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Biosynthesis of 13-Desmethyl Spirolide C by the Dinoflagellate *Alexandrium ostenfeldii*

Shawna L. MacKinnon, Allan D. Cembella,† Ian W. Burton, Nancy Lewis, Patricia LeBlanc, and John A. Walter*

Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford Street, Halifax NS, Canada B3H 3Z1

john.walter@nrc-cnrc.gc.ca

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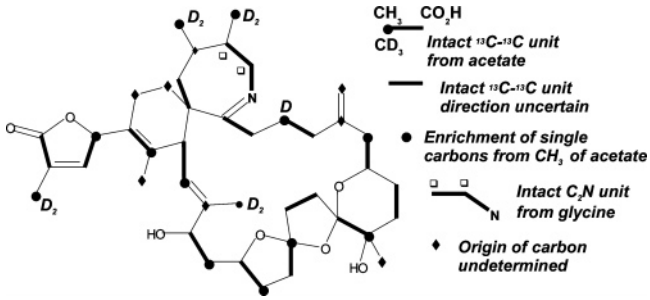

Biosynthetic origins of the cyclic imine toxin 13-desmethyl spirolide C were determined by supplementing cultures of the toxigenic dinoflagellate *Alexandrium ostenfeldii* with stable isotope-labeled precursors [1,2-$^{13}C_2$]acetate, [1-^{13}C]acetate, [2-$^{13}CD_3$]acetate, and [1,2-$^{13}C_2$,^{15}N]glycine and measuring the incorporation patterns by ^{13}C NMR spectroscopy. Despite partial scrambling of the acetate labels, the results show that most carbons of the macrocycle are polyketide-derived and that glycine is incorporated as an intact unit into the cyclic imine moiety. This work represents the first conclusive evidence that such cyclic imine toxins are polyketides and provides support for biosynthetic pathways previously defined for other polyether dinoflagellate toxins.

Introduction

Spirolides are members of a class of macrocyclic imine toxins[1,2] (Figure 1) that also includes pinnatoxins,[3] gymnodimine,[4] pteriatoxins,[5] and spiro-prorocentrimine.[6] Most, if not all, of these types of imine toxins are believed to originate from marine dinoflagellates,[7] based upon confirmatory evidence from pure cultures of the producing organism[4,6] or arguments regarding structural homology,[8] although the origin of pinnatoxins, found thus far only in shellfish, remains elusive. These macrocyclic imines are accumulated in suspension-feeding bivalve shellfish and can cause rapid toxic responses in routine mouse bioassay tests for lipophilic toxins, but toxicity to humans has not yet been demonstrated.

Several spirolides, including spirolides B and D[1] as well as two related, but biologically inactive compounds, spirolides E and F[2], were first structurally characterized from the polar lipid fraction of extracts of scallop hepatopancreas from the Atlantic coast of Nova Scotia. A pattern of six major related compounds

† Present address: Alfred Wegener Institute for Polar and Marine Research, Bremerhaven, Germany.

(1) Hu, T.; Curtis, J. M.; Walter, J. A.; Wright, J. L. C. *J. Chem. Soc., Chem. Commun*. **1995**, 2159−2161.

(2) Hu, T.; Curtis, J. M.; Walter, J. A.; Wright, J. L. C. *Tetrahedron Lett*. **1996**, *37*, 7671−7674.

(3) (a) Uemura, D.; Chou, T.; Haino, T.; Nagatsu, A.; Fukuzawa, S.; Zheng, S.; Chen, H. *J. Am. Chem. Soc.* **1995**, *117*, 1155−1156. (b) Chou, D.; Kamo, O.; Uemura, D. *Tetrahedron Lett.* **1996**, *37*, 4023−4026. (c) Chou, D.; Haino, T.; Kuramoto, M.; Uemura, D. *Tetrahedron Lett.* **1996**, *37*, 4027−4030. (d) Takada, N.; Umemura, N.; Suenaga, K.; Chou, T.; Nagatsu, A.; Takeharu, H; Yamada, K.; Uemura, D. *Tetrahedron Lett.* **2001**, *42*, 3491−3494.

(4) (a) Seki, T.; Satake, M; Mackenzie, L.; Kaspar, H. F.; Yasumoto, T. *Tetrahedron Lett*. **1995**, *36*, 7093−7096. (b) Miles, C. O.; Wilkins, A. L.; Stirling, D. J.; MacKenzie, A. L. *J. Agric. Food Chem*. **2000**, *48*, 1373−1376.

(5) Takada, N.; Umemura, N.; Suenaga, K.; Uemura, D. *Tetrahedron Lett*. **2001**, *42*, 3495−3497.

(6) Lu, C.-K.; Lee, G.-H.; Huang, R.; Chou, H.-N. *Tetrahedron Lett*. **2001**, *42*, 1713−1716.

(7) Wright, J. L. C.; Cembella, A. D. In *Physiological Ecology of Harmful Algal Blooms*; Anderson, D. M.; Cembella, A. D.; Hallegraeff, G. M., Eds.; Springer-Verlag: Heidelberg, 1998; pp 427−452.

(8) Falk, M.; Burton, I. W.; Hu, T.; Walter, J. A.; Wright, J. L. C. *Tetrahedron* **2001**, 57, 8659−8665.

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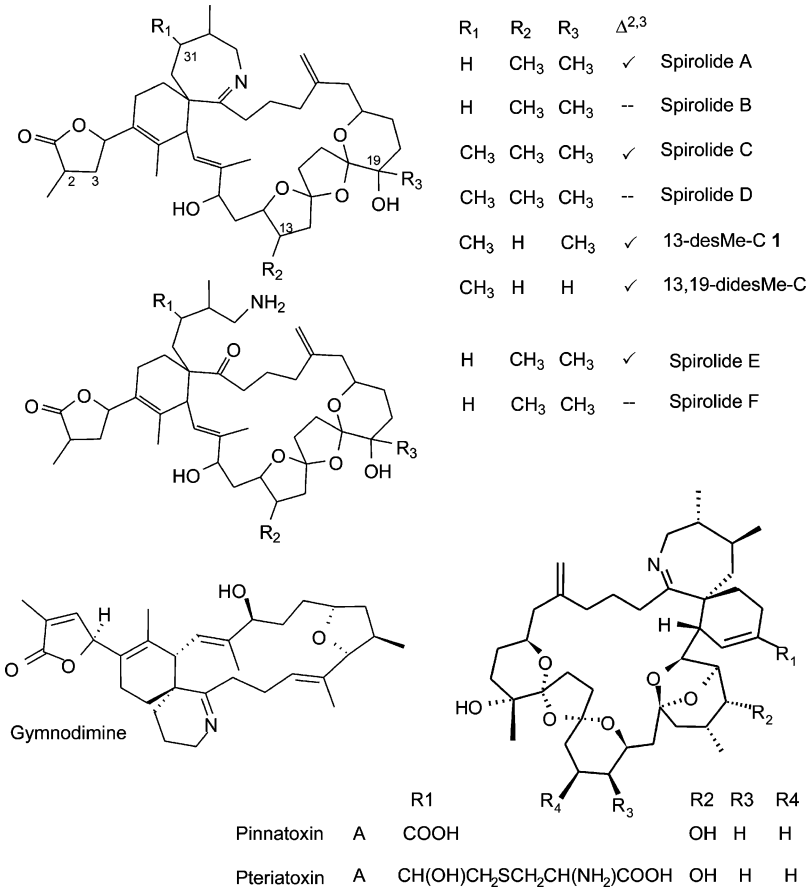


FIGURE 1. Structures of representative macrocyclic imine toxins.

