Biosynthesis of Scorpinone, a 2-Azaanthraquinone from *Amorosia littoralis*, a Fungus from Marine Sediment[⊥]

Ryan M. Van Wagoner,[†] Peter G. Mantle,[‡] and Jeffrey L. C. Wright*,[†]

Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin K Moss Lane, Wilmington, North Carolina 28409, and Centre for Environmental Policy, Imperial College London, London SW7 2AZ, U.K.

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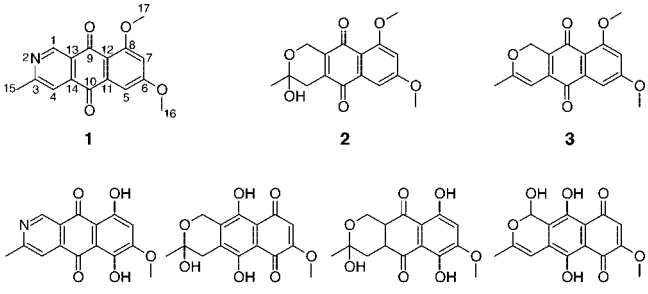
The biogenetic origin of the carbon atoms in the 2-azaanthraquinone scorpinone (**1**), produced by the rare fungus *Amorosia littoralis* isolated from marine sediment, was explored through isotopic enrichment studies utilizing [2-^{13}C]-acetate and [1,2-^{13}C]-acetate. The labeling results reveal a heptaketide precursor is involved in the biosynthesis of **1**, as has been found for the structurally related naphthoquinone dihydrofusarubin. The previously identified naphthoquinone herbarin (**2**) was also isolated and appears to bear the same biogenetic relationship to **1** as the fusarubins do to the fungal 2-azaanthraquinone bostrycoidins.

2-Azaanthraquinones are a class of polyketide-derived heterocycles produced in nature primarily by fungi or lichens. Such compounds are reported to display a variety of biological activities including phytotoxic effects and activity against various microorganisms.[1–4] 2-Azaanthraquinone has also been isolated from the plant *Mitrocarpus scaber* and was found to be active against *Trypanosoma congolense*.[5] Other reported activities include cAMP phosphodiesterase inhibition.[6]

All reported naturally occurring 2-azaanthraquinones from fungi are structurally similar to bostrycoidin (**4**),[7–13] which in turn shares many structural features with the fusarubin (**5**) family of metabolites.[14] Indeed, members of both families of compounds are often produced simultaneously by the same fungal isolate.[12,15,16] Within both families, most variation occurs in the number of *O*-methyl groups, the presence or absence of oxygenation at C-5, or the oxidation state of the heterocyclic ring. Previous stable isotope enrichment studies with labeled acetate have shown dihydrofusarubin (**6**) to result from a heptaketide precursor via an aromatizing condensation.[17] Other studies have shown the production of **4** and **5** to be similarly sensitive to the carbon to nitrogen ratio and pH of the culture conditions,[15,16,18–21] suggesting that **4** and **5** share a common biosynthetic precursor.



1 **2** **3** **4** **5** **6** **7**

Another 2-azaanthraquinone, scorpinone (**1**), was reported recently from a newly described fungus isolated from marine sediment, *Amorosia littoralis*.[10,22] Compound **1** exhibits methylation of both phenolic groups, lack of oxygenation at C-5, and a fully aromatic heterocyclic ring. The novelty of the organism producing **1** led us to investigate whether the biosynthesis of **1** is similar to that of **4** or whether the biosynthetic pathway is distinct, possibly incorporating an amino acid as a precursor. Scheme 1 shows two possible pathways leading to production of **1**, the second of which incorporates amino acids in the main chain from a hypothetical hybrid polyketide synthase/nonribosomal peptide synthetase pathway. Two distinct patterns of acetate incorporation are predicted from the pathways, making stable isotopic enrichment studies utilizing acetate an ideal first experiment toward elucidating the biosynthetic pathway.

