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Registry No. 1-S^+·OTf^-, 113109-87-8; 1-A^+·OTf^-, 113214-87-2; 1-S^+·BF_4^-, 113215-02-4; 1-A^+·BF_4^-, 113299-11-9; 1-S-SPh, 113109-92-5; 1-A-SPh, 113215-04-6; 1-S-$SCOCH_3$, 113109-93-6; 1-A-$SCOCH_3$, 113215-07-9; 2-S^+·OTf^-, 113109-89-0; 2-A^+·OTf^-, 113214-89-4; 2-S^+·BF_4^-, 113215-03-5; 2-A^+·BF_4^-, 113299-12-0; 2-S-SPh, 113109-96-9; 2-A-SPh, 113215-06-8; 2-S-$SCOCH_3$, 113109-97-0; 2-A-$SCOCH_3$, 113215-08-0; 3-S^+·OTf^-, 113214-91-8; 3-A^+·OTf^-, 113214-93-0; 3-S-SPh, 113109-94-7; 3-A-SPh, 113299-13-1; 3-S-$SCOCH_3$, 113109-95-8; 3-A-$SCOCH_3$, 113215-05-7; 4-S^+·OTf^-, 113214-95-2; 4-A^+·OTf^-, 113214-97-4; 4-S-OCD_3, 113109-98-1; 4-A-OCD_3, 113215-00-2; 4-S-OCH_3, 113350-82-6; 4-A-OCH_3, 104832-41-9; 5-S^+·OTf^-, 113109-91-4; 5-A^+·OTf^-, 113214-99-6; 5-S-OCH_3, 113109-99-2; 5-A-OCH_3, 113215-01-3; NaSPh, 930-69-8; $KSCOCH_3$, 10387-40-3; $NaOCD_3$, 6552-73-4; $NaOCH_3$, 124-41-4.

Supplementary Material Available: IR, ^1H, ^{13}C NMR, and elemental analysis data for 1-S-SPh, 1-A-SPh, 1-S-$SCOCH_3$, 2-S-SPh, 2-A-SPh, 2-S-$SCOCH_3$, 2-A-$SCOCH_3$, 3-S-$SCOCH_3$, 3-A-$SCOCH_3$, 5-S-OCH_3, and 5-A-OCH_3; IR, ^1H NMR, and elemental analysis data for 1-A-$SCOCH_3$; ^1H NMR data for 4-A-OCH_3, 1, and 2; IR, ^1H, and ^{13}C NMR data for 3-S-SPh and 3-A-SPh; ^1H and ^{13}C NMR data for 4-S-OCH_3 and 5 (12 pages). Ordering information is given on any current masthead page.

Isonitrile Biosynthesis in the Cyanophyte *Hapalosiphon fontinalis*

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In 1957 Hagedorn and Tonjes reported the isolation of the first naturally occurring isonitrile, xanthocillin from the fungus *Penicillium notatum*.[1] Since that time isonitriles have been found in other fungi,[2] bacteria,[3] and marine organisms[4] and more recently in blue-green algae.[5] The biosynthetic origin of the isonitrile group has intrigued chemists for three decades, but the data obtained to date with fungi, bacteria, and sponges do not present a simple picture. Studies on the biosynthesis of xanthocillin have suggested that L-tyrosine is the primary source of the isonitrile nitrogen,[6] but C_1 donors linked to tetrahydrofolate metabolism (methionine, formate, C-2 of glycine, and C-3 of serine)[7] as well as other C_1 donors (e.g., cyanide and carbamoyl phosphate)[8] are not sources of the isonitrile carbon. Puar et al.[9] have found, however, that L-[methyl-^{13}C]methionine labels the isocyano group of the hazimicins in the bacterium *Micromonospora echinospora* var. *challisensis*, and Garson[10] has discovered that [^{14}C]cyanide is incorporated into the isonitrile carbons of the marine sponge metabolite diisocyanoadociane. Garson's result is most interesting, since sponges frequently possess symbiotic microorganisms and certain bacteria and blue-green algae generate inorganic cyanide from amino acids.[11] Our interest in the possible role of symbiotic