(spirolides A−F) was observed annually during late spring to early summer from hepatopancreas extracts of shellfish and from net-haul plankton fractions from the water column.[9] The causative organism of spirolide toxicity was identified as *Alexandrium ostenfeldii*,[10] a planktonic marine dinoflagellate previously suspected to have been associated only with paralytic shellfish poisoning (PSP) toxins. Structural elucidation of spirolides A, C and 13-desmethyl C **1**[11] was accomplished by accumulation of material from toxic shellfish and from unialgal culture of a toxic clone of *A. ostenfeldii* (AOSH1) isolated from Ship Harbour, NS. This AOSH1 isolate, and bulk plankton samples from Ship Harbour, have proven to be a rich source of **1**, which is the dominant component of the spirolide profile in this area.[12]

The macrocyclic imines are unusual, even among other dinoflagellate polyether toxins, in that the toxic response elicited in mouse bioassays by intraperitoneal injection[13] is considered to be "fast acting" (within minutes) but also subject to a narrow threshold. Below that critical threshold no effect is observed, whereas dosages above the threshold typically yield a rapid death. The cyclic imine portion of the molecule is responsible for the toxicity,[2] but the mode of action has not been fully established.[13]

The polyketide origin of certain dinoflagellate polyether toxins, e.g., the toxins associated with diarrhetic shellfish poisoning (DSP), has now been clearly ascertained by means of stable isotope labeling studies.[14−19] A speculative polyketide biogenetic pathway has also been proposed recently for pinnatoxins.[20] Nevertheless, biosynthetic labeling experiments have not been previously undertaken for any of the macrocyclic imine toxins. Here, we report biosynthetic studies in which 13-desmethyl spirolide C **1** (Figure 1) was harvested from cultures of the dinoflagellate *Alexandrium ostenfeldii*,[10] separately supplemented with [1,2-$^{13}C_2$]acetate, [1-^{13}C]acetate, [2-$^{13}CD_3$]-acetate, and [1,2-$^{13}C_2$,^{15}N]glycine, and the labeling pattern determined from ^{13}C NMR spectroscopy.

(9) Cembella, A. D.; Bauder, A. G.; Lewis, N. I.; Quilliam, M. A. In *Harmful Algal Blooms 2000*; Hallegraeff, G. M., Blackburn, S. I., Bolch, C. J., Lewis, R. J., Eds.; Intergovernmental Oceanographic Commission (UNESCO): Paris, 2001; pp 173−176.

(10) Cembella A. D.; Lewis, N. I.; Quilliam, M. A. *Phycologia* **2000**, *39*, 67−74.

(11) Hu, T.; Burton, I. W.; Cembella, A.; Curtis, J. M.; Quilliam, M. A.; Walter J. A.; Wright, J. L. C. *J. Nat. Prod.* **2001**, *64*, 308−312.

(12) Cembella, A. D.; Lewis, N. I.; Quilliam, M. A. *Natural Toxins* **1999**, *8*, 1−10.

(13) Richard, D.; Arsenault, E.; Cembella, A. D.; Quilliam, M. A. In *Harmful Algal Blooms 2000*; Hallegraeff, G. M., Blackburn, S. I., Bolch, C. J., Lewis, R. J., Eds.; Intergovermental Oceanographic Commission (UNESCO): Paris, 2001; pp 383−386.

(14) Needham, J.; McLachlan, J. L.; Walter, J. A.; Wright, J. L. C. *J. Chem. Soc., Chem. Commun.* **1994**, 2599−2600.

(15) Needham, J.; Hu, T.; McLachlan, J. L.; Walter, J. A.; Wright J. L. C. *J. Chem. Soc., Chem. Commun.* **1995**, *42*, 1623−1624.

(16) Wright, J. L. C.; Hu T.; McLachlan, J. L.; Needham, J.; Walter, J. A. *J. Am. Chem. Soc.* **1996**, *118*, 8757−8758.

(17) Macpherson, G. R.; Burton, I. W.; LeBlanc, P.; Walter, J. A.; Wright, J. L. C. *J. Org. Chem.* **2003**, *68*, 1659−1664.

(18) Rawlings, B. J. *Nat. Prod. Rep.* **1999**, *16*, 425−484.

(19) Moore, B. S. *Nat. Prod. Rep.* **2005**, *22*, 580−593.

(20) Kuramoto, M.; Arimoto, H.; Uemura, D. *Mar. Drugs* **2004**, *1*, 39−54.

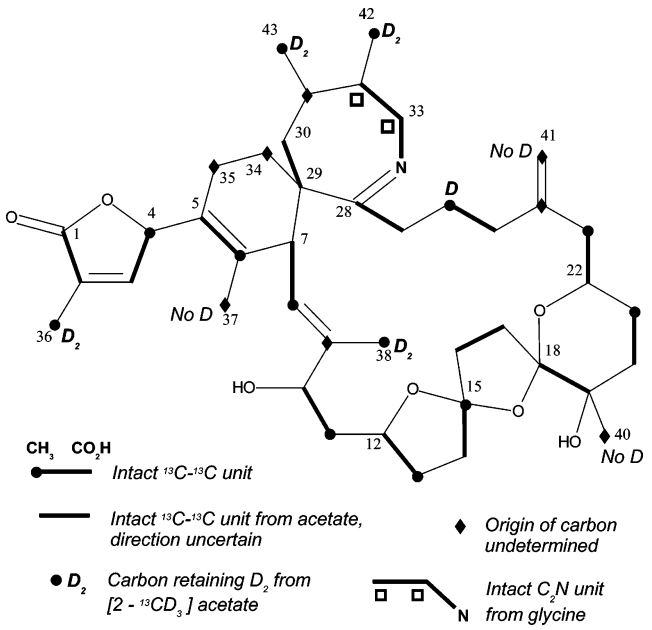


FIGURE 2. Structure of 13-desmethyl spirolide C **1**, showing incorporation of stable isotope labels from [1,2-$^{13}C_2$]acetate, [1-^{13}C]acetate, [2-$^{13}CD_3$]acetate, and [2,3-$^{13}C_2$,^{15}N]glycine.