Results and Discussion

Cultures of *A. littoralis* were supplemented with [2-^{13}C]-acetate, and the effect on enrichment levels of individual carbon atoms of **1** as determined by ^{13}C NMR spectroscopy with inverse gated decoupling is shown in Table 1. The results suggested a need to revise the ^{13}C NMR assignments for **1** as originally reported, specifically in reversing the assignments for C-13 and C-14, which appear to have been incorrectly transcribed in the original report.[10] This modification has the dual benefit of providing a pattern of labeling that is more straightforward to interpret and of matching other reported assignments of similar compounds.[11–13] The data indicate that seven carbons are enriched over natural abundance, consistent with a polyketide origin for all carbon atoms in **1**, as shown in the first pathway in Scheme 1, but are not consistent with incorporation of the carbon atoms of alanine.

Further feeding studies were undertaken using [1,2-^{13}C]-acetate to identify the groupings of intact acetate units within **1**. One estimate of the level of ^{13}C incorporation was derived from integration of the signals in the ^{1}H NMR spectrum and their associated ^{13}C−^{1}H coupled satellite signals. The combined integrated intensities of the satellite peaks varied between 3.4% and 4.7% of the sum of the peak integrations with their respective satellite integrations for the aromatic ^{1}H signals and were 1.9% for the *O*-methyl signals. This latter ratio differs from the expected natural abundance level of 1.1% most likely due to metabolic incorporation of C-1 of labeled acetate into the cellular C_1 pool. By this method of measurement, the level of enrichment of **1** with labeled acetate was determined to be around 2–3%.

A quantitative ^{13}C NMR spectrum using inverse gated ^{1}H decoupling and relaxation delays of 5.0 s was collected to provide more information on the level and pattern of incorporation of doubly labeled acetate at each atomic position. For these studies, CH_2Cl_2 was used as an internal mass calibrant to allow an estimation of the degree of nonspecific incorporation of ^{13}C via metabolic scrambling of the acetate precursor. On the basis of integration of

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* To whom correspondence should be addressed. Tel: 910-962-2397. Fax: 910-962-2410. E-mail: wrightj@uncw.edu.

[†] University of North Carolina at Wilmington.

[‡] Imperial College London.

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Scheme 1. Some Possible Biogenetic Pathways for **1**

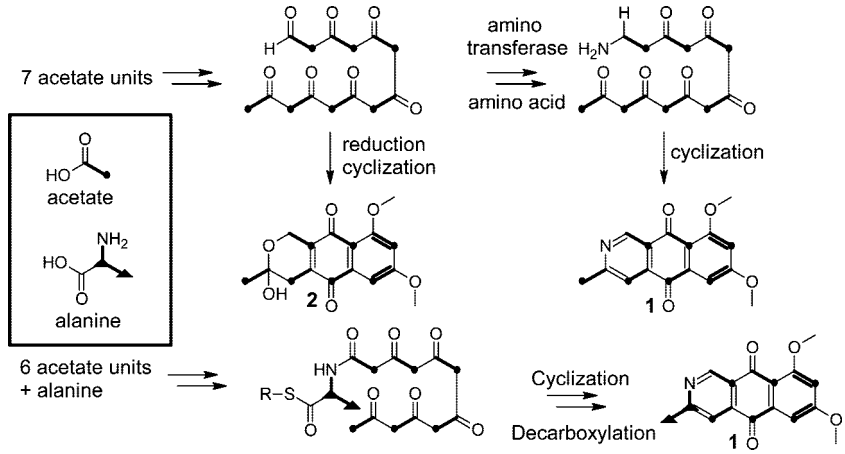

Table 1. ^{13}C NMR Peak Areas for [2-^{13}C]-Acetate-Enriched **1**

position	δ (ppm)	peak area[a]
1	149.2	0.79
3	164.1	0.66
4	117.9	2.26
5	103.6	2.66
6	164.9	0.68
7	105.4	2.47
8	162.7	0.81
9	180.5	0.60
10	183.5	2.13
11	136.9	0.85
12	115.6	1.57
13	125.4	2.30
14	136.5	0.85
15	24.7	2.89
16	56.1	1.04
17	56.6	0.96

[a] Standard deviation of baseline peak area: 0.15.

the ^1H NMR spectrum, CH_2Cl_2 was present at a molar ratio of 1.1:1 compared to **1**.