Table I. Incorporation Experiments for Hapalindole A

precursor	amount fed (μCi)	total incorpn (%)	specific incorpn (%)	loss of ^{14}C upon acid hydrolysis of 1 to 2 (%)
[^{14}C]cyanide	24.5	0.16	1.21	94.8
[2-^{14}C]glycine	45.1	0.33	1.00	99.0
L-[3-^{14}C]serine	27.6	0.34	0.87	97.0
[^{14}C]formate	50.0	0.11	0.26	96.3
L-[*methyl*-^{14}C]methionine	27.3	0.05	0.08	97.6
[1,2-^{14}C]acetate	30.5	0.15	0.57	5.7
DL-[*methylene*-^{14}C]tryptophan	49.9	0.13	0.36	0.06

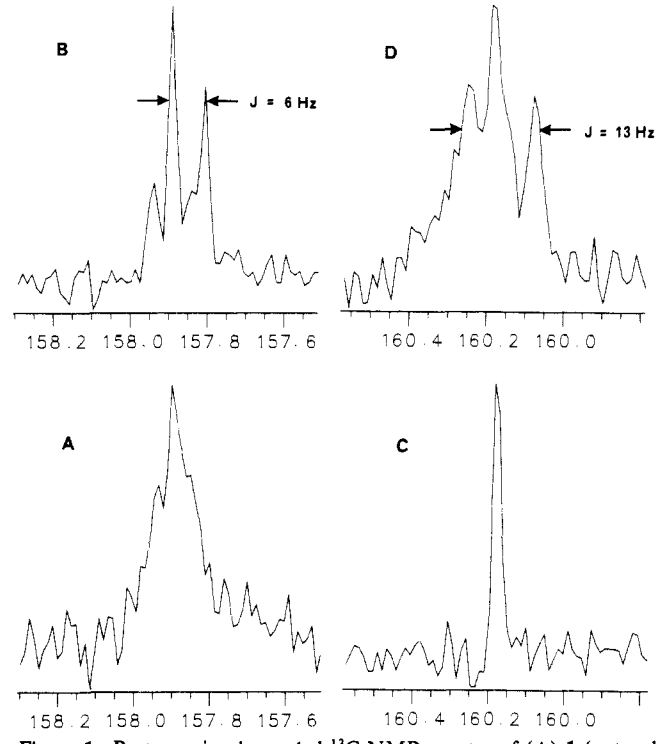

Figure 1. Proton noise-decoupled ^{13}C NMR spectra of (A) 1 (natural abundance); (B) 1 obtained from feeding [2-^{13}C,^{15}N]glycine to *H. fontinalis*; (C) 3 (natural abundance); and (D) 3 obtained from hydrolysis of 1 showing spectrum B.

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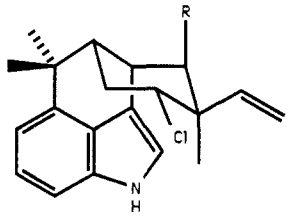
blue-green algae in the biosynthesis of sponge metabolites[12] has prompted us to examine isonitrile biosynthesis in the terrestrial cyanophyte *Hapalosiphon fontinalis*. We report here our preliminary findings with hapalindole A (**1**), the major isonitrile in *H. fontinalis*.



1 R = NC

2 R = NH_2

3 R = NHCHO

For our biosynthetic experiments, *H. fontinalis* (strain V-3-1, ATCC 39694) was cultured in 4.5-L glass vessels containing an inorganic medium[5a,b] adjusted to pH 7.2. Each culture was incubated at 24 ± 2 °C, continuously illuminated at an incident intensity of 150 μEinstein m^{-2} s^{-1} with cool-white fluorescent lighting and aerated at approximately 1 L/min with air (no added CO_2). The alga was harvested by filtration after 18–20 days (hapalindole A production had ceased), lyophilized, and extracted with 7:3 ethanol/water (500 mL) for 12 h. Flash chromatography followed by isocratic HPLC of the extract on C-18 silica gel with 7:3 methanol/water gave 8-15 mg of pure **1**.

