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<https://doi.org/10.1021/jo0204754>

The Journal of Organic Chemistry, 68, 5, pp. 1659-1664, 2002-12-19

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Studies of the Biosynthesis of DTX-5a and DTX-5b by the Dinoflagellate *Prorocentrum maculosum*: Regiospecificity of the Putative Baeyer–Villigerase and Insertion of a Single Amino Acid in a Polyketide Chain

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Received July 17, 2002

The biosynthetic origins of the diarrhetic shellfish poisoning toxins DTX-5a and DTX-5b have been elucidated by supplementing cultures of the producing organism *Prorocentrum maculosum* with stable isotope labeled precursors and determining the incorporation patterns by ¹³C NMR spectroscopy. The amino acid residue in the sulfated side chain is found to originate from glycine, and oxygen insertion in the chain is shown to occur after polyketide formation.

Introduction

The diarrhetic shellfish poisoning (DSP) toxins are a complex family of compounds^{1,2} that includes okadaic acid (OKA) (1)^{2,3} and dinophysins DTX-1 (2)² and DTX-2 (3),⁴ which are all potent inhibitors of the essential eukaryotic serine/threonine phosphatases PP1 and PP2A. These toxins, responsible for shellfish poisoning episodes around the world,^{2,4} are produced by several temperate and subtropical benthic dinoflagellate species belonging to the genera *Dinophysis* and *Prorocentrum*.^{5–7} Somewhat later, other groups found a series of OKA derivatives containing a short ester chain ranging in size from C₇ to C₉.^{8–10} These compounds appeared to be formed by esterification of OKA with a short diol moiety and were

subsequently named “diol esters”. Later it was found that in toxin-producing *Prorocentrum* spp., OKA and DTX-1, as well as the diol esters, are generated by hydrolysis of larger sulfated diesters such as DTX-4 (4)¹¹ and DTX-5a (5) and -5b (6).¹² These sulfated esters are essentially inactive toward PP1 and PP2A in vitro, and it has been proposed that the sulfated diesters are the initial biosynthetic products of the dinoflagellate.¹³

The unique chemical structure and bioactivity of the DSP toxins have prompted extensive biosynthetic studies that have revealed much complexity. Initially it was observed that the toxins and two okadaic acid diol esters are labeled extensively from acetate in an unexpected pattern and some positions did not appear to be labeled.^{9,10} Later labeling studies of okadaic acid and various sulfated diesters^{13,14} revealed a series of unusual biosynthetic steps. For example, DTX-4 (4) was shown to be assembled from two polyketide (PK) chains (Figure 1), each having a glycolate starter unit followed by a series of acetate additions, presumably in the usual PK manner.^{13,14} However, the nascent PK chain of the OKA portion of 4 is heavily modified during this process. For example, a series of Favorski rearrangements are invoked to eject the carbonyl carbon of an intact PK acetate unit, resulting in a series of “isolated” backbone carbons derived from the methyl group of a cleaved acetate unit.^{13,15} Furthermore, all of the pendant methyl groups are derived from the methyl group of acetate (Figure 1),

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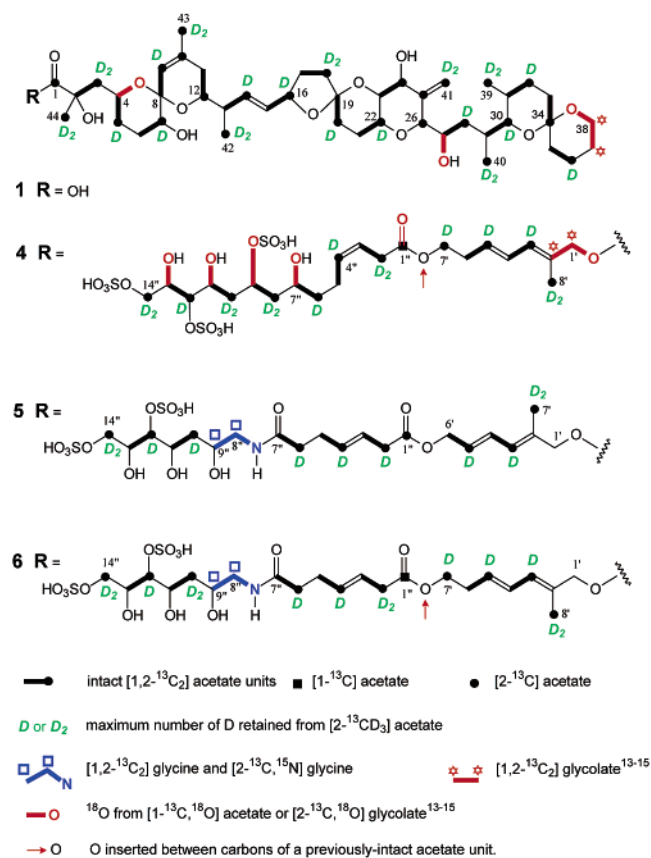