The effects of enrichment on peak areas and $^{13}C-^{13}C$ scalar couplings as determined by ^{13}C NMR spectroscopy are shown in Table 2. The data are again consistent with a linear heptaketide precursor as shown in Scheme 1. Several features apparent from the data are worth noting. First, three sets of signals (a singlet, a flanking doublet, and a smaller doublet of doublets that are partially overlapped with the central peak) were observed for every non-terminal carbon position in the aromatic nucleus of **1** (Figure 1). Table 2 includes the peak areas for each of the signals associated with each resonance. The central peaks correspond to natural abundance ^{13}C, and their observed integrated areas, when compared to the area measured for the mass calibrant CH_2Cl_2, are consistent with there being little to no random incorporation of ^{13}C via metabolic scrambling. The two main satellite peaks noted in Table 2 result from coupling between enriched ^{13}C atoms of an intact acetate unit, and additional peaks in the spectrum result from coupling between enriched carbon atoms of adjacent acetate units.

When the primary route of incorporation of ^{13}C-enriched atoms is through intact acetate units, then doublet couplings (as opposed to doublets-of-doublets) will only occur between atoms that are part of the same acetate unit. Thus, it is possible to determine whether a given atom that has significantly different ^1J values for each of its neighbors is part of an intact acetate unit. This is based on whether a single doublet is present or if a superposition of two doublets, each having a distinct ^1J value, is present instead. One such case occurs for C-3, which has a ^1J value of 50 Hz with C-15 and 58 Hz with C-4. The doublet associated with C-3 has a ^1J value of 50 Hz with no apparent overlapping doublet with a ^1J of 58 Hz, the pattern expected if C-3 is part of an intact acetate starter unit (see Figure 1). However, this analysis cannot be easily applied to labeled atoms in the rest of the molecule since the coupling values for most positions show very little difference from one neighbor to another.

Additional evidence supporting intact incorporation of [1,2-^{13}C]-acetate comes from mass spectrometric measurements (Figure 2). The envelope of molecular masses representing molecules with multiple heavy isotopes clearly shows a spacing of 2 Da between adjacent peaks. This is exactly what one would expect from intact incorporation, as incorporation of ^{13}C via metabolic processing of acetate into other precursors would not favor spacings of 2 Da over spacings of 1 Da as strongly. Moreover, the width and center of the heavy isotope envelope in the mass spectrum is consistent with a model in which each individual acetate unit has a 50–60% probability of arising from [1,2-^{13}C]-acetate, a similar level to that seen in a previous study with **6**,[17] which will be further discussed below. This correlates well with the NMR data in that there is roughly a 1:1 ratio of doublets-of-doublets (i.e., two adjacent labeled units) to simple doublets (i.e., a single labeled unit with a natural abundance neighbor).

Thus, a consistent picture emerges from the combined NMR and mass spectrometric data concerning the manner of enrichment of the carbon atoms of scorpinone (**1**) by labeled acetate. The overall enrichment of the sample is 2–3%, as suggested both by integration of the ^{13}C satellite peaks in the ^1H NMR spectrum and by integration of the quantitative ^{13}C NMR spectrum. If this enrichment was distributed uniformly across all of the molecules in solution, one would expect the occurrence of neighboring labeled acetate units (the doublets-of-doublets in the ^{13}C NMR spectrum) to be on the order of 0.1%. However, both the ^{13}C NMR and mass spectrometric data indicate that the enrichment in labeled molecules is around 50%. The sample therefore consists of a mixture in which 95% of the molecules are at natural abundance of ^{13}C and 5% of the molecules are enriched with labeled acetate to a level of 50%.

