm] to give pure barbamide (**1**, 3.86 mg/L). For each feeding experiment, barbamide identity and purity was established from TLC, PDA-HPLC, ^1H and ^{13}C NMR spectroscopy.

Calculation of the results of ^{13}C-labeled precursor feeding experiments on barbamide

The percentage ^{13}C incorporation into barbamide from exogenously supplied substrates was calculated as follows. The ^{13}C NMR spectral data and integrations for natural abundance and enriched samples were listed in a database for both *N*-methyl amide conformers of barbamide (**1**). Normalization factors for every carbon atom in barbamide were calculated by sequentually dividing the integral for each natural abundance carbon atom into the integration values of all carbon atoms in the natural abundance spectrum in turn (e.g. in this case, 20 columns of normalization factors were generated). Multiplication of the normalization factors for each resonance by the integrated value of the carbon atom being used for normalization in the ^{13}C enriched sample provided 'expected integration values' for each resonance in the enriched spectrum (20 columns of data). These were used to calculate the percentage ^{13}C enhancement of each signal by dividing the integrated area of each carbon peak in enriched barbamide by the above calculated 'expected integration values', and multiplying by 100 (20 columns of calculated percentages). Finally, the average percentage enhancement for each carbon signal was calculated by considering all values except those expected to show ^{13}C enrichment, and then rounding to the nearest 10%.

A. Feeding L-[1-^{13}C]Leucine and L-[2-^{13}C]Leucine. L-[1-^{13}C]Leucine (180 mg) and L-[2-^{13}C]leucine (120 mg), were provided to 3×1 L cultures on days 3, 6 and 8, and all three cultures were harvested on day 10 (12 g wet wt., 0.8 g dry wt., 446 mg lipid extract). A total of 9.0 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (DMSO-d_6) from this feeding experiment showed 230% increase in signal integral for C-4 whereas no enrichment was observed for C-5; C-1 80%, C-2 80%, C-3 100%, C-4 230%, C-5 100%, C-6 110%, C-7 110%, C-8 130%, C-9 70%, C-10 100%, C-11 100%, C-12 110%, C-13 100%, C-14 110%, C-15 100%, C-16 110%, C-17 80%, C-18 120%, *O*-methyl 140%, *N*-methyl 100%. Based on the ^{13}C integrals for this sample, a *t*-statistic for C-4 was found equal to 8.02, giving a >99.95% confidence that its ^{13}C content lies outside of the integral values for the natural abundance population.[37]

B. Feeding (2*S*,4*R*)-[5-^{13}C]leucine. Synthetic (2*S*,4*R*)-[5-^{13}C]Leucine (180 mg) was added to 3×1 L cultures on days 3, 6, and 8, and all three cultures were harvested on day 10 (20.5 g wet wt., 806 mg lipid extract). A total of 10.9 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (in DMSO-d_6) from this feeding experiment showed 530% increase in signal integral for C-9; C1 130%, C-2 90%, C-3 120%, C-4 150%, C-5 90%, C-6 100%, C-7 100%, C-8 90%, C-9 530%, C-10 100%, C-11 90%, C-12 90%, C-13 100%, C-14 90%, C-15 90%, C-16 110%, C-17 90%, C-18 100%, *O*-methyl 160%, *N*-methyl 80%. Based on the ^{13}C integrals for this sample, a *t*-statistic for C-9 was found equal to 3.41, giving a >99.75% confidence that its ^{13}C content lies outside of the integral values for the natural abundance population.[37]