FIGURE 1. Structures of okadaic acid (**1**), DTX-4 (**4**), DTX-5a (**5**) and DTX-5b (**6**) showing incorporation of stable isotope labels determined from previous experiments (for **1** and **4**)^{13–15} and from the experiments in this report (for **5** and **6**).

an unusual feature in PK biosynthesis in which such pendant methyl groups are usually derived from methionine or by incorporation of a propionate unit. It is postulated that these acetate-derived pendant methyl groups are attached to an electrophilic carbon of the PK chain through an aldol-like condensation involving acetate or malonate.¹⁵ Further proof for this proposal came from the observed retention of a maximum of two deuteriums at each of these methyl groups following the incorporation of [2-¹³CD₃] sodium acetate¹⁵ (Figure 1). The cyclization processes to yield the characteristic polycyclic ether structure are unknown, though an interesting mechanism has been proposed.¹⁶

The biosynthesis of the sulfated diester side chain in **4** (Figure 1) showed further intriguing complexity. As in the OKA portion of the molecule, the pendant methyl groups of the side chain are derived from the methyl group of acetate, and a Favorski-like elimination of backbone carbons is also observed. However, the oxygen of the ester bond in the sulfated side chain was not derived from ¹⁸O-acetate.¹⁵ Instead, it was found to arise by direct insertion of an oxygen atom between two carbons of an intact acetate unit by a Baeyer–Villiger-like mechanism, presumably catalyzed by flavin monooxygenase or a cytochrome P450.¹⁵

The discovery of two nitrogen-containing DSP derivatives, DTX-5a (**5**) and DTX-5b (**6**), isolated from the subtropical dinoflagellate *Prorocentrum maculosum*¹² poses some additional biosynthetic questions. The first is the generation of the “diol moiety” portion of the sulfated side chain in each compound. The diol portion of **5** contains seven carbons, whereas in **6** it is composed of eight carbons, as found in DTX-4 (**4**). Of further significance is the origin of the amide link in the side chain in **5** and **6** and specifically how the nitrogen is incorporated. A plausible hypothesis to account for this would be the involvement of an amino acid in the construction of the side chain, as biosynthetic pathways involving a combination of amino acid and acetate precursors are known.^{17–19}

We therefore sought to establish the origin of the diester side chain in each compound, to examine further the characteristics of the enzymatic reactions responsible for oxygen insertion in the side chain to create the ester bond, and to determine the origin of the amide link in **5** and **6**. The experiments reported here establish the biosynthetic origins of **5** and **6** via feeding experiments with sodium [1,2-¹³C₂]acetate, sodium [2-¹³CD₃]acetate, [1,2-¹³C₂]glycine, and [2-¹³C, ¹⁵N]glycine.

