Biosynthesis of the Marine Cyanobacterial Metabolite Barbamide. 1. Origin of the Trichloromethyl Group

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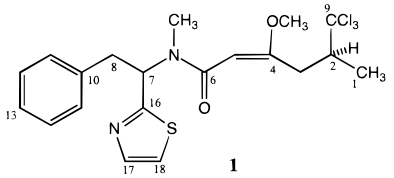
A remarkable structural feature of many marine-derived natural products is the covalent inclusion of chlorine and bromine atoms.[1] A majority of these halogen atoms are incorporated into positions which are suggestive of their reaction as X^+ species, although there are a few examples wherein passive incorporation of a X^- species appears rational.[2] Haloperoxidase enzymes responsible for the formation of the X^+-halogenating species have been isolated from several marine organisms and have been an area of intense interest.[3] In contrast, a number of sponge and cyanobacterial metabolites possess halogenated functional groups which make uncertain the electronic nature of the halogenating species.[1] One such example is barbamide (**1**), a molluscicidal metabolite we isolated from the marine cyanobacterium *Lyngbya majuscula*.[4]



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Discovery of barbamide is exceptional in several regards: (1) it clarifies the origin of related molecules from sponge−cyanobacterial−bacterial complexes[5] and, because *L. majuscula* is potentially culturable, (2) it provides an experimental system in which to examine these remarkable biochemical chlorination reactions.

To this end, we have brought the producing strain of *L. majuscula* from Curaçao into laboratory culture in Oregon, and in culture it retains its capacity to produce barbamide in good yield (ca. 2.4% of extractable lipids).[6] Herein we report experiments conducted with this cultured marine cyanobacterium which show that barbamide biosynthesis involves chlorination of the unactivated *pro-S* methyl group of leucine (or a leucine-derived intermediate), a result which leads to our speculation that novel biochemistry, possibly of a radical nature, may be involved in the chlorination mechanism.

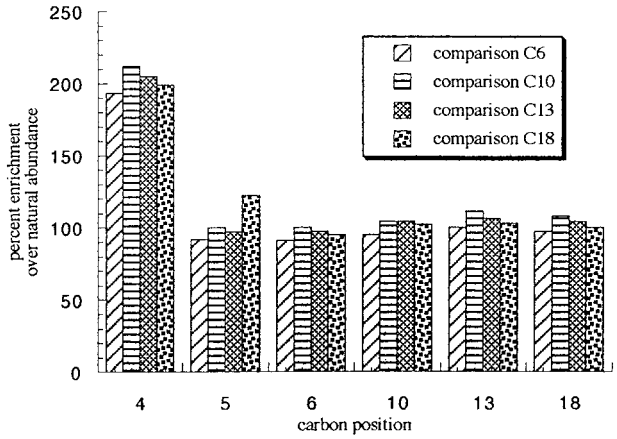

Figure 1. A panel of selected carbon atom intensities in barbamide produced during supplementation with similar quantities of [1-^{13}C]leucine and [2-^{13}C]leucine (see text) normalized to various carbon positions in the natural abundance spectrum.

Establishment that L-leucine contributed to a portion of the atoms in the lipid-like section of barbamide (C1−C6, C9) was initially shown by adding two portions (50 mg each) of L-[2-^{13}C]-leucine on days 3 and 6 to 5 × 1 L cultures. These cultures were grown 9 days and then harvested (see the Supporting Information).[6] Barbamide was isolated by a combination of VLC and C18 SepPak and characterized by TLC and ^{1}H NMR, and the degree and specificity of ^{13}C incorporation was evaluated by 100 MHz ^{13}C NMR. With L-[2-^{13}C]leucine as precursor, a 373% increase in signal intensity for C4 was observed (4.10% ^{13}C at C4) when normalized to several expectedly unlabeled positions of similar hybridization in barbamide.[7]

Having established that leucine contributed at least C2−C6 to form atoms C1−C4 and C9 of barbamide, it remained in question whether C5 in barbamide derived from C1 of leucine or from another source. To probe this feature and be able to draw firm conclusions from even a negative incorporation result, a similar amount of both L-[1-^{13}C]leucine (60 mg) and L-[2-^{13}C]leucine (40 mg) were provided on days 3, 6, and 8 to 3 × 1 L cultures and harvested on day 10. The isolated barbamide produced under these conditions was examined by ^{13}C NMR in comparison with a natural abundance sample (Figure 1). Again, C4 showed an expected enhancement when normalized to four unenriched signals (203%) whereas C5 showed no enhancement (102%), clearly indicating that C1 of leucine is lost in the biosynthetic process.