The labeling patterns observed are consistent with a biosynthetic route involving seven intact acetate units as shown in Scheme 1. This overall architecture is consistent with the F folding mode typically associated with polyaromatic polyketides from fungi as noted by Thomas.[23] Although other, more complicated folding schemes cannot be ruled out by our data, precedent and simplicity favor the folding pattern as depicted.

In the course of purification of **1**, it became apparent that there were other minor compounds present in the initial extracts that appeared to be related to **1** based on similarity in the UV–vis absorption spectra and molecular masses as determined by LC-MS. Several compounds were isolated, but only one was recovered in sufficient quantities to allow full structural determination. On the basis of both mass spectrometric and 2D NMR data, this compound was identified as herbarin (**2**), a compound previously

Table 2. ^{13}C NMR Spectrum of [1,2-^{13}C]-Acetate-Enriched **1**

position	δ (ppm)	s area[a]	d ^1J (Hz)	d area[b]	dd ^1J (Hz)	dd area[c]	labeled/unlabeled	% dd
1	149.2	0.83	59	1.78	n/a	n/a	2.13	n/a
3	164.1	0.59[d]	50	0.96[d]	50, 58	0.79[d]	3.0[d]	45[d]
4	117.4	0.76	59	0.82	59, 56	0.89	2.26	52.1
5	103.5	0.84	66	0.96	66, 62	0.7	1.98	42.3
6	165	0.93[d]	67	0.66[d]	67, 69	0.29[d]	1.0[d]	31[d]
7	105.4	0.69	70	0.95	70, 69	0.84	2.59	47.1
8	162.8	0.6	70	0.84	70, 68	0.94	2.97	52.8
9	180.5	0.57	59	0.63	59, 55	0.51	2.01	44.7
10	183.5	0.57	53	0.72	53, 53	0.69	2.45	49.1
11	137.5	0.62[d]	59	0.66[d]	n/d[e]	0.4[d]	1.7[d]	38[d]
12	115.6	0.41	59	0.52	59, 63	0.63	2.82	54.9
13	125.4	0.62	59	0.69	59, 58	0.47	1.87	40.4
14	137	0.61[d]	55	0.94[d]	n/d[e]	0.66[d]	2.6[d]	41[d]
15	25.1	0.89	51	1.78	n/a	n/a	2	n/a
16	56.1	0.92	n/a	n/a	n/a	n/a	n/a	n/a
17	56.6	0.85	n/a	n/a	n/a	n/a	n/a	n/a

[a] Estimated uncertainty: 0.05. [b] Estimated uncertainty: 0.10. [c] Estimated uncertainty: 0.20. [d] Peak area estimated due to spectral overlap. [e] Could not be estimated due to low signal intensity and spectral overlap.

isolated from the yeast *Torula herbarum.*[24] The structures of **1** and **2** differ in that **2** possesses a cyclic hemiketal structure where the nitrogen at position 2 is replaced by oxygen. Nevertheless, compound **2** exhibited the same pattern of enrichment from [2-^{13}C]-acetate as observed in **1**. There was an additional compound that had a molecular mass and a UV–vis absorption spectrum consistent with dehydroherbarin (**3**).[24] The remaining minor compounds appeared to be congeners of **1** or **2** modified by oxidation or reduction, but, unfortunately, it was not possible to definitively assign structures to them due to the small amounts recovered.

The structural similarity between **1** and **2** suggests that both result from a similar or common biosynthetic pathway. Indeed the presence of both compounds in *A. littoralis* is an important observation. The situation is directly analogous to the co-occurrence of the bostrycoidins and fusarubins in *Fusarium* cultures discussed above and reinforces the suggestion that bostrycoidin (**4**) and scorpinone (**1**) share a similar biosynthetic pathway. This view is further supported by the similarity in patterns of stable isotopic enrichment by labeled acetate for **1** and **6**.[17] In *A. littoralis*, as in *Fusarium* and other fungal genera, 2-azaanthraquinone production appears to be coupled with the production of other naphthoquinone compounds.[21]