Results

Incorporation of [1,2-¹³C₂]Acetate into DTX-5a and DTX-5b. ¹³C NMR spectra from samples enriched from [1,2-¹³C₂]acetate showed (Figure 2) that most ¹³C resonances in both **5** and **6** contained ¹³C–¹³C doublets, indicating either intact incorporation from an acetate unit (doublets of high intensity) or adjacent incorporation of labels originating from separate units (doublets of lower intensity such as those found in the resonances of methyl groups). Quantitative measurement of percent ¹³C enrichment at each position showed that positions C1–C36, C39–C40, C3′–C7′ (in **5**) or C3′–C8′ (in **6**), C1′–C7′ and C10′–C14′ were uniformly enriched (av total percent ¹³C for these positions 4.8 ± 0.6% SD for **5**, 3.7 ± 0.3% SD for **6**). Analysis of ¹J_{CC} coupling constants and 2D ¹³C INADEQUATE spectra of **5** and **6** indicated, in the OKA portion (C1–C44), a pattern of incorporation of intact acetate units and single labels derived from acetate, which were identical to those previously found in **4**^{13,15} (Figure 1). The isotopic labeling pattern for the diol portion C1′–C8′ of **6** was also identical to that in **4**. However, the labeling pattern for the diol moiety of **5** was different and provided evidence that an acetate carboxyl-derived carbon (corresponding to C7′ of **4** and **6**), which previously formed an intact acetate unit with C1′, was deleted from the carbon backbone. Otherwise, the labeling pattern of **5** was the same for the corresponding carbons C1′–C7′ of **4** and **6** (note that the numbering system adopted for this report differs from that used originally¹² for **4**). The remainder of the side chain (C1′′–C14′′) was labeled in the same manner in both **5** and **6**, showing intact ¹³C–¹³C pairs from C2′′ to C7′′ and from C10′′ to C13′′ and single carbons derived from acetate at C1′′ and C14′′ (Figure 1).

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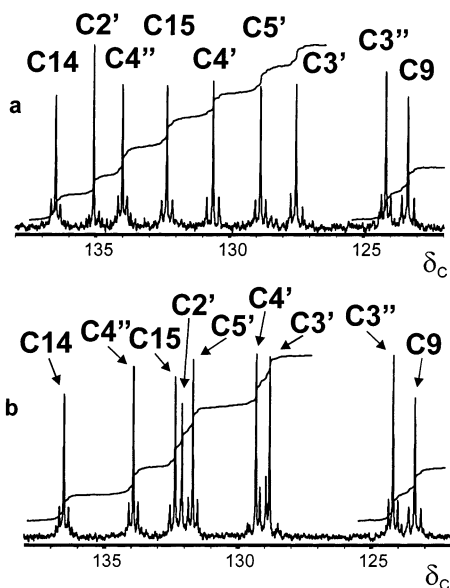


FIGURE 2. Portions of the 125.7 MHz ^{13}C NMR spectra of (a) **5** and (b) **6** enriched from $[1,2-^{13}\text{C}_2]$ acetate and satellites due to incorporation of intact ^{13}C – ^{13}C units, at C14, C4'', C15, C4', C5', and C3'. Integrals show uniform ^{13}C enrichment at these carbons and a lower enrichment at C2', corresponding to incorporation of scrambled label only. In **4**, C2' was shown previously^{13,15} to originate from glycolate. Spectrum a was recorded under “quantitative” conditions with suppressed NOE, whereas spectrum b was obtained without NOE suppression. The latter spectrum yielded relative enrichments of proton-bearing carbons close to those from a “quantitative” spectrum of the same compound, indicating that the former in particular was yielding reliable enrichment values.

Positions C37, C38, C1', and C2' showed lower levels of incorporation of ^{13}C from $[1,2-^{13}\text{C}_2]$ acetate (av $3.3 \pm 1.0\%$ SD for **5**, $3.0 \pm 0.1\%$ SD for **6**), and no doublet components corresponding to coupling within these pairs, consistent with the finding for **4** that these positions are only indirectly labeled from scrambled acetate via glycolate, without retention of an intact ^{13}C – ^{13}C unit. Positions C8'' and C9'' similarly showed low incorporation (av $3.4 \pm 0.6\%$ SD for **5**, $2.0 \pm 0.3\%$ SD for **6**) and lack of ^{13}C – ^{13}C coupling between them. Total ^{13}C enrichments were lower in **5** and **6** obtained from *P. maculosum* compared with **4** from *P. lima*, while the proportion of enrichment due to scrambling of the $[1,2-^{13}\text{C}_2]$ acetate label was higher, amounting to $2.0 \pm 0.4\%$ SD ^{13}C in **5** and $1.7 \pm 0.2\%$ SD ^{13}C in **6**. This was determined from the singlet intensity of ^{13}C resonances at positions where a proportion of intact double label had been incorporated, after subtraction of the singlet component from the calculable fraction of natural abundance (NA) acetate. Thus, the low levels of acetate-derived ^{13}C at positions C37, C38, C1', C2', C8'', and C9'' can be attributed to scrambled label. The average relative intensity $I_d/(I_s + I_d)$ of doublet to total (singlet + doublet) intensity, after subtraction of the NA contribution to the singlet resonances but including the scrambled component, was 0.45 ± 0.06 SD (**5**) or 0.37 ± 0.06 SD (**6**) for carbons that were members of intact ^{13}C – ^{13}C units, and 0.23 ± 0.05 SD (**5**) or 0.16 ± 0.03 SD (**6**) for pendant methyl and vinyl carbons. This is consistent with synthesis of a proportion of the compounds from a highly ^{13}C -enriched pool (>23%