Results and Discussion

The ^{13}C NMR spectrum of **1** from cultures supplemented with [1,2-$^{13}C_2$]acetate (99% ^{13}C) (experiment 1) showed ^{13}C−^{13}C coupling satellites about each of the resonances, providing direct evidence that ^{13}C from the label had been incorporated (Figure 3). Coupling constants J_{CC} for each carbon pair are shown in Table 1 and in Figure S1 (Supporting Information). However, quantitative measurements by NMR showed that the % ^{13}C incorporation at each position was uniform within error and sufficiently large (average 12.6 ± 2%) that most of the intensity in ^{13}C coupling multiplets could be accounted for by the adjacent incorporation of single ^{13}C units generated by scrambling of the ^{13}C−^{13}C label. The high degree of scrambling was confirmed by the ^{13}C spectra of the samples derived from [1-^{13}C]acetate (experiment 3, Figure 3) and [2-$^{13}CD_3$]acetate (experiment 4, Figure 4), both of which also contained ^{13}C−^{13}C satellites with relative intensities similar to those found with [1,2-$^{13}C_2$]acetate. Resonances of carbon nuclei bonded to two or more carbon atoms showed ^{13}C−^{13}C couplings along all bonds (Figure 3 and Figures S2A−S2G, Supporting Information) indicating that, in experiment 1, there had been high ^{13}C enrichment (at least 30%) in the precursor pool from which **1** was synthesized.

Nevertheless, close comparison of multiplet patterns showed that at many positions in **1** a small proportion of ^{13}C−^{13}C pairs were incorporated intact from [1,2-$^{13}C_2$]acetate (Figure 3). This was clearly demonstrated at the resonances of carbons for which $^1J_{CC}$ varied along different bonds. At many of these carbon resonances (C2, C3, C4, C5, C6, C8, C10, C12, C14, C16, C17, C18, C19, C20, C21, C25, C27), consistent increases of intensity for one satellite pair occurred relative to others on the same carbon resonance (Figure 3 and Figures S2A−S2G, Supporting Information) when resonances of a [1,2-$^{13}C_2$]acetate-derived sample (experiment 1) were compared with those from samples derived from [1-^{13}C]acetate (experiment 3) and [2-$^{13}CD_3$]acetate (experiment 4). The increase was a factor of ca. 1.2−1.8. These observations, together with the $^1J_{CC}$ values, enabled the position of incorporation of some intact ^{13}C−^{13}C pairs to be plotted on the molecular structure (Figure 2 and Figure S1, Supporting Information). The same pattern (Figure 3 and Figures S2A−S2G, Supporting Information) was found when **1** was derived from a mixed precursor consisting of [1,2-$^{13}C_2$] acetate (99% ^{13}C) and natural ^{13}C-abundance (NA) acetate in a ratio of 1/3 (experiment 2). The mixed precursor was used to test the possibility that the sample in experiment 1 contained a mole fraction (ca. 0.12) of adjacently incorporated double-labeled units at ^{13}C enrichments approaching 100%, with the remaining mole fraction (ca. 0.88) being from NA acetate. In experiment 2, the mixed precursor would limit the adjacent incorporation of double-labeled units to 25% probability and would increase the prominence of the satellites due to intraunit couplings. As the mixed precursor produced a pattern very similar to the fully double-labeled precursor used in experiment 1, scrambling of a large proportion of the label to single ^{13}C units, prior to incorporation in **1**, is the only explanation for the observations. The overall % ^{13}C in experiment 2 was lower, ca. 3.5%, in keeping with the 1:3 dilution of the [1,2-$^{13}C_2$]acetate label with NA material.

In experiment 5, undiluted [1,2-$^{13}C_2$]acetate (99%^{13}C), with harvest of the biomass after 24 h, was used to determine whether incorporation of intact units could be detected before significant scrambling occurred. This experiment resulted in a low level of ^{13}C incorporation with barely detectable ^{13}C satellites and no indication of a lower level of scrambling than before; therefore, the results were not further analyzed.

More evidence for incorporation of intact ^{13}C−^{13}C pairs from [1,2-^{13}C] acetate, as well as confirmation of spectral assignment and connectivity, was obtained from ^{13}C 2D-INADEQUATE spectra of **1**. Comparing spectra from experiments 1 and 2 to those from experiment 3 (Figure 5 and Figures S3A−S3L, Supporting Information), the intensities of peaks at double-quantum transition frequencies for some of the ^{13}C−^{13}C pairs were enhanced relative to others in both samples derived from [1,2-$^{13}C_2$]acetate. In contrast, the peak intensities for the scrambled [1-^{13}C]acetate-derived sample (experiment 3) were much more uniform between all possible ^{13}C−^{13}C pairs. These observations took into account intensity differences arising from coupling constant variations and spin−lattice relaxation. INADEQUATE experiments were optimized for ^{13}C-^{13}C coupling constants (J_{CC}) of 40 Hz for experiment 1, 30, 40, and 60 Hz for experiment 2, and 40 and 60 Hz for experiment 3. The relative intensities of double-quantum peaks, corresponding to J_{CC} ranging from 0.8 to 1.3 of the optimum, should all be more than 88% of their maximum intensities, but those corresponding to $2J_{CC}$ would be nullified because there is a sinusoidal variation of intensity with J_{CC}.[21] The intensities of the peaks are also dependent on the relaxation times T_1 of the ^{13}C nuclei: those bonded to H should all be well-relaxed, whereas quaternary carbons have longer T_1 and reduced resonance intensity.

The INADEQUATE spectra showed strong correlations for all C−C pairs denoted as "intact ^{13}C−^{13}C units" in Figure 2 and Figure S1 (Supporting Information), except those corresponding to J_{CC} ca. 76 Hz or those having quaternary carbons at both ends and large couplings (C1−C2, C18−C19). In the latter cases, there was clear evidence from the 1D ^{13}C spectrum that the intact units were arranged as shown. Experiment 2 provided more clear-cut distinction between intact and adjacent ^{13}C−^{13}C units than experiment 1, an expected consequence of dilution of the

(21) Homans, S. W. *A Dictionary of Concepts in NMR*; Clarendon Press: Oxford, 1992.