On the basis of the current labeling data, the nitrogen atom in **1** could derive conceivably from either inorganic nitrogen or a nitrogen-containing organic precursor. An early study showed that anhydrofusarubin lactol (**7**) was converted quantitatively into **4** by heating to 100 °C in benzene containing excess ammonia.[25] At room temperature, the reaction was reported to proceed to 50% completion in 72 h. Although the conditions used for the conversion are very different from the culture medium, a similar mechanism may operate for **1** and a putative homologue of **2** that resembles **7**. There is other evidence that **6** may be converted to **4** in the presence of ammonia in the culture medium.[15] It is interesting to note, however, that **1** was first isolated from mycelium surface-cultured on the same medium as at present,[10] reducing the likelihood of spontaneous conversion, whereas **4** and co-occurring **5** have been isolated from the culture medium.[12,13,15,16,18,20] Alternatively, introduction of the nitrogen atom into scorpinone (**1**) may be mediated by an amino transferase that utilizes pyridoxal phosphate as a cofactor and an amino acid as a nitrogen source. Such domains have been found integrated in polyketide synthase modules for the microcystins,[26] the prodigiosins,[27] and mycosubtilin,[28,29] for example.

In summary, the biosynthesis of scorpinone (**1**) by the rare fungus *A. littoralis* follows a conventional polyketide pathway involving a linear heptaketide chain. The means of subsequent incorporation of the nitrogen atom into the 2-azaanthraquinone **1** and related compounds remains a subject of speculation.

Experimental Section

General Experimental Procedures. NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer or on a Bruker Avance 400 MHz spectrometer (quantitative ^{13}C NMR). Chemical shifts were referenced internally to $CDCl_3$. Experiments requiring integration of ^{13}C NMR peaks were acquired with inverse gated decoupling and relaxation times of 5.0–7.0 s. Low-resolution mass spectra were acquired on a Waters ZQ instrument using electrospray ionization and interfaced to an Agilent 1100 HPLC system with a Waters SunFire C_{18} column (4.6 × 150 mm, 5 μm). High-resolution mass spectra for the determination of isotope patterns were acquired on an Applied Biosystems QStar XL MS/MS instrument using electrospray ionization, tandem quadrupole/time-of-flight mass analyzers, and an Agilent 1100 HPLC system. Preparative HPLC was carried out using a system consisting of two Waters 515 pumps and a Waters 2487 UV–vis detector with a Waters SunFire C_{18} Prep column (10 × 250 mm, 5 μm). UV–vis spectra were acquired on a Beckman DU640B spectrophotometer.

Fungal Material. The fungus was first isolated from intertidal sediment in the Bahamas. Its novel aza-anthraquinone scorpinone (**1**)[10] was discovered via a microscale screen for incorporation of radiolabeled precursors into fungal secondary metabolites.[30] The fungus has been described and assigned to a new genus[22] and is deposited in the NovoNordisk (Bagsværd, Denmark) collection (NN 6654) and at the Centraalbureau de Schimmelcultures, Utrecht, The Netherlands (CBS 120339).

Production of Labeled Metabolites. *A. littoralis*, scraped from the surface of cultures on potato dextrose agar, was inoculated into a 500 mL Erlenmeyer flask containing 100 mL of Czapek Dox broth (Difco) supplemented with 0.5% (w/v) yeast extract (Sigma). The flask was incubated on a rotary shaker (200 rpm, 10 cm eccentric throw) for 2 weeks and homogenized aseptically, and 10 mL amounts were distributed to each of six flasks containing the same medium. [2-^{13}C]-Acetate or [1,2-^{13}C]-acetate (sodium salt, 99 atom %, Sigma-Aldrich) in sterile water was added on days 8, 11, 12 (morning), 12 (evening), 13, 14, and 15 amounting cumulatively to 10, 20, 30, 40, 60, 80, and 100%, respectively, of 85 mg for each flask. On day 20 the black submerged mycelial pellets and the black encrustations on flask walls were separated from broth and combined for each acetate treatment. The tissue was extracted overnight in absolute EtOH and then exhaustively with $CHCl_3$. Combined solutes were subjected to preparative chromatography using silica gel (SILG UV_{254}, 2 mm layer, Camlab) with toluene−EtOAc−formic acid (15:4:1). The major yellow band (**1**) and other UV-absorbing bands were excised for purification.