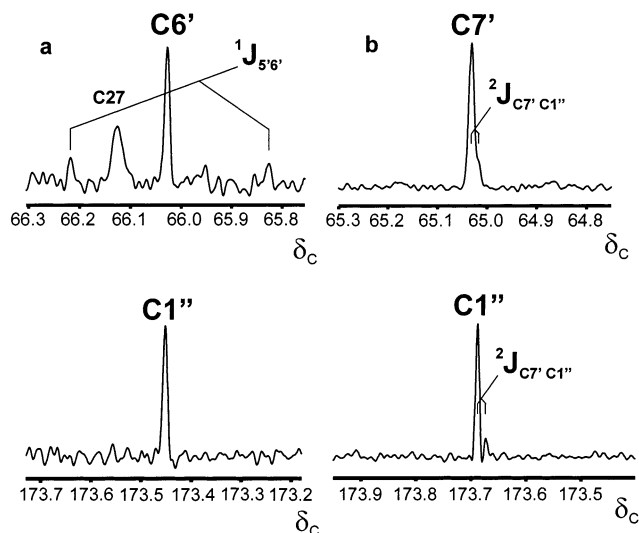


FIGURE 3. Portions of the Lorentz–Gauss resolution-enhanced 125.7 MHz ^{13}C NMR spectra of **5** and **6** enriched from $[1,2-^{13}\text{C}_2]$ acetate, showing resonances for (a) C6' and C1'' of **5** and (b) for C7' and C1'' of **6**. The latter peaks show isotopically shifted ^{13}C – ^{13}C doublets ($^2J_{\text{COC}} \approx 2.5$ Hz) indicating that O has been inserted in **6** between two carbons of a previously intact unit derived from acetate. The relative intensity of these doublets is the same as for $^1J_{\text{CC}}$ doublets for carbons derived from intact acetate units.

^{13}C in **5**, >16% in **6**), with subsequent (or prior) dilution by material containing lower levels of label, down to and including NA. The differences in pool enrichments probably reflect synthesis of **5** and **6** over different time intervals. Other non-methyl carbons bearing single acetate-derived labels (C1, C10, C25, C26, C7' (in **6**), C1'', and C14'') had resonances with satellites indicating probabilities of adjacent incorporation similar to the methyl carbons. As amounts of compound were smaller, enrichments lower, and the extent of scrambling greater than that for **4**, multiplets (doublets of doublets) due to adjacent incorporation of three or more ^{13}C s were not observed in the spectra of **5** and **6**.

The pattern of $[1,2-^{13}\text{C}_2]$ acetate incorporation along the side chains of **5** and **6** is interesting to compare with that of **4**.^{13,15} In particular, the differing lengths of the C1'–C6' moiety in **5** and the C1'–C7' moiety in **6** prompted an examination of the spectra for evidence of Baeyer–Villiger (BV) insertion of oxygen between C6' and C1'' of **5** or between C7' and C1'' of **6**.¹⁵ In the latter molecule, which mimics the DTX-4 (**4**) case, the acetate labeling pattern suggested that oxygen would be inserted between two carbons of an intact acetate unit as in **4**. This would result in doublet satellite peaks ($^2J_{\text{COC}} \approx 2.6$ Hz) of the C7' and C1'' resonances, having the same relative intensity to the singlet resonances as other $^1J_{\text{CC}}$ doublets arising from incorporation of intact acetate units. Indeed, such satellites were found for **6** (Figure 3b), supporting the hypothesis that oxygen is inserted between two carbons of an intact acetate unit. However, this was not observed with the C6' and C1'' resonances of **5** (Figure 3a), as these carbons arise from *separate* acetate units. This was confirmed by the quantitative ^{13}C spectra of **5** and **6** labeled from $[2-^{13}\text{C}_2]$ acetate (see below), which