Each precursor (25–50 μCi, diluted to 10 mg with unlabeled material) was pulse fed over a 4-day period, 12–14 days after inoculation. The amount of label in the isonitrile carbon was determined by degrading **1** to hapalindole A amine (**2**) by acid hydrolysis (1 N HCl, 80 °C, 4 h).[13] The reaction product was purified by isocratic HPLC on C-18 silica gel with a 15:85 water/methanol mixture.

The results of the feeding experiments are shown in Table I.[14-16] The incorporation levels are low but significant considering the high dilution of the precursors by the photosynthetic carbon pool. The incorporation of [2-^{14}C]glycine, L-[3-^{14}C]serine, L-[methyl-^{14}C]methionine, and [^{14}C]formate clearly shows that the isonitrile carbon in hapalindole A originates from a C_1 donor related to tetrahydrofolate (THF) metabolism. The high specific incorporation of [^{14}C]cyanide is comparable to the result reported by Garson.[10]

The specific incorporation levels of cyanide and glycine suggested that stable isotope incorporation experiments were feasible. An attempt to feed 500 mg of $K^{13}CN$ (99 atom% enriched) in a pulsed feeding experiment, however, was unsuccessful (toxic). Analysis of the proton noise-decoupled ^{13}C NMR spectrum of the labeled **1** (15 mg) resulting from feeding [2-^{13}C,^{15}N]glycine (500 mg) showed a 100% enhancement of the isonitrile carbon signal (157.89 ppm). This corresponds to a specific incorporation of about 1%. In the natural abundance ^{13}C NMR spectrum of **1** (Figure 1A), the signal at 157.89 ppm is split into a broad triplet ($J_{^{13}C,^{14}N} \approx 4$ Hz).[17] In the ^{13}C NMR spectrum of the labeled **1** (Figure 1B) a relatively sharp doublet ($J_{^{13}C,^{15}N} \approx 6$ Hz)[17] at 157.85 ppm is superimposed onto the broad triplet at 157.89 ppm. To confirm the NMR interpretation, the labeled **1** was converted into the corresponding formamide **3** (1:1 EtOH/HOAc, room temperature, 3 h). The natural abundance ^{13}C NMR spectrum of **3** (Figure 1C) shows sharp signals at 160.18 and 165.31 ppm (relative intensities 3:1, respectively) for the two conformations of the formamide group. The labeled **3** shows two 1:2:1 triplets instead (Figure 1D shows the triplet for the major conformer). The center peak of each triplet is attributed to the natural abundance carbon-13, whereas the satellite peaks of each triplet, which are doublets centered at 160.16 and 165.29 ppm ($J_{^{13}C,^{15}N} \approx 13$ Hz),[17] are assigned to carbon-13 that has been incorporated, along with nitrogen-15, from the [2-^{13}C,^{15}N]glycine. The results indicate clearly that C(2)–N of glycine is incorporated intact into the isonitrile group, with no significant loss of ^{15}N-label due to transamination of the glycine prior to isonitrile formation. This is in contrast to the results obtained for xanthocillin, where the isonitrile nitrogen stems from tyrosine and the isonitrile carbon from a yet to be determined carbon source.