C. Feeding (2*S*,4*S*)-[5-^{13}C]leucine. (2*S*,4*S*)-[5-^{13}C]Leucine (180 mg) was added to 3×1 L cultures on days 3, 6, and 8, and all three cultures were harvested on day 10 (16.3 g wet wt., 1.1 g dry wt., 257 mg lipid extract). A total of 12.3 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (in DMSO-d_6) from this feeding experiment showed 610% increase in signal integral for C-1; C1 610%, C-2 100%, C-3 90%, C-4 90%, C-5 100%, C-6 100%, C-7 110%, C-8 110%, C-9 270%, C-10 90%, C-11 100%, C-12 100%, C-13 110%, C-14 100%, C-15 100%, C-16 90%, C-17 100%, C-18 100%, *O*-methyl 90%, *N*-methyl 120%. Based on the ^{13}C integrals for this sample, *t*-statistics for C-1 and C-9 were found equal to 70.49 and 23.07, respectively, giving a >99.95% confidence that the ^{13}C content of both lie outside of the integral values for the natural abundance population.[37]

D. Feeding L-[$^2H_{10}$]leucine. L-[$^2H_{10}$]Leucine (160 mg) was added to 2×1 L cultures on days 3, 6 and 8, and both cultures were harvested on day 10 (5.6 g wet wt., 0.55 g dry wt., 157 mg lipid extract). A total of 6.70 mg of labeled **1** was isolated from the crude organic extract. The ^2H NMR spectrum of **1** (in toluene) showed two ^2H bands, one centered at δ 3.13 (for H-2 and H_2-3) and the other at δ 1.22 (for H_3-1). Integration of these two peaks showed the ratio of $^2H_3$-1 to ^2H-2+$^2H_2$-3 to be 3.00:2.77.

E. Feeding [2-^{13}C]-5,5,5-trichloroleucine HCl. [2-^{13}C]-5,5,5-trichloroleucine (160 mg total) was supplied to 2×1 L cultures on day 3, 6, and 8, and then both flasks were harvested on day 10 (7.9 g wet wt., 0.63 g dry wt., 67 mg lipid extract). A total of 4.6 mg of labeled **1** was isolated. The ^{13}C NMR spectrum (toluene-d_8] showed a 2970% enrichment in C-4 (in toluene-d_8); C-1 90%, C-2 90%, C-3 90%, C-4 2,970%, C-5 70%, C-6 obscured, C-7 obscured, C-8 90%, C-9 80%, C-10 – C-15 obscured, C-16 150%, C-17 80%, C-18 80%, *O*-methyl obscured, *N*-methyl 110%. Based on the ^{13}C integrals for this sample, a *t*-statistic for C-4 was found equal to 125.45, giving a >99.95%

confidence that its ^{13}C content lies outside of the integral values for the natural abundance population.[37]

F. Feeding sodium [1,2-$^{13}C_2$]acetate. Sodium [1,2-$^{13}C_2$]acetate (208 mg) was mixed with unlabelled sodium acetate (415 mg) and supplied to 3×1 L cultures on days 3, 6 and 8, and all three cultures were harvested on day 10 (15.2 g wet wt., 1.2 g dry wt., 521 mg lipid extract). A total of 15.2 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (DMSO-d_6) from this feeding experiment showed an additional pair of doublets for the C-5 ($^1J_{cc}$=71.8 Hz) and C-6 ($^1J_{cc}$=71.8 Hz) resonances.

G. Feeding sodium [1-^{13}C,^{18}O]acetate. Sodium [l-^{13}C,^{18}O]acetate (375 mg) was mixed with unlabeled acetate (622 mg) and supplied to 3×1 L cultures on days 3, 6 and 8, and all three cultures were harvested on day 10. A total of 10.4 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (in DMSO-d_6) revealed an isotopic shift ($\Delta\delta$=0.03 ppm) at the C-6 resonance (δ 167.04). Integration of this signal revealed that 38% of the ^{13}C at C-6 carried an ^{18}O atom.