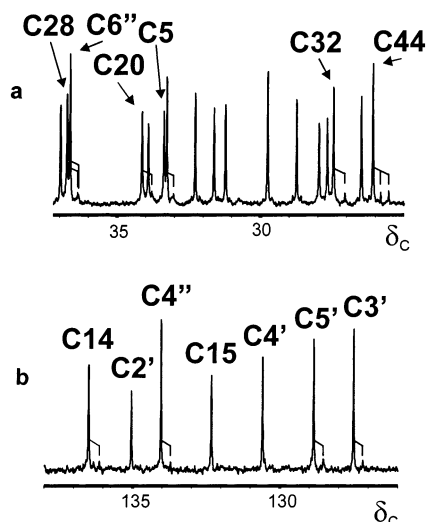


FIGURE 4. Portions of the 125.7 MHz ^{13}C NMR spectra of (a) **6** and (b) **5** enriched from $[2\text{-}^{13}\text{CD}_3]\text{acetate}$. Isotopically shifted peaks indicate D-retention.

showed that C1'' in **5** is derived from the methyl and C1'' in **6** is derived from the carboxyl carbon of acetate (Figure 1).

Incorporation of $[2\text{-}^{13}\text{CD}_3]\text{Acetate}$ into DTX-5a and DTX-5b. Examination of the spectra of labeled **5** and **6** following incorporation of $[2\text{-}^{13}\text{CD}_3]\text{acetate}$ provided evidence of D-retention (and hence the fate of acetate hydrogen) as indicated by isotopically shifted peaks in the $\{^1\text{H}, \text{D}\}$ -decoupled ^{13}C spectra (Figure 4). Despite lower enrichments than were obtained previously with **4** and a greater degree of scrambling of the label, the patterns of D-retention in the backbone of the OKA moiety of **5** and **6** were found to be identical with that for **4**¹⁵ (Figure 1). All the pendant methyl groups retained up to two Ds. The side chain carbons C8', C2'', C10'', and C14'' of **6** each retained up to two Ds showing that, as in corresponding positions in **4**, dehydration does not occur at these positions following β -keto reduction. In **6**, as in **4**, the other acetate-methyl-derived positions on the side chain retained only one D following the enoyl reduction. The results for **5** were similar, although it was not possible to detect peaks indicating D₂ retention at C18, C2'', or C10'', probably owing to insufficient signal to noise ratio (average enrichments were higher for **5** than for **6**, but the quantity of material was smaller). Nevertheless, apart from these differences, the remaining pattern of D retention was fully consistent with **4** and **6**.

The quantitative NMR data for **5** and **6** labeled from $[2\text{-}^{13}\text{CD}_3]\text{acetate}$ showed a higher average ^{13}C enrichment ($4.6 \pm 0.5\%$ SD ^{13}C for **5** or $2.5 \pm 0.3\%$ SD ^{13}C for **6**) at carbons originating from the 2-position of acetate (Figure 1), compared to averages for all other positions of $3.5 \pm 0.5\%$ SD ^{13}C (for **5**) or $2.0 \pm 0.3\%$ SD ^{13}C (for **6**). The difference in % ^{13}C between the acetate-derived positions and the other positions, 1.1% (for **5**) or 0.5% (for **6**), is the average enrichment due to incorporation of unscrambled label. Another $1.108[1.0 - 0.046]\% = 1.1\%$ ^{13}C (for **5**) or $1.108[1.0 - 0.025]\% = 1.1\%$ ^{13}C (for **6**), originates from endogenous acetate at natural isotopic abundance. The remainder, av 2.4% ^{13}C (for **5**) or 0.9%