To examine the chirality of chlorine addition to the prochiral methyl groups of leucine and, hence, the C2 chirality of barbamide, chirally ^{13}C-labeled leucines, 4(*S*)-L-[5-^{13}C]leucine and 4(*R*)-L-[5-^{13}C]leucine, were synthetically prepared[8] and separately provided to cultures. Cultures (3 × 1 L for each labeled leucine) were provided 60 mg of the chirally ^{13}C-labeled leucines on days 3, 6, and 8 (180 mg total for each leucine), incubated a total of 10 days, and harvested, and the barbamide was isolated. Figure 2 displays the ^{13}C NMR spectra obtained from these two experiments as well as a natural abundance spectrum for barb-

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(7) Assignment of ^{1}H and ^{13}C NMR shifts for barbamide followed published data with one correction. HMQC data for **1** indicated a reversal of assignments for C4 and C6;[4] by this new analysis, C4 of the major tertiary amide geometrical isomer is at δ166.8 and C6 is at δ167.0.

(8) The synthesis of 2(*S*), 4(*S*)-[5-^{13}C]leucine (with total stereocontrol at C-2 and 85% de at C-4) was an adaptation of the chemoenzymatic approach (Kelly, N. M.; Reid, R. G.; Willis, C. L.; Winton, P. I. *Tetrahedron Lett.* **1995**, *36*, 8315). 2(*S*), 4(*R*)-[5-^{13}C]Leucine (with total stereocontrol at C-2 and 70% de at C-4) was also prepared by a chemoenzymatic approach but the stereogenic center at C-4 was created via a conjugate addition reaction. Further details of these syntheses will be published elsewhere.

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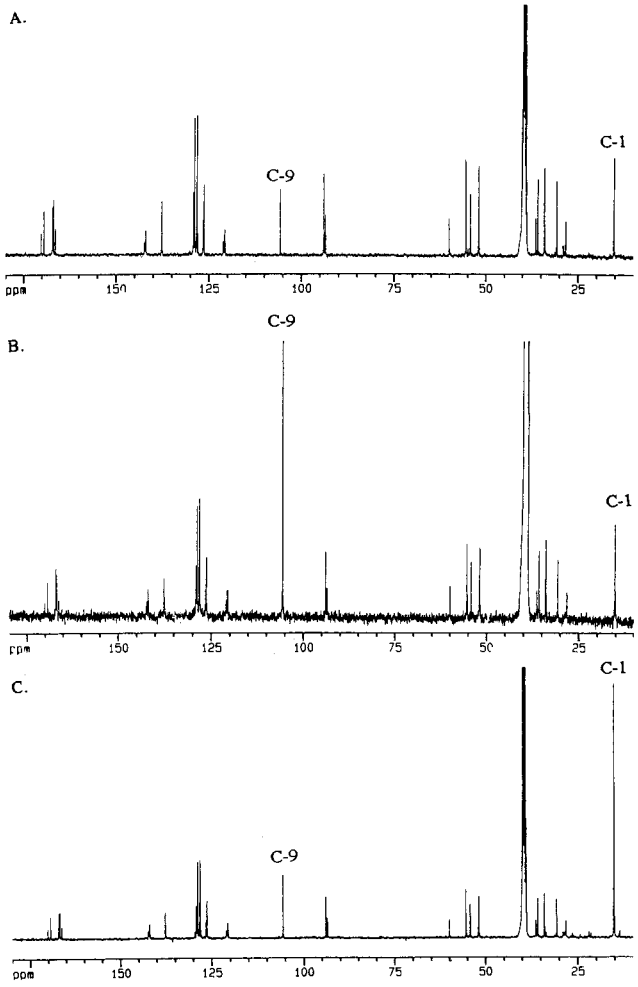

Figure 2. Comparison of the ^{13}C NMR of (a) natural abundance barbamide, (b) barbamide produced during supplementation with 4(*S*)-L-[5-^{13}C]leucine, and (c) barbamide produced during supplementation with 4(*R*)-L-[5-^{13}C]leucine.

amide which demonstrate that 4(*S*)-L-[5-^{13}C]leucine selectively enhanced the signal for the C9 trichloromethyl group of barbamide 790% (8.7% ^{13}C at C9) whereas 4(*R*)-L-[5-^{13}C]leucine selectively enhanced the signal for the C1 methyl group 655% (7.2% ^{13}C at C1).[9] Hence, the *pro-S* methyl group of leucine is chlorinated resulting in 2(*S*) stereochemistry in barbamide.