H. Feeding L-[3-^{13}C]phenylalanine. L-[3-^{13}C]Phenylalanine (250 mg) was supplied to 3×1 L cultures on days 3 and 6, and all three cultures were harvested on day 9 (19.8 g wet wt., 0.9 g dry wt.). A total of 15.1 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (DMSO-d_6) from this feeding experiment showed a 290% increase in signal integral for C-8; C-1 120%, C-2 100%, C-3 140%, C-4 50%, C-5 130%, C-6 120%, C-7 130%, C-8 290%, C-9 60%, C-10 160%, C-11 90%, C-12 100%, C-13 120%, C-14 100%, C-15 90%, C-16 80%, C-17 150%, C-18 120%, *O*-methyl 100%, *N*-methyl 110%. Based on the ^{13}C integrals for this sample, a *t*-statistic for C-8 was found equal to 6.39, giving a >99.95% confidence that its ^{13}C content lies outside of the integral values for the natural abundance population.[37]

I. Feeding [2-^{13}C,^{15}N]glycine. [2-^{13}C,^{15}N]Glycine (225 mg) was supplied to 3×1 L cultures on days 3, 6 and 8, and all three cultures were harvested on day 10 (14.2 g wet wt., 236 mg lipid extract). A total of 18 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** showed 200 and 190% increase in signal intensities of *N*-CH_3 and *O*-CH_3, respectively. The incorporation of intact ^{13}C–^{15}N bond was detected by a modified GHNMBC experiment (in toluene-d_8).[10] The ^{13}C NMR spectrum of **1** (toluene-d_8) from this feeding experiment showed the following enrichments relative to natural abundance: C-1 90%, C-2 90%, C-3 90%, C-4 80%, C-5 90%, C-6 100%, C-7 90%, C-8 90%, C-9 90%, C-10 80%, C-11 100%, C-12 100%, C-13 90%, C-14 100%, C-15 100%, C-16 100%, C-17 150%, C-18 130%, *O*-methyl 190%, *N*-methyl 200%. Based on the ^{13}C integrals for this sample, *t*-statistics for C-17, C-18, and the N–CH_3 and O–CH_3 groups were found equal to 7.65, 6.03, 14.57, and 15.49, respectively, giving a >99.95% confidence that the ^{13}C content of all four lie outside of the integral values for the natural abundance population.[37]

J. Feeding L-[methyl-^{13}C]methionine. L-[methyl-^{13}C]-Methionine (60 mg) was supplied to 2×1 L cultures on days 3, 6, and 8, and both cultures were harvested on day 10 (1.75 g wet wt., 345 mg lipid extract). A total of 19.7 mg of labeled **1** was isolated from the crude organic extract. The ^{13}C NMR spectrum of **1** (in DMSO-d_6) showed 140 and 150% increase in signal intensities of the N–CH_3 and *O*–CH_3 signals, respectively; C-1 80%, C-2 90%, C-3 90%, C-4 100%, C-5 80%, C-6 100%, C-7 80%, C-8 100%, C-9 100%, C-10 100%, C-11 110%, C-12 110%, C-13 100%, C-14 110%, C-15 110%, C-16 110%, C-17 100%, C-18 90%, *O*-methyl 150%, *N*-methyl 140%. Based on the ^{13}C integrals for this sample, *t*-statistics for the O–CH_3 and N–CH_3 groups were found equal to 5.19 and 3.84, respectively, giving a >99.95% confidence for the O–CH_3 and a >99.90% confidence for the N–CH_3 that the ^{13}C content of both lie outside of the integral values for the natural abundance population.[37] A modestly higher amount of exogenously provided L-[methyl-^{13}C]methionine (90 mg to 2×1 L) was found to be toxic to the organism.