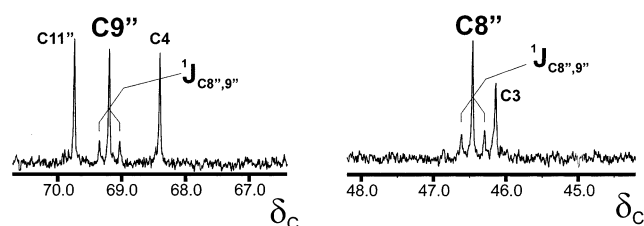


FIGURE 5. Portions of the 125.7 MHz ^{13}C NMR spectrum of **6** enriched from $[1,2\text{-}^{13}\text{C}_2]\text{glycine}$, showing ^{13}C - ^{13}C coupling satellites ($^1J_{\text{CC}} = 40.2$ Hz) due to incorporation of the intact C₂ unit of glycine into the C-8'' and C-9'' positions of **6**.

^{13}C (for **6**), represents a high degree of scrambling of the $[2\text{-}^{13}\text{CD}_3]\text{acetate}$ label.

After subtraction of the average scrambled label and NA contributions to the nonisotopically shifted component of each resonance, the retention $R\%$ of D from unscrambled $[2\text{-}^{13}\text{CD}_3]\text{acetate}$ label was calculated from the relative peak heights of the isotopically shifted (intensity $I_{\text{D1}}, I_{\text{D2}}$) and the remaining unshifted (residual intensity I_0) components. Thus for a CH group, $R\% = 100I_{\text{D1}}/(I_{\text{D1}} + I_0)$; for a CH₂, $R\% = 100(I_{\text{D1}} + 2I_{\text{D2}})/(2(I_0 + I_{\text{D1}} + I_{\text{D2}}))$; and for a CH₃ group, $R\% = 100(I_{\text{D1}} + 2I_{\text{D2}} + 3I_{\text{D3}})/(3(I_0 + I_{\text{D1}} + I_{\text{D2}} + I_{\text{D3}}))$. The average D-retention (R_{av}) was similar for both compounds and for different moieties within them: For labeled positions in the OKA moiety of **5** and **6**, R_{av} was $31 \pm 10\%$ SD and $32 \pm 11\%$ SD, respectively; for all Me groups, R_{av} was $39 \pm 6\%$ SD and $34 \pm 3\%$ SD, respectively; and for labeled positions in the side chains R_{av} was $34 \pm 6\%$ SD and $32 \pm 10\%$ SD. Owing to the difficulties in performing quantitative measurements with small quantities of material at low enrichment in the presence of a high degree of scrambling, the errors for D-retention at individual positions are large (ca. $\pm 15\%$ D). Nevertheless, the assumption of uniform ^{13}C enrichment from acetate across the molecule is justified by the results obtained from the $[1,2\text{-}^{13}\text{C}_2]\text{acetate}$ precursor experiments.

Incorporation of $[1,2\text{-}^{13}\text{C}_2]\text{Glycine}$ and $[2\text{-}^{13}\text{C}, ^{15}\text{N}]\text{Glycine}$ into DTX-5a and DTX-5b. Neither of the positions C8'' and C9'' in **5** and **6** incorporated unscrambled acetate directly (Figure 1), indicating that they originated from another biosynthetic source. Inspection of the structure suggested glycine as a likely precursor for these carbon positions and the contiguous N. To test this hypothesis, cultures of *P. maculosum* were supplemented with $[1,2\text{-}^{13}\text{C}_2]\text{glycine}$ and $[2\text{-}^{13}\text{C}, ^{15}\text{N}]\text{glycine}$ in separate experiments. Incorporation of $[1,2\text{-}^{13}\text{C}_2]\text{glycine}$ resulted in exclusive labeling at C8'' and C9'' in both **5** and **6**, as indicated by satellite doublets at the C8'' and C9'' resonances ($^1J_{\text{CC}} = 40.2$ Hz, enrichment to $1.6 \pm 0.1\%$ SD ^{13}C for **5**, $1.6 \pm 0.1\%$ SD ^{13}C for **6**) (Figure 5). Following incorporation of $[2\text{-}^{13}\text{C}, ^{15}\text{N}]\text{glycine}$ only the C8'' resonance in each compound displayed an additional doublet ($^1J_{\text{CN}} = 10.4$ Hz, enrichment to $1.9 \pm 0.1\%$ SD ^{13}C for **5**, $1.8 \pm 0.1\%$ SD ^{13}C for **6**) (Figure 6). No other carbons were enriched from these precursors, and there was no measurable scrambling of label, the measured percent ^{13}C for all other carbons corresponding to NA ($1.1 \pm 0.2\%$ SD).