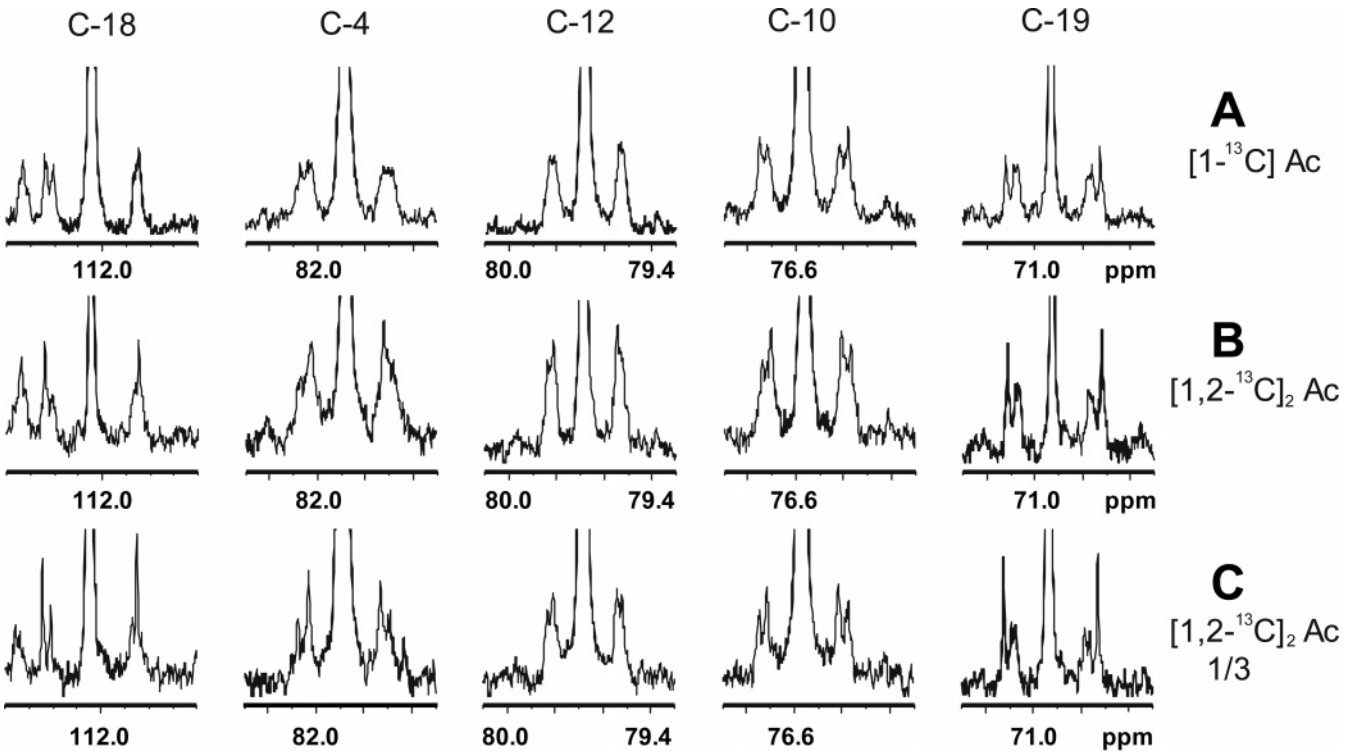


FIGURE 3. Expanded segments of 125.7 MHz ^{13}C NMR spectra of **1** showing differences in $^{13}C-^{13}C$ satellite resonance intensities for samples enriched from labeled acetate. Panels: (A) [1-^{13}C]acetate (experiment 3); (B) [1,2-$^{13}C_2$]acetate (experiment 1); (C) [1,2-$^{13}C_2$]acetate/natural ^{13}C-abundance (NA) acetate (molar ratio1/3, experiment 2). Chemical shift scale is constant.

TABLE 1. Coupling Constants J_{CC} and J_{CN} (Hz) for $^{13}C-^{13}C$ and $^{13}C-^{15}N$ Pairs in 1[a]

C no.	C no.	J_{CC} (Hz)	C no.	C no.	J_{CC} (Hz)	C no.	C no.	J_{CC} (Hz)
1	2	61.9	11	12	39.6	24	41	72.3
2	3	68.5	12	13	33.5	25	26	35.2
2	36	48.0	13	14	31.4	26	27	34.4
3	4	37.7	14	15	42.6	27	28	37.1
4	5	49.0	15	16	42.2	27	28	37.0
5	6	75.4	16	17	32.8	29	30	34.0
5	35	42.0	17	18	43.0	29	34	32.0
6	7	39.2	18	19	49.5	30	31	33.7
6	37	45.6	19	20	36.7	31	32	30.3
7	8	45.1	19	40	41.2	31	43	35.4
7	29	36.2	20	21	32.7	32	33	34.0
8	9	74.7	21	22	35.8	32	42	35.6
9	10	45.6	22	23	39.4	34	35	30.0
9	38	43.5	23	24	41.0			J_{CN} (Hz)
10	11	37.4	24	25	40.3	33	N	5.5

[a] Error ± 0.5 Hz.

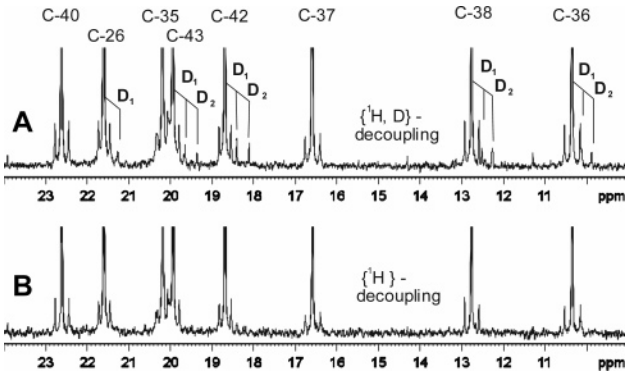


FIGURE 4. Expanded segment of ^{13}C spectrum of **1** enriched from [2-$^{13}CD_3$]acetate (experiment 4). Panels: (A) recorded with {^1H, D}-decoupling showing isotopically shifted peaks indicating D-retention; (B) recorded with {^1H} decoupling only.

label with NA acetate. This clarified the distribution of label around the spiroimine ring, particularly the intact unit at C29−C30 (Figure 5 and Figures S3A−S3L, Supporting Information). Where couplings "between units" were seen in experiment 1, the INADEQUATE peaks had intensities 0.4−0.7 that of the "intact unit" peaks. This level of intensity difference is in accord with the relative intensities of intact-unit and between-unit couplings where these were resolved in the 1D spectrum.

Although the amount of spirolide determined by NMR quantitation in experiment 1 was low (244 ± 50 μg), the high uniform ^{13}C concentration (12.6 ± 2%) meant that the sample was equivalent in NMR ^{13}C sensitivity to a 3 mg NA sample. An INADEQUATE experiment would normally be impossible with such an amount, and the fact that $^{13}C-^{13}C$ couplings are observed at all indicates that much of the enriched sample consisted of intact $^{13}C-^{13}C$ units incorporated adjacent to each other with a probability of the order of 40−70%. The contribution to the resonance intensities from natural abundance material (ca. 1% ^{13}C) cannot account for all the singlet peak intensity observed in the multiplets. Thus, the results also indicate some incorporation of scrambled single label from the original double-labeled precursor, ca. 5% ^{13}C. This leaves ca. 7% ^{13}C associated with intact double-labeled units plus adjacently incorporated ^{13}C's not originating from the same acetate unit.