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References

1. Faulkner, D. J. *Nat. Prod Rep.* **2000**, *17*, 7–55 (and previous articles in this series).
2. Butler, A.; Walker, J. V. *Chem. Rev.* **1993**, *93*, 1937–1944.
3. Orjala, J. O.; Gerwick, W. H. *J. Nat. Prod.* **1996**, *59*, 427–430.
4. von Hofheinz, H.; Oberhansli, W. E. *Helv. Chem. Acta* **1977**, *60*, 660–669.
5. Dumdei, E. J.; Simpson, J. S.; Garson, M. J.; Byriel, K. A.; Kennard, C. H. L. *Aust. J. Chem.* **1997**, *50*, 139–144.
6. (a) Flowers, A. E.; Garson, M. J.; Webb, R. I.; Dumdei, E. J.; Charan, R. D. *Cell Tissue Res.* **1998**, *292*, 597–607. (b) Bewley, C. A.; Holland, N. D.; Faulkner, D. J. *Experientia* **1996**, *52*, 716–722.
7. Pettit, G. R.; Kamano, Y.; Herald, C. L.; Tuinman, A. A.; Boettner, F. E.; Kizu, H.; Schmidt, J. M.; Baczynskyj, L.; Tomer, K. B.; Botems, R. J. *J. Am. Chem. Soc.* **1987**, *109*, 6883–6895.
8. Harrigan, G. G.; Luesch, H.; Yoshida, W. Y.; Moore, R. E.; Nagle, D. G.; Paul, V. J.; Mooberry, S. L.; Corbett, T. H.; Valeriote, F. A. *J. Nat. Prod.* **1998**, *61*, 1075–1077.
9. Sitachitta, N.; Rossi, J.; Roberts, M. A.; Gerwick, W. H.; Fletcher, M. D.; Willis, C. L. *J. Am. Chem. Soc.* **1998**, *120*, 7131–7132.