Isolation. The scrapings from preparative SiO_2 TLC were extracted with several portions of MeOH and $CHCl_3$, and the extract was subjected to C_{18} HPLC using a mobile phase consisting of 68% 15 mM NH_4OAc (aq) and 32% CH_3CN, which provided **1** (4.9 mg) and **2** (2.0 mg).

Scorpinone (1): yellow solid; UV (MeOH) λ_{max} (log ϵ) 239 (4.36), 283 (4.06), 322 (sh; 3.69), 405 (3.63) nm; ^1H NMR ($CDCl_3$) δ 9.38 (1H, s, H-1), 7.78 (1H, s, H-4), 7.40 (1H, d, J = 2.4 Hz, H-5), 6.81

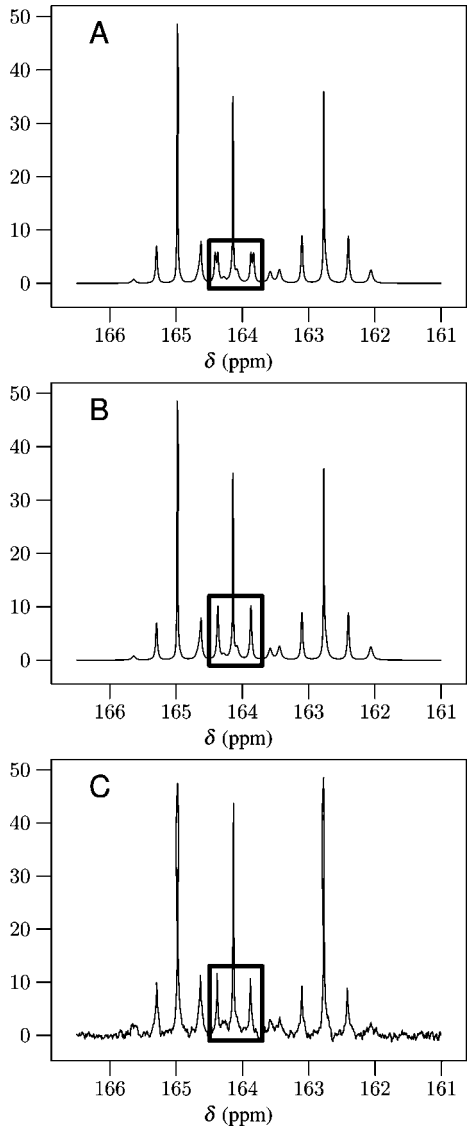

Figure 1. Comparison of simulated (A, B) and experimental (C) ^{13}C NMR spectra for C-3 of [1,2-^{13}C]-acetate-enriched **1**. (A) Simulation for the case where the coupling of the doublet can be either 50 or 58 Hz. (B) Simulation for the case where the coupling of the doublet can only be 50 Hz. (C) Experimental spectrum. In the simulated spectra, linewidths of the singlet, doublet, and doublet-of-doublet signals were set at 1.0, 3.0, and 6.0 Hz, respectively.

(1H, d, J = 2.4 Hz, H-7), 3.99 (3H, s, H_3-16), 3.97 (3H, s, H_3-17), 2.72 (3H, s, H_3-15); ^{13}C NMR Table 2; ESIMS *m/z* 284.