The direction of incorporation of $^{13}C-^{13}C$ units in **1** (Figure 2) was deduced from quantitative ^{13}C spectra of samples derived from [1 -^{13}C]acetate and [2-$^{13}CD_3$]acetate (experiments 3 and 4, respectively; Figure S4, Supporting Information). The spectra

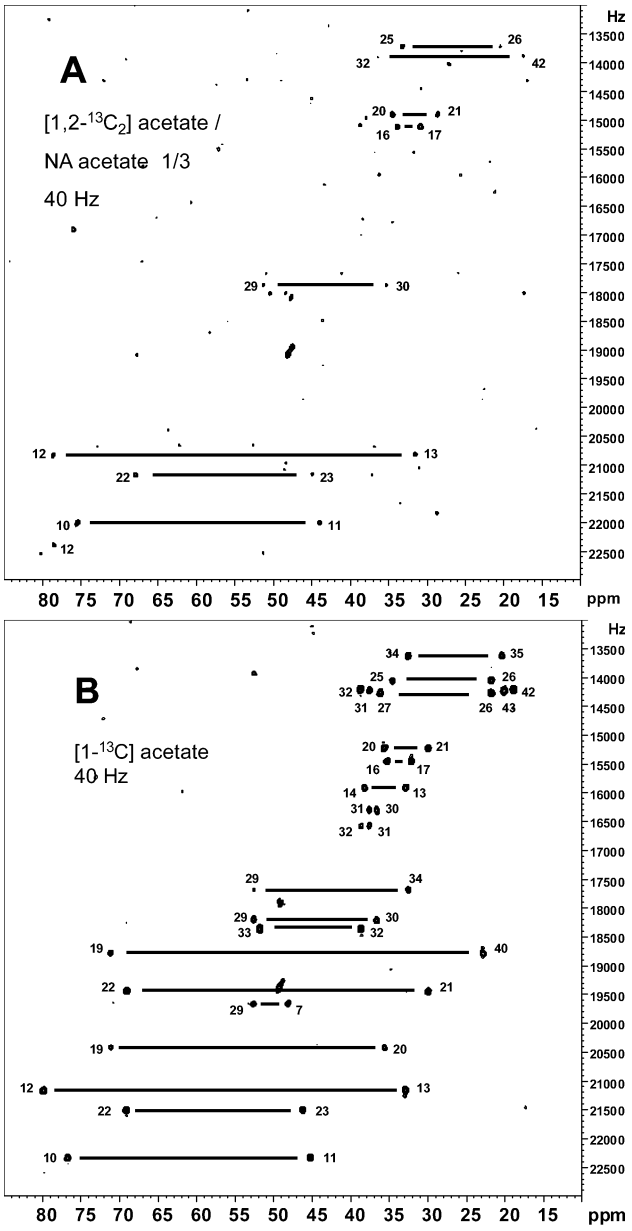


FIGURE 5. Portion of the ^{13}C 2D INADEQUATE spectrum of **1** enriched from: (A) [1,2-$^{13}C_2$]acetate/ NA acetate (molar ratio 1/3, experiment 2); (B) [1-^{13}C]acetate (experiment 3). Spectra are optimized for J_{CC} = 40 Hz. Spectrum A shows enhanced pairs corresponding to incorporation of intact $^{13}C-^{13}C$ units, whereas enrichment in B is uniform owing to scrambling. Note: A slight change of frequency offsets has caused the (C-11, C-12) peaks of B to occur in a region of the plane that is not displayed (see Figure S3A, Supporting Information). For remaining examples, see Figures S3A−S3L (Supporting Information).

showed $^{13}C-^{13}C$ coupling doublets similar to those in spectra from [1,2-$^{13}C_2$]acetate-labeled samples, indicating a high degree of scrambling.

In experiment 3, the average % ^{13}C from two separate measurements was 6.2% (cf. ca. 12.6% found for experiment 1), consistent with scrambling from a single label, which contains half the total amount of ^{13}C of a double label. Where incorporation of intact $^{13}C-^{13}C$ units was indicated between C3 and C26 (see above), within each unit the carbon resonance at lower position number was slightly more intense (avg 6.4% ± 0.2% SD) than that at the higher number (avg 6.0% ± 0.3% SD), consistent with head-to-tail condensation around the macrocycle up to C26, as shown on the diagram.

