Pergamon

Tetrahedron Letters 40 (1999) 5175–5178

TETRAHEDRON LETTERS

Biosynthesis of the Marine Cyanobacterial Metabolite Barbamide. 2: Elucidation of the Origin of the Thiazole Ring by Application of a New GHNMBC Experiment

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Received 30 March 1999; accepted 21 May 1999

Abstract: A new NMR experiment is presented for the detection of intact ^{13}C-^{15}N units in biosynthetic studies. Its use is demonstrated through a feeding experiment utilizing [2-^{13}C,^{15}N] glycine which confirmed the origin of the thiazole ring in the marine cyanobacterial metabolite barbamide as originating from cysteine.

A Curaçao collection of the marine cyanobacterium *Lyngbya majuscula* has yielded several structurally-intriguing and biologically-active natural products, including the antitumor compound curacin A,[1] the exceedingly ichthyotoxic metabolite antillatoxin,[2] and the molluscicidal substance barbamide (**1**).[3] Barbamide contains several very interesting structural motifs that have stimulated our interest into its biosynthetic origins. For example, our examination of the origin of barbamide's trichloromethyl group provided insight into the nature of the halogenation process in this prokaryote.[4] Another structural feature of interest in barbamide is the thiazole ring which likely derives from condensation of the amino acids cysteine and phenylalanine. However, direct examination of this hypothesis is problematic as isotopically labeled cysteine is very expensive, is metabolically labile, and is likely toxic to the organism.[5] These concerns prompted us to explore other approaches to provide information on the origin of the thiazole moiety in barbamide (**1**, figure 1).

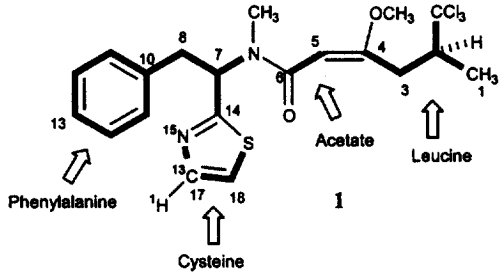

Figure 1. Structure of barbamide indicating origin of most biosynthetic units.

Because cysteine metabolically derives from serine, a feeding experiment utilizing this latter amino acid was attempted. Unfortunately, serine was toxic to the organism.[5] Conceived as an alternative to feeding cysteine or serine, we reasoned that glycine (e.g. [2-^{13}C, ^{15}N] glycine), the metabolic precursor of serine and cysteine, might be better tolerated by this organism (figure 2).[6] While conversion of glycine into cysteine involves loss of C-1 as CO_2, the C-2 carbon and nitrogen atom are incorporated intact into cysteine. Hence, observation of intact incorporation of the ^{13}C-^{15}N doubly-labeled glycine fragment would provide strong evidence for a cysteine origin to the thiazole ring of barbamide. Detection of intact ^{13}C-^{15}N bond incorporation has been used previously in studying the biosynthesis of alkaloids and other amino acid derived natural products.[6,7]

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PII: S0040-4039(99)01008-4

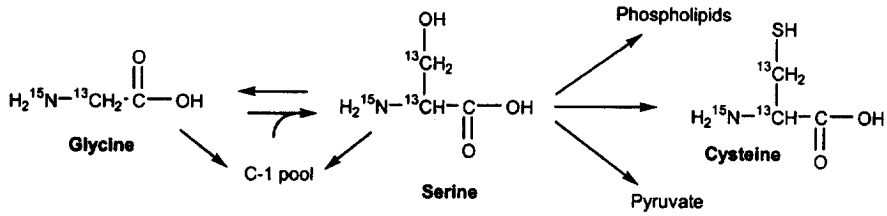

Figure 2. Metabolic relationship of glycine, serine, and cysteine.