10. Williamson, R. T.; Sitachitta, N.; Gerwick, W. H. *Tetrahedron Lett.* **1999**, *40*, 5175–5178.
11. We erroneously assigned the *pro-S* and *pro-R* methyl groups in unlabeled leucine. Before chlorination, C-3 of leucine is the first priority substituent on the C-4 chiral center; hence, the methyl group which becomes chlorinated is in the *pro-R* position.
12. (a) Hartung, J. *Angew. Chem., Int. Ed.* **1999**, *38*, 1209–1211. (b) MacMillan, J. B.; Molinski T. F. *J. Nat. Prod.* **2000**, *63*, 155–157.
13. Fu, X.; Zeng, L. -M.; Su, J. -Y.; Pais, M. *J. Nat. Prod.* **1993**, *56*, 637–642.
14. Shaw-Reid, C. A.; Kelleher, N. L.; Losey, H. C.; Gehring, A. M.; Berg, C.; Walsh, C. T. *Chem. Biol.* **1999**, *6*, 385–400.
15. Hurd, R. E. *J. Magn. Reson.* **1990**, *87*, 422–428.
16. (a) Palmer, A. G., III; Cavanagh, J.; Write, P. E.; Rance, M. *J. Magn. Reson.* **1991**, *93*, 151–170. (b) Kay, L. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663–10665. (c) Schleucher, J.; Schwendinger, M.; Sattler, M.; Schmidt, P.; Schedletzky, O.; Glaser, S. J.; Sorenson, O. W.; Griesinger, C. *J. Biomol. NMR* **1994**, *4*, 301–306.
17. (a) Bax, A.; Summers, F. *J. Am. Chem. Soc.* **1986**, *108*, 2093–2094. (b) Wilker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. *Magn. Res. Chem.* **1993**, *31*, 287–292.
18. Scott, K.; Keeler, J.; Van, Q. V.; Shaka, A. J. *J. Magn. Reson.* **1997**, *125*, 302–324.
19. McDonald, L. A.; Ireland, C. M. *J. Nat. Prod.* **1992**, *55*, 376–379.
20. Williamson, R. T. Isolation and synthesis of bioactive marine galactolipids. M.S. Thesis, University of North Carolina at Wilmington, Wilmington, NC, 1996.
21. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591–596.
22. Rossi, J. V.; Roberts, M. A.; Yoo, H. D.; Gerwick, W. H. *J. Appl. Phycol.* **1997**, *9*, 195–204.
23. Kelly, N. M.; Reid, R. G.; Willis, C. L. *Tetrahedron Lett.* **1995**, *36*, 8315–8318.
24. Kelly, N. M.; Sutherland, A.; Willis, C. L. *Nat. Prod. Rep.* **1996**, 205–219.
25. In naming this isotope-labeled leucine, we erroneously assigned higher priority to the ^{13}C-containining methyl group than to the C-3 methylene group. However, re-evaluation of the priority rules [Hanson, K. *J. Am. Chem. Soc.* **1966**, *88*, 2731–2742] identifies C-3 as the priority group because atomic number is considered a higher priority than is atomic mass. Atomic mass is considered only if the groups are otherwise identical (in this case, the C-5 and C-6 methyl groups).
26. Fletcher, M. D.; Harding, J. R.; Hughes, R. A.; Kelly, N. M.; Schmalz, H.; Sutherland, A.; Willis, C. L. *J. Chem. Soc., Perkin Trans. 1* **2000**, 43–51.
27. (a) Kazlauskas, R.; Ligard, R. O.; Wells, R. J.; Vetter, W. *Tetrahedron Lett.* **1977**, 3183–3186. (b) Kazlauskas, R.; Murphy, P. T.; Wells, R. J. *Tetrahedron Lett.* **1978**, 4945–4948. (c) Fu, X.; Zeng, L.-M.; Su, J.-Y.; Pais, M. *J. Nat. Prod.* **1997**, *60*, 695–696. (d) Fu, X.; Ferriera, M. L.; Schmidtz, F. J.; Kelly-Borges, M. *J. Nat. Prod.* **1998**, *61*, 1226–1231. (e) Gebreyesus, T.; Yosief, T.; Carmely, S.; Kashman, Y. *Tetrahedron Lett.* **1988**, *29*, 3863–3864. (f) Hofheinz, W.; Oberhansli, W. E. *Helv. Chim. Acta* **1977**, *60*, 660–669. (g) Lee, G. M.; Molinski, T. F. *Tetrahedron Lett.* **1992**, *33*, 7671–7674.
28. Bender, D. A. *Amino Acid Metabolism*, Wiley: London, 1975.
29. (a) See for example: Williard, P. G.; Laszlo, S. E. *J. Org. Chem.* **1984**, *49*, 3489–3493. (b) Helmchen, G.; Wegner, G. *Tetrahedron Lett.* **1985**, *26*, 6047–6050. (c) Brantley, S. E.; Molinski, T. F. *Org. Lett.* **1999**, *13*, 2165–2167.
30. This is within the reported 0.03–0.55 ppm range for ^{18}O-labeled carbonyl compounds [Vederas, J. C. *Nat. Prod. Rep.* **1987**, 277–337].
31. Carmeli, S.; Moore, R. E.; Patterson, G. M. L.; Yoshida, W. Y. *Tetrahedron Lett.* **1993**, *34*, 5571–5574.
32. Seto, H.; Watanabe, H.; Furihata, K. *Tetrahedron Lett.* **1996**, *37*, 7979–7982.
33. Martin, G. E.; Crouch, R. S. *J. Heterocyclic Chem.* **1995**, *32*, 1665–1669.
34. Buckingham, J.; Donaghy, S. M.; Cadogan, J. I. G.; Raphael, R. A.; Rees, C. W. *Dictionary of Organic Compounds*, Chapman & Hall: New York, 1982.
35. Shimojima, Y.; Hayashi, H.; Ooka, T.; Shibuawa, M. *Tetrahedron* **1984**, *40*, 2519–2527.
36. Huffman, W. A.; Ingersoll, A. W. *J. Am. Chem. Soc.* **1951**, *73*, 3366–3369.
37. Ramsey, F. L.; Schafer, D. W. *The Statistical Sleuth: A Course in Methods of Data Analysis*, Duxbury Press: Belmont, CA, 1997 (pp 36–42).