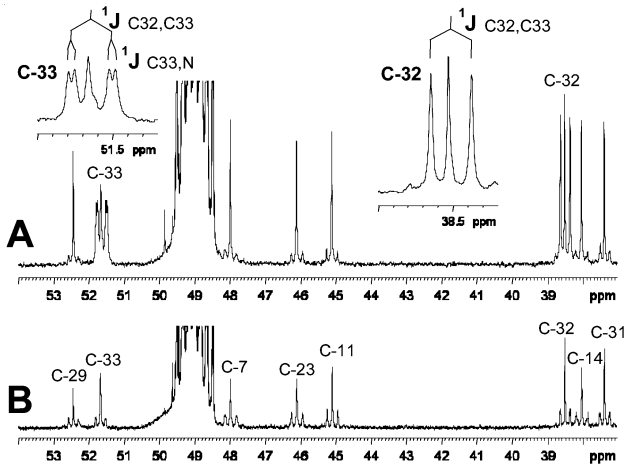


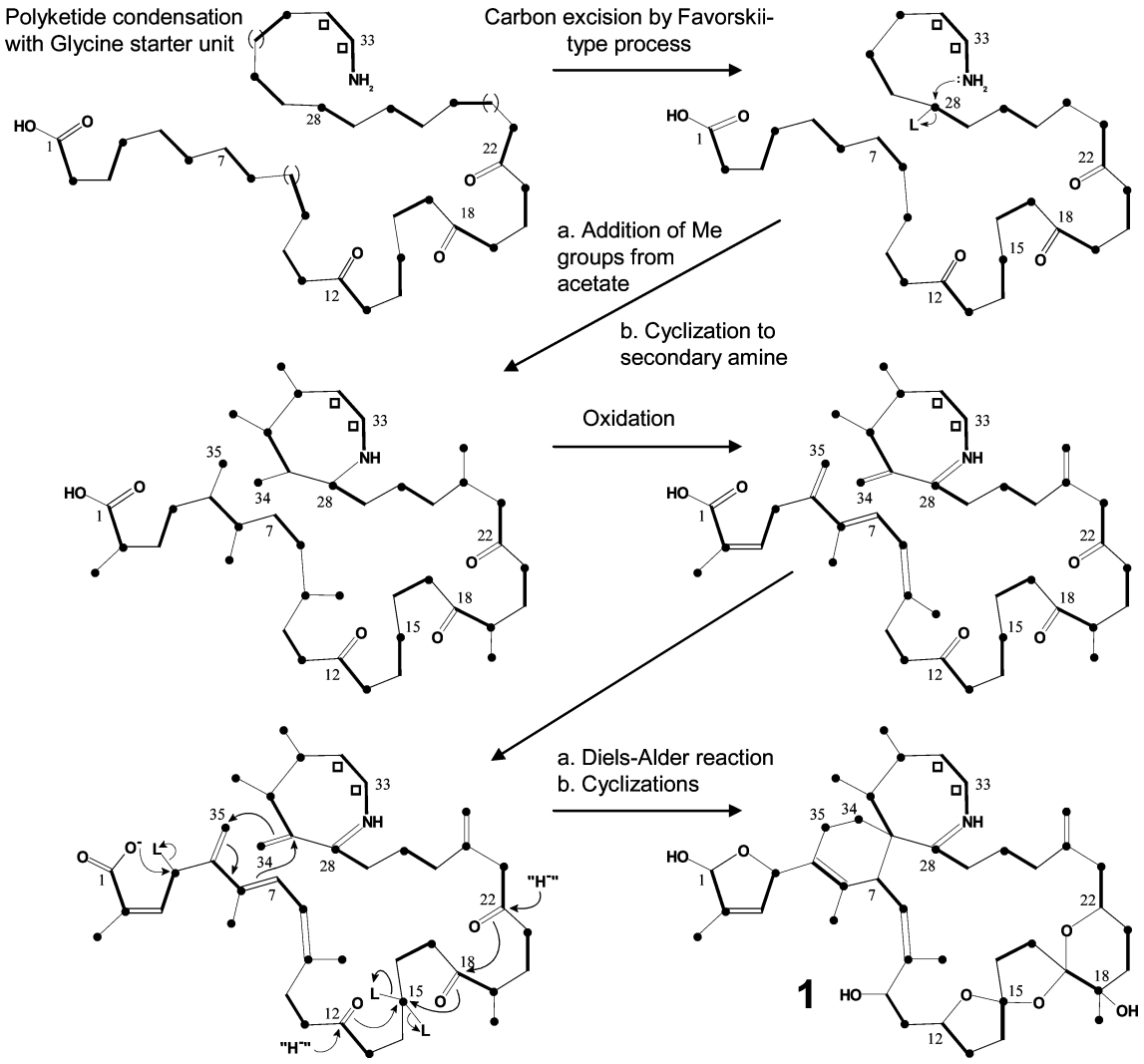
FIGURE 6. Portions of the 125.7 MHz ^{13}C NMR spectrum of **1** enriched from: (A) [1,2-$^{13}C_2$,^{15}N]glycine (experiment 6); (B) [1,2-$^{13}C_2$]-acetate (experiment 1). Spectrum A shows $^{13}C-^{13}C$ coupling satellites ($^1J_{CC}$ 34.1 Hz) and $^{13}C-^{15}N$ coupling satellites ($^1J_{CN}$ 5.5 Hz) due to incorporation of an intact C_2N unit from glycine into the spiroimine moiety (C32, C33, and N).

For experiment 4, the average % ^{13}C was 5.2%, similar to that found with [1-^{13}C]acetate, but the measurements were more difficult owing to noise introduced by D-decoupling. There was a tendency for carbon pairs associated with $^{13}C-^{13}C$ units to show a higher % ^{13}C at the higher position number (5.41% ± 0.45% SD) than the lower position number of a pair (5.06% ± 0.25% SD), but within individual pairs the polarity of the label was not always opposite to that found with the [1-^{13}C]acetate label. The direction of labeling is indicated in Figure 2 and Figure S4 (Supporting Information) where there is agreement between samples from experiments 3 and 4; at other positions the high degree of scrambling, combined with noise and inefficient relaxation at some positions, obscured the result. For these samples, the proportions of doublets in the total intensity of each resonance indicated that incorporation of the scrambled label occurred when the % ^{13}C was considerably higher (e.g., ca. 24% ^{13}C in experiment 3) than the eventual average % ^{13}C (6.2%), indicating dilution of the labeled (scrambled) spirolide with natural abundance material.

Sites of D-retention from [2-$^{13}CD_3$]acetate in experiment 4 were determined from ^{13}C spectra with {^{1}H}- and {^{1}H, D}-decoupling (Figure 4). Isotopically shifted peaks in the {^{1}H, D}-decoupled spectrum of the [2-$^{13}CD_3$]acetate-labeled sample showed retention of D_2 and D_1 at several methyl groups (C36, $\Delta\delta_C$ 0.25, 0.50; C38, C42 and C43 $\Delta\delta_C$ 0.29, 0.58), as well as D_1 retention at C26 ($\Delta\delta_C$ 0.34), confirming that these originated from C2 of acetate. D-retention was not found at C37, C40, or C41.

Consideration of the structure of the imine ring, the evidence (above) for an acetate-derived chain, and other studies of biosynthesis in dinoflagellates,[14−17] suggested that glycine would be a likely starter unit for polyketide condensation. ^{13}C spectra of **1** from a culture supplemented with [1,2-$^{13}C_2$,^{15}N] glycine (Experiment 6, Figure 6) clearly showed incorporation of the intact C_2N unit at C32 (d, J_{CC} 34.1 Hz) and C33 (dd, J_{CC} 34.1 Hz, J_{CN} 5.5 Hz) at a high level (3.0 ± 0.5% ^{13}C) compared to all other positions, for which there was a low ^{13}C incorporation

SCHEME 1. Biosynthetic Scheme for 1 Consistent with the Labeling Pattern Determined Experimentally and with Enzyme-Mediated Processes Known To Occur or Postulated in Other Dinoflagellates[a]



[a] Symbols are as for Figure 2. Position numbers refer to carbons retained in **1**. The only O atoms shown are those that may later be involved in ring formation; their biosynthetic origins and those of OH groups were not sought in the current experiments. The origins of some carbons including C34 and C35 could not be determined experimentally (see text).

from scrambled glycine label (ca. 0.2% ^{13}C), analogous to that from scrambled acetate.