However, there are a number of challenges involved in the detection of intact ^{13}C-^{15}N bonds. The most common method has been to directly observe one bond ^{13}C-^{15}N couplings in the 1D ^{15}N NMR spectrum.[7] This method is plagued by the low magnetogyric ratio, low natural abundance, long spin lattice relaxation, and negative NOE effect of ^{15}N. Alternatively, the one bond ^{13}C-^{15}N couplings have been analyzed in the 1D ^{13}C NMR spectrum.[6] This requires that enough biosynthetically labeled material be available to acquire sufficient S/N to confidently assign these relatively small satellite peaks. Additionally, because ^{13}C–^{15}N coupling constants are quite small (usually 2-10 Hz), any spurious peaks, such as those produced from long range inter-unit ^{13}C-^{13}C couplings (2-10 Hz) or from multiple conformations of the molecule under analysis, can make their detection difficult. For example, in the current case of barbamide (1) biosynthetically labeled from [2-^{13}C, ^{15}N] glycine, both of these problems were realized. First, C-2 of glycine contributes to the C-1 pool which is used in turn to convert glycine into serine and in doing so enriches C-3 of serine/cysteine (figure 2); second, barbamide exists in two different N-methyl amide conformations.[3] In summary, use of 1D ^{13}C NMR spectroscopy to detect heteronuclear couplings in biosynthetically labeled samples can be both time consuming and equivocal.

Our proposal for the origin of the thiazole ring of barbamide involves an N-methylated phe-cys dipeptide in which the cys sulfur condenses with the phe carbonyl, dehydrates, and decarboxylates to form the aromatic ring (figure 3). To this end, feeding experiments with [3-^{13}C] phe (83 mg, 3 x 1.0 L) showed an expected enhancement in C-8 of barbamide (219%). Next, [2-^{13}C, ^{15}N] glycine was administered on days 3, 6, and 8 to 1 L of barbamide-producing *L. majuscula* in culture (75 mg to each of 3 flasks), cultured for 10 days, the alga harvested and the barbamide isolated. If our hypothesis was correct, we expected intact incorporation of a ^{13}C-^{15}N unit between the C-17 and N-15 positions of barbamide (figures 2 and 3). Improvement in the detection of this intact incorporation was obtained in two ways. First, the N-methyl amide rotamer population in barbamide was modified from an unfavorable 3:1 ratio in DMSO-d_6 at 298 K to a more favorable 5:1 ratio in toluene-d_8 at 305 K. Second, we developed a new method for observing the coupling within intact ^{13}C-^{15}N units based on a modification of the HMBC experiment, a modification which should find broad applicability in assessing the incorporation of intact bonds from biosynthetic precursors to natural products.

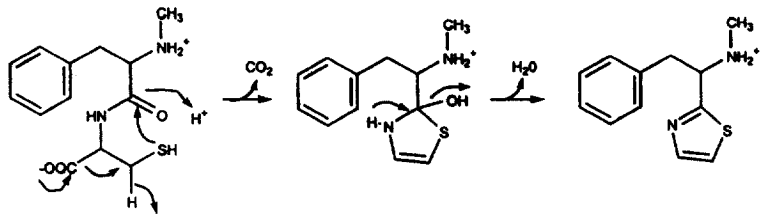

Figure 3. Proposed biosynthetic pathway of the "dolaphenine" portion of barbamide.

This new method of detecting ^{13}C-^{15}N couplings is based on a method proposed by Seto, Watanabe, and Furihata for the detection of long range ^{13}C-^{13}C couplings, a method they used to discriminate between incorporation patterns indicative of the mevalonate and non-mevalonate pathway.[8] As in Seto's experiment, our method relies on an HMBC pulse sequence with no low-pass ^1J filter. In our experiment, the indirectly observed nucleus is ^{15}N.[9] Protons attached to ^{13}C will be split by the $^1J_{CH}$ coupling of 160-210 Hz for an sp^2 hybridized carbon. In the case of the incorporation of ^{13}C-^{15}N doubly labeled precursors (formed *via* the route shown in figure 2), the proton that resides on the ^{13}C-labeled glycine-derived carbon will appear as a doublet of *ca.* 180 Hz. When this proton long-range couples to the thiazole ^{15}N, a *ca.* 180 Hz doublet will be observed 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 correlation pathways are observed in the spectrum presented in figure 4, confirming the incorporation of doubly ^{13}C-^{15}N-labeled glycine. Other protons long-range coupled to the two nitrogen resonances of barbamide (1) include H-18 to thiazole–N and H-7 to both thiazole-N and phe-N. Since C-18 derives from the C-1 pool, it serves as an internal standard to confirm that the C-17-^{15}N coupling does not originate from excessive random ^{13}C enrichment at that position. To improve sensitivity and in consideration of the large spectral dispersion of ^{15}N, no initial TANGO or BIRD building block is incorporated before the HMBC pulse sequence.