Since the radiotracer experiments suggest the involvement of the tetrahydrofolate (THF) metabolism in the isonitrile biosynthesis, 5-formimino-THF could be an intermediate in the pathway to the isocyanide.[18] The formimino-THF intermediate could also explain the retention of one methylene proton from glycine in the biosynthesis of the formamide in tuberin;[19] however, no reports of ^{15}N-incorporation experiments with tuberin could be found. The role of cyanide in the isonitrile biosynthesis remains obscure. It is possible that cyanide is formed intracellularly by amino acid oxidation, as has been reported for the blue-green alga *Anacystis nidulans*.[20] Free cyanide (>1 mg/L) could not be detected in the culture medium of *H. fontinalis*. Cyanide could enter THF metabolism via the formation of serine from glycine as suggested by Knowles[21] or, more likely, as a substrate for formation of formimino-THF or a related THF derivative. Studies to evaluate the intact incorporation of [^{13}C,^{15}N]cyanide into the isonitrile group of **1** as well as experiments to evaluate amino acid catabolism to free cyanide are currently in progress.

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Registry No. 1, 92219-95-9; Met, 63-68-3; Gly, 56-40-6; Ser, 56-45-1; Trp, 73-22-3; formate, 64-18-6; cyanide, 57-12-5; acetate, 64-19-7.

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(12) There are several examples in the literature that provide circumstantial evidence. For instance, a symbiotic blue-green alga is present in the sponge *Theonella swinhoei* from the Red Sea [Wilkinson, C. R. In *Biology des Spongiaires*; Levi, C., Boury-Esnault, N., Eds.; Centre National de la Recherche Scientifique: Paris, France, 1979; pp 373–380]. Two sesquiterpenes of the bisabolene class, theonellin isothiocyanate and theonellin formamide, have been isolated from Okinawan *T.* cf. *swinhoei* [Nakamura, H.; Kobayashi, J.; Ohizumi, Y.; Hirata, Y. *Tetrahedron Lett.* **1984**, *25*, 5401–5404]. Isothiocyanates and formamides in sponges are believed to be derived from the corresponding isonitriles [Hagadone, M. R.; Scheuer, P. J.; Holm, A. *J. Am. Chem. Soc.* **19848** *106*, 2447–2448]. Although theonellin isonitrile was not present in Okinawan *T.* cf. *swinhoei*, it has been found in a nudibranch from Sri Lanka [Gulavita, N. K.; de Silva, E. D.; Hagadone, M. R.; Karuso, P.; Scheuer, P. J.; Van Duyne, G. D.; Clardy, J. *J. Org. Chem.* **1986**, *51*, 5136–5139]. The source of the isonitrile in the nudibranch is presumably dietary, since nudibranchs feed on sponges.

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(14) Radiochemical purity was determined by HPLC analysis, by using simultaneous radioactivity and UV detection.

(15) Total incorporation = (μCi isolated/μCi fed) × 100; specific incorporation = (μCi $mmol^{-1}$ isolated/μCi $mmol^{-1}$ fed) × 100.

(16) Incorporation of [1,2-^{14}C]acetate into the monoterpene portion of **1** was found to be inefficient. The small amount of label lost in the acid hydrolysis can be explained by experimental error and/or scrambling of label via the citric acid cycle and photosynthetic CO_2 fixation. The feeding of DL-[*methylene*-^{14}C]tryptophan shows efficient incorporation into the indole portion of **1**, despite the large size and poor solubility of the precursor. Virtually no label is found in the isonitrile carbon. The specific incorporation shown in Table I is based on racemic tryptophan. The actual specific incorporation could be 0.72% since L-tryptophan may be the only form utilized by the organism.

(17) The $^1J_{C,N}$ coupling constants have negative signs. Witanowski, M.; Stefaniak, L.; Webb, G. A. In *Annual Reports on NMR Spectroscopy*; Webb, G. A., Ed.; Academic Press: New York, 1981 and 1986; Vols. 11B and 18.

(18) The formiminotetrahydrofolate could be formed from glycine in a variation of the reversible glycine cleavage mechanism in which C-2 of glycine is transferred to 5,10-methylene-THF and the nitrogen is lost [Kochi, H.; Kikuchi, G. *J. Biochem.* **1974**, *75*, 1113–1127].

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