Considered together, the results provide substantial evidence that most carbons of the macrocycle are polyketide-derived from successive linking of acetate units with subsequent modifications. The origins of some carbons which were not part of a doubly labeled unit, or did not retain D (C9, C24, C31, C34, C35, C37, C40, C41), could not be decided, partly as a consequence of the extensive scrambling which obscured the measurement of differences in ^{13}C levels retained from the various acetate precursors. Uniform scrambling throughout **1** was encountered in all experiments with acetate labels despite efforts to vary the conditions of labeling. In contrast, the [1,2-$^{13}C_2$,^{15}N] glycine label was incorporated with minimal scrambling as an intact unit in the cyclic imine moiety, suggesting that glycine is a starter unit for the polyketide condensation. This manner of glycine incorporation resembles that of the glycine and/or glycolate starter units in the biosynthesis of diarrhetic shellfish poisoning (DSP) toxins by marine dinoflagellates: okadaic acid, DTX-1[14] and DTX-4[15,16] by *Prorocentrum lima* and DTX-5a,5b[17] by *P. maculosum*.

The pattern of assembly of the nascent polyketide chain suggested by the combined evidence is shown in Scheme 1, where numbers correspond to carbon positions retained in **1**. Although the direction of incorporation is uncertain for some $^{13}C-^{13}C$ units, those that could be determined were consistent with a glycine starter unit, followed by unidirectional acetate condensation from a single pool containing a high proportion of acetate scrambled to single ^{13}C-labeled units, with subsequent elimination from the nascent chain of some carbonyl-derived carbons (indicated by brackets). These observations are entirely analogous to those found in other dinoflagellate products such as DTX-4 and DTX-5a,5b.[16,17] For these compounds, enrichment from [1,2-$^{13}C_2$]acetate was uniform for *all* carbons not derived from glycolate or glycine. "Isolated" backbone carbons derived from the methyl carbon of acetate, remaining after elimination of some carbonyl carbons from intact acetate units, were attributed to Favorski-type rearrangements,[16] and pendant methyl groups, also derived from the methyl group of acetate and retaining up to two deuterium atoms from a [2-$^{13}CD_3$]acetate label, were ascribed to an aldol-like condensation of acetate or malonate to an electrophilic backbone carbon.[16,17] The observa-

tions for **1** are consistent with these processes, except that it was not possible, owing to scrambling, to establish that the "isolated" carbons in the putative polyketide chain (Scheme 1: C9, C24, and C31) or the pendant methyl/methylene groups that did not show measurable retention of deuterium (C37, C40, C41) were derived from the methyl group of acetate. The four remaining methyl groups (C36, C38, C42, and C43) each retained up to two deuterium atoms, proving their origin from the [2-$^{13}CD_3$] acetate label.

Further enzyme-mediated processes proposed for **1** are cyclization of the seven-membered ring to form a secondary amine, followed by oxidation at several sites to form the iminium group and some C═C bonds, including putative exomethylene groups at C5 and C29 that subsequently participate in a Diels−Alder reaction (activated by the C═N group) to form, respectively, C35 and C34 of the cyclohexene ring. The current experiments did not prove that the latter carbons originated from acetate methyl groups; nevertheless, experimentally it was clear that they were not derived from an intact $^{13}C-^{13}C$ unit. Formation of the remaining rings can reasonably be attributed to a series of enzyme-induced reductive cyclizations. The biosynthetic origins of the O atoms were not sought in these experiments so their participation in ring formation is speculative and no attempt is made in Scheme 1 to account for the hydroxyl substituents. There was no evidence for biosynthesis from larger fragments involving citric acid cycle or other intermediates analogous to that proposed for DSP toxins by Norte et al.[22] or for brevetoxins by Shimizu.[23] In that case, differing levels of enrichment and/or scrambling would be anticipated for different moieties, as found in the marine diatom product domoic acid.[24]

The degree of scrambling in our experiments with the AOSH1 (Nova Scotian) strain of *A. ostenfeldii* was unaffected by the length of time that the label was supplied to the culture prior to harvest (from 24 h to 2 weeks). Monitoring of acetate in the culture medium by ^{1}H NMR showed that the $^{13}C-^{13}C$ label was not scrambled in the medium and that the uptake correlated with the amount of spirolide produced. Scrambling therefore occurred within the dinoflagellate and was not associated with bacterial contamination.

Further experiments conducted at a later date with an isolate of *A. ostenfeldii* from Limfjord, Denmark (unpublished observations), yielded the same degree of acetate scrambling. Label incorporation and interpretation was consistent with that found with the Nova Scotian strain, but no new information was provided on the labeling pattern. Previous labeling experiments with tropical and temperate strains of *Prorocentrum* spp. have shown considerable variations in acetate scrambling, but the intact incorporation of glycine and/or glycolate labels with little scrambling has been consistent with all our dinoflagellate cultures.[14−17]

Spiroimine-containing compounds, such as spirolides, gymnodimine, and pinnatoxins, are a unique group of marine natural products with potent ion-channel and/or neurotoxic activity.[20] To our knowledge, no previous biosynthetic labeling experiments have been performed with any macrocyclic spiroimines. A likely reason is the difficulty of isolating and culturing the organisms to generate sufficient biomass for the harvest of these labeled metabolites. The origin of pinnatoxins remains unknown; hence, labeling studies are not feasible. Nevertheless, Kuramoto et al.[20] have noted that pinnatoxins and their analogues could be produced from a single polyketide chain. This proposed pathway does not indicate the origin of the imine nitrogen or the details of polyketide condensation but resembles our proposed Scheme 1. This is not surprising, given the high degree of structural homology (about 70% in the case of spirolides and gymnodimine[7]) and presumed common dinoflagellate origin among the spiroimine toxins. Evidence on the biosynthesis of polyether toxins in dinoflagellates[7,18,19,23] suggests that they are derived by the successive addition of acetate units to a growing polyketide chain; hence, the reactions are mediated by the enzyme (or enzyme complex) polyketide synthase (PKS). Limited genomic studies on the brevetoxin-producing dinoflagellate *Karenia brevis* (= *Gymnodinium breve*) have localized PKS genes within the dinoflagellate that are clearly distinct from bacterial genes for polyketides.[25] Further gene expression studies have shown that brevetoxin synthesis in this species is coupled to expression of PKS in the cell cycle. Some recent evidence from molecular genetics also supports the proposed polyketide pathway for spirolides. At least four putative PKS genes have been identified from expressed sequence tags (ESTs) generated from a normalized cDNA library of our AOSH1 strain of *A. ostenfeldii* from Nova Scotia.[26] Gene homologies are consistent with type 1 modular PKS, a type found among the dinoflagellates and their protistan allies. Future work will be directed toward confirming the specific nature of these PKS genes and their role in the biosynthesis of spirolides.