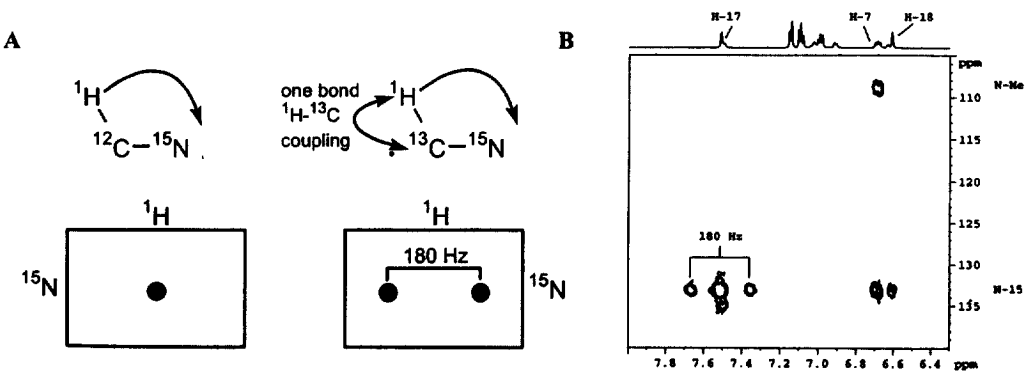

Figure 4. a) Schematic representation of the expected coupling patterns from the modified GHNMBC experiment. b) Expansion of the spectrum acquired for barbamide (1) biosynthetically labeled from [2-^{13}C, ^{15}N] glycine (see discussion in text).

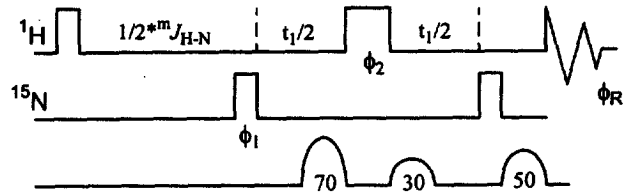

Figure 5. GHNMBC pulse sequence used to detect intact ^{13}C-^{15}N bond incorporation from [2-^{13}C, ^{15}N] glycine into barbamide (1) (ϕ_1 = 0, 2; ϕ_2 = 0, 0, 2, 2; ϕ_R = 0, 2, 0, 2, 2, 0, 2, 0).

As shown above in Figure 4, this NMR method provides an unambiguous result that does not require the interpretation of small ^{13}C-^{15}N coupling constants. In the case of barbamide (1), a coupling between C-17 and N-15 was detected, indicating intact bond incorporation consistent with a glycine > serine > cysteine origin (figure 2). Because the C-1 pool was substantially enriched in ^{13}C from the applied [2-^{13}C, ^{15}N] glycine, the side chain of serine and cysteine, which derives from the C-1 pool, was also substantially enriched. In turn, this led to an observed ^{13}C-enrichment at C-18 in barbamide (1). Overall, these results are consistent with our hypothesis that the thiazole ring derives from a decarboxylated cysteine residue that condenses with the C-1 carbonyl carbon of phenylalanine (figure 3).

The data for this GHNMBC experiment were acquired in a relatively short experiment time (*ca.* 1 hr), partly because of the isotopic labeling of ^{15}N provided by the feeding experiment, and partly because of the large magnetogyric ratio between ^{1}H and ^{15}N and the large spectral dispersion of ^{15}N. Additionally, the modified GHNMBC experiment is relatively facile to program and can subsequently be used in a straight-forward manner. This method should find broad applications in biosynthetic studies of alkaloids and other amino acid-derived natural products.

Acknowledgments: We gratefully acknowledge the assistance of the CARMABI research station, Curaçao, in making cyanobacterial collections, V. Hsu and the Dept. of Biochemistry and Biophysics, OSU for assistance with NMR, and G. Martin for critically reading the manuscript. This work was supported by the Sea Grant Program (NOAA) under grant R/BT-09.

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