Herbarin (2): yellow solid; UV (MeOH) λ_{max} (log ϵ) 218 (4.32), 269 (4.00), 285 (sh; 3.82), 411 (3.36) nm; ^1H NMR (DMSO-d_6) δ 7.11 (1H, d, J = 2.4 Hz, H-5), 6.95 (1H, d, J = 2.4 Hz, H-7), 6.04 (1H, br s, OH), 4.49 (1H, dd, J = 19.0, 2.0 Hz, H-1a), 4.43 (1H, dt, J = 19.0, 2.8 Hz, H-1b), 3.94 (3H, s, H_3-16), 3.90 (3H, s, H_3-17), 2.55 (1H, dd, J = 18.2, 2.4 Hz, H-4a), 2.39 (1H, dt, 18.2, 2.8 Hz, H-4b), 1.43 (3H, s, H_3-15); ^{13}C NMR (DMSO-d_6) δ 183.2 (C-10, HMBC H-4a, H-5), 182 (C-9), 164.1 (C-6, HMBC H-7, H_3-16), 161.5 (C-8, HMBC H-7, H_3-17), 142.7 (C-13, HMBC H-1a, H-1b, H-4a, H-4b), 136.5 (C-14, HMBC H-1a, H-1b, H-4a, H-4b, H_3-15), 136 (C-11), 112.9 (C-12, HMBC H-5, H-7), 103.8 (C-5, HMBC H-7), 103.7 (C-7, HMBC H-5, H_3-17), 93.0 (C-3, HMBC H-1a, H-1b, H-4a, H-4b, H_3-15, OH), 57.1 (C-1), 56.2 (C-17), 55.8 (C-16), 32.0 (C-4, HMBC H_3-15, OH), 28.0 (C-15); ESIMS *m/z* 305 $[M + H]^+$, 287, 245.

Quantitative ^{13}C NMR Spectroscopy of 1. Quantitative ^{13}C NMR data were acquired by addition of 1.5 μL of CH_2Cl_2 to a solution of the sample in $CDCl_3$. Comparison of the integrated peak areas in the ^1H NMR spectrum was used to estimate the molar ratio of CH_2Cl_2 to **1**. This ratio was used to calibrate the integrated peak area of the CH_2Cl_2 signal in the ^{13}C NMR spectrum. Peak areas in the ^{13}C NMR spectrum were integrated and the underlying signal intensities for each coupling pattern were estimated by manual deconvolution of the expected multiplet shapes.

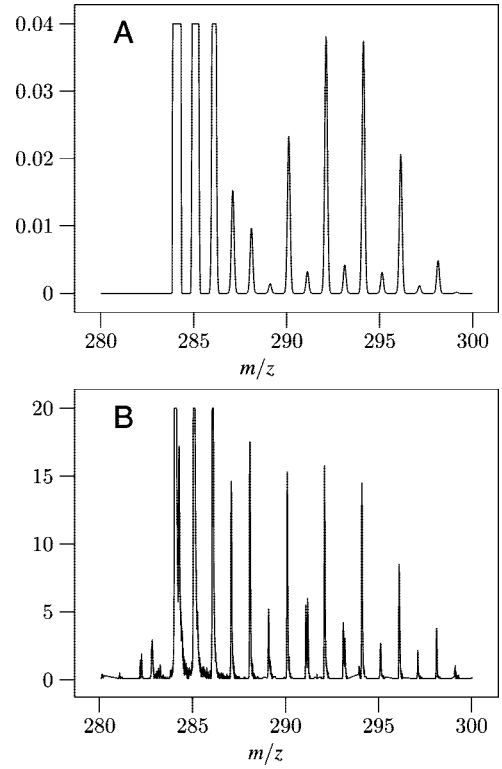

Figure 2. Comparison of simulated (A) and experimentally determined (B) mass spectra for [1,2-^{13}C]-acetate-enriched **1**. (A) Simulated mass spectrum for **1** assuming 2% of the molecules are hyperenriched. For the enriched molecules, the probability of incorporation of a labeled acetate unit is 0.62. (B) Experimental mass spectrum for **1**.

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Supporting Information Available: NMR spectra for [1,2-^{13}C]-acetate-enriched **1** and for **2**; UV–vis and MS data for **1**, **2**, and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

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