Experimental Section

Culture, Labeling, and Harvest of *A. ostenfeldii*. Cultures of *A. ostenfeldii* (AOSH1), originally isolated from Ship Harbour, Nova Scotia, were grown in L-1-enriched seawater medium[27] at 14 °C under a photon flux density of 80 μmol quanta m^{-2} s^{-1} on a 14:10 h light/dark photocycle. Acetate-labeling solutions were autoclaved before being added to the cultures in exponential growth phase. Unialgal cultures were maintained using a sterile technique to minimize bacterial growth. Samples were taken aseptically and filtered by centrifugation (ca. 1 min at 2000*g*) through a 0.45 μm spin-filter (Ultrafree-MC, Durapore membrane) for NMR and toxin analysis or preserved in Lugol's iodine solution for cell counts. Cultures were harvested for spirolide extraction by concentration on a 20 μm Nitex mesh filter followed by centrifugation for 15 min at 4 °C (3500*g*).

For the initial labeling experiment (experiment 1), a culture was grown in a 15 L glass vessel with a Teflon paddle impeller. The culture was supplemented with [1,2-$^{13}C_2$]acetate (99% ^{13}C) (500 mg, 0.43 mM) at 7 d after inoculation, and samples of growth medium were taken every 2 days to monitor incorporation by quantitative ^{1}H NMR and LCMS. When the acetate was depleted after 142 h of uptake, 500 mg was added aseptically to the culture, and harvesting took place 192 h after inoculation.

Cultures for subsequent experiments were grown in 2.8 L Fernbach flasks, each containing 1.5−2.0 L of L-1 medium, after establishing conditions for uptake with unlabeled acetate. These

(22) (a) Norte, M.; Padilla, A.; Fernández, J. J. *Tetrahedron Lett.* **1994**, *35*, 1441−1444. (b) Norte, M.; Padilla, A.; Fernández, J. J.; Suoto, M. L. *Tetrahedron* **1994**, *30*, 9175−9180.

(23) Shimizu, Y. *Ann. Rev. Microbiol*. **1996**, *50*, 431−465.

(24) Ramsey, U. P.; Douglas, D. J.; Walter, J. A.; Wright, J. L. C. *Nat. Toxins* **1998**, *6*, 137−146.

(25) Snyder V. R.; Guerrero, M. A.; Sinigalliano, C. D.; Winshell, J.; Perez, R.; Lopez, J. V.; Rein, K. S. *Phytochemistry* **2005**, *66*, 1767−1780.

(26) Cembella, A. D.; John, U.; Singh, R.; Walter, J.; MacKinnon, S. *Proc. of the 11th International Conference on Harmful Algae*, Cape Town, South Africa; Nov 14−19, 2004.

(27) Guillard, R. R. L.; Hargraves, P. E. *Phycologia* **1993**, *32*, 234−236.

cultures were each supplemented with one of the following labeled precursors at 0.4 mM in the medium: [1,2-$^{13}C_2$]acetate (99% ^{13}C)/natural ^{13}C-abundance (NA) acetate mixture in molar ratio 1/3 (experiment 2); [1-^{13}C]acetate (99% ^{13}C) (experiment 3); [2-$^{13}CD_3$]-acetate (99% ^{13}C, 99% ^{2}H) (experiment 4). The cultures were resupplemented with another 0.2 mM of labeled acetate after 53 h growth and 0.6 mM at 100 and 120 h. A similar culture was supplemented with 0.4 mM [1,2-$^{13}C_2$]acetate (99% ^{13}C) and harvested after 24 h (experiment 5), and another was supplemented with [1,2-$^{13}C_2$,^{15}N]glycine (99% ^{13}C, 99% ^{15}N) at 1.02 mM and harvested after 7 d (experiment 6).

Extraction and Purification of Spirolide. Desmethyl spirolide C was isolated from centrifuged cell pellets of *A. ostenfeldii* cultures by a procedure similar to that of Hu et al.[11] The wet cells were extracted four times by adding methanol followed by sonication and centrifugation. The methanolic supernatants were pooled, evaporated to dryness, dissolved in water, and partitioned twice with dichloromethane to yield the spirolide-containing dichloromethane portion, which was fractionated on a Sephadex LH-20 column eluted with methanol. Fractions containing spirolides were identified using thin-layer chromatography, pooled, evaporated to dryness, redissolved in 30% methanol/water, and then subjected to a C18 flash-chromatography column conditioned and eluted with 40% acetonitrile/water (0.1% trifluoroacetic acid). Spirolides were further concentrated and purified on a Vydac 201TP510 C18 HPLC column eluted isocratically with 30% acetonitrile/water (0.1% trifluoroacetic acid) and monitored by absorbance at 210 nm.

NMR Spectroscopy. Samples of purified 13-desmethyl spirolide C **1** were dissolved in CD_3OD (0.5 mL in 5 mm Wilmad 535pp tubes) for NMR spectroscopy at 500.13 MHz (^{1}H) or 125.7 MHz (^{13}C) at 20 °C. ^{1}H and ^{13}C resonances of **1** in CD_3OD have been assigned previously[11] (Figure S1, Supporting Information). Procedures used for recording spectra under quantitative conditions for measurement of absolute ^{13}C enrichment at each position, and calculation methods, were similar to those previously described.[15−17,24] Quantitative ^{13}C spectra with {^{1}H}-Waltz, or {^{1}H, D}-Waltz/Garp, decoupling were recorded with 5 or 10 s delay and with no ^{1}H irradiation between acquisitions, to suppress nuclear Overhauser enhancement. A few microliters of CH_2Cl_2 at natural ^{13}C abundance (assumed 1.108% ^{13}C) were added to the solution as a NA "standard", the molar ratio r of **1** to CH_2Cl_2 being measured from the ^{1}H spectrum. The % ^{13}C at position *i* of **1** is 1.108 I_i/rI_{CH2Cl2}, where I_i and I_{CH2Cl2} are the respective ^{13}C integrated intensities of position *i* and of the $^{13}CH_2Cl_2$ resonance. ^{13}C spectra were also recorded with 40° pulses and 0.1 s delay between 2.18 s acquisition periods, to maximize S/N. Patterns of intact acetate unit incorporation were determined from the intensities of ^{13}C singlet, doublet and multiplet resonances, and from matching of $^{13}C-^{13}C$ coupling constants (Table 1, Figure S1, Supporting Information). Two-dimensional ^{13}C INADEQUATE spectra optimized for $^{1}J_{CC}$ of 30, 40, or 60 Hz were also recorded to confirm the positions of incorporation of intact $^{13}C-^{13}C$ units. Acquisition conditions were: f2 spectral width (SW2) 198.8 ppm, 1K data points; f1 SW1 25 kHz, 512 increments; 320 scans/increment.

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Supporting Information Available: Structure of **1** showing ^{13}C assignments, labeling pattern, and $^{13}C-^{13}C$ coupling constants; expanded segments of ^{13}C resonances and ^{13}C 2D INADEQUATE spectra for experiments 1, 2 and 3; structure of **1** showing %^{13}C incorporation for experiments 3 and 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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