Next, we examined possible oxidations or desaturations of the leucine-derived fragment (C1−C4 plus C9) during barbamide biosynthesis. Activation of the C9 methyl group of barbamide to chlorination might arise through the leucine catabolic pathway wherein a double bond between C2 and C3 of barbamide is conceivably followed by carboxylation of C9 (the *pro-S* methyl group). L-[UL-$^{2}H_{10}$]leucine (120 mg total) was added to 2 L of *L. majuscula* culture on days 3, 6, and 8, with harvest on day 10. The barbamide isolated from this experiment showed greater than 2% ^{2}H content by FABMS. Analysis by ^{2}H NMR showed two ^{2}H bands, one centered at δ3.13 (for H-2 and/or H_2-3) and the other at δ1.22 (for H_3-1) (Figure 3).[10] Integration of these two peaks showed the ratio of $^{2}H_3$-1 to ^{2}H-2 + $^{2}H_2$-3 to be 3.00:2.77, indicating that no losses of protons from C3 or C4 of leucine occurred during its incorporation into barbamide. Therefore, the

(9) The slight enrichment in C9 from the latter feeding experiment (256% increase in signal intensity; 2.8% ^{13}C at C9) is likely due to the 70% de at C4 in this synthetic leucine preparation.

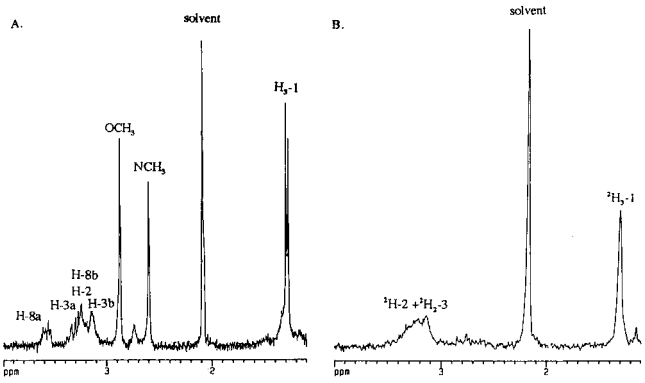

Figure 3. Comparison of selected regions of a) the 400 MHz ^{1}H NMR (toluene-d_8) with (b) the ^{2}H NMR (toluene-d_8) of 6.7 mg of barbamide (**1**) produced during supplementation of cultures with [*UL*-^{2}H]leucine.

C9 methyl group of barbamide is apparently not activated to electrophilic chlorine additions via a leucine catabolic pathway (e.g., a C2−C3 double bond is not formed).

These results are notable for several reasons. First, successful biosynthetic experiments using stable isotope methods with marine organisms are rare, particularly so in marine cyanobacteria.[11] Of the many conceivable biosynthetic origins for the C1−C4 plus C9 portion of barbamide, this work has conclusively shown that it arises from L-leucine. Results from incorporation of the two chirally ^{13}C-labeled leucines unequivocally establishes the 2(*S*) stereochemistry in barbamide; chlorination occurs at the *pro-S* methyl group of leucine. Incorporation experiments using L-[UL-^{2}H]leucine showed that the leucine *pro-S* methyl group is not activated via the catabolic pathway because a double bond does not form between C2 and C3 of barbamide. Because chlorination occurs at a stage after leucine biosynthesis and with no detectable methyl group activation, novel mechanisms of chlorination, perhaps involving radicals, are implicated.

Chlorinated leucine moieties possessing the same stereochemistry at comparable centers have been observed as marine "sponge" metabolites;[5] by this work, there is strengthening of the proposal that these chlorinated "sponge" metabolites actually derive from cyanobacterial metabolism.[5b] On the basis of the structures of these "sponge" metabolites, it is likely that L-leucine itself is the substrate for these novel biochemical chlorinations.

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Supporting Information Available: Barbamide production and isolation protocols; natural abundance ^{13}C NMR spectra of **1** and of **1** produced in cultures supplemented with [1-^{13}C]leucine and [2-^{13}C]leucine; and panels of selected carbon atom intensities in **1** produced from 4(*S*)-L-[5-^{13}C]leucine or 4(*R*)-L-[5-^{13}C]leucine (4 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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(10) Spiking this barbamide sample with a known quantity of CH_2Cl_2 and re-recording the ^{2}H NMR spectrum showed by integration of the $C^{2}H_2Cl_2$ and $^{2}H_3$-1 signals that it was enriched 67 times over natural abundance (ca. 1% in ^{2}H content).

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