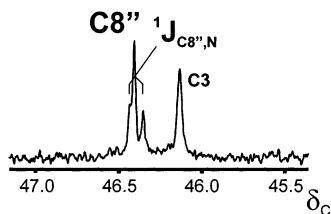


FIGURE 6. Portion of the 125.7 MHz ^{13}C NMR spectrum of **6** enriched from $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine, showing ^{13}C – ^{15}N coupling satellites ($^1J_{\text{CN}} = 10.4$ Hz) due to incorporation of the intact ^{13}C – ^{15}N unit of glycine into the N and C-8'' positions of **6**.

Discussion

Assembly of DTX-5a (**5**) and DTX-5b (**6**) by the subtropical dinoflagellate *P. maculosum* is entirely consistent with the biosynthesis of DTX-4 (**4**) produced by the temperate-water species *P. lima* (Figure 1)^{13,15} and establishes a PK mechanism for their biosynthesis. In both types of sulfate ester, an identical process involving a Favorski-like (or Tiffeneau–Demyanov-like²⁰) elimination of carboxyl acetate carbons from the nascent polyketide chain and addition of pendant methyl groups from the methyl carbons of acetate is followed in the assembly of the okadaic acid moiety. Combinations of these processes occur fairly frequently in dinoflagellate biosynthesis, yet are rare elsewhere.

The most significant difference between DTX-4 (**4**) and the DTX-5a/5b pair (**5**, **6**) resides in the structure of the sulfated side chains (Figure 1). The earlier labeling work with **4** clearly established that this portion of the molecule is a PK chain incorporating a glycolate starter unit, into which an oxygen atom is inserted to create an ester link.¹⁵ The ester link is also present in the side chain of **5** and **6**. Whereas the length of the so-called diol ester moiety in **4** and **6** is the same, in **5** it is one carbon shorter. The labeling data reveal that this is accounted for by the deletion of a carboxyl acetate carbon, presumably by a Favorski or Tiffeneau–Demyanov mechanism, to yield a shorter chain (by one carbon) in **5**. As we have noted, this in itself is not remarkable, but underscores a precise substrate specificity in which oxygen is inserted in the chain at a predetermined length (14 carbons and a nitrogen atom) from the terminus, regardless of the length of the side chain. Thus, the enzymatic system that catalyzes the insertion reaction (a putative Baeyer–Villigerase) uses this end of the side chain as a reference point to determine the point of oxygen insertion. Furthermore in **5**, oxygen insertion must occur *after* elimination of a carbon that previously formed a two-carbon unit with C1''. If the BV step is post-PKS, it strongly implies that the Favorski elimination step is an integral part of the polyketide process. This idea is supported by the results of Moore and co-workers on the biosynthesis of enterocins and wailupemycins in *Streptomyces maritimus*.²¹ During biosynthesis of the enterocin molecule, the polyketide chain undergoes a key Favorski rearrangement in which the carboxyl acetate carbon is oxidatively

excised from the polyketide backbone but is still retained as a pendant carboxyl group. Two genes, which are an integral part of the enterocin biosynthetic cluster, have been associated with this rearrangement step.²²

Another important difference between **4** and the **5/6** pair (Figure 1) is the presence of an amide link in the side chain of both **5** and **6**. The failure of $[1,2-^{13}\text{C}_2]$ acetate to label either C-8'' or C-9'' in either **5** or **6** clearly indicates participation of another precursor. This two-carbon unit with contiguous nitrogen suggested that the amino acid glycine might be incorporated directly into the nascent PK chain. This was confirmed in two separate feeding experiments in which DTX-5-producing cultures of *P. maculosum* were supplemented with $[1,2-^{13}\text{C}_2]$ - and $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine in separate feeding experiments. In both compounds, incorporation of $[1,2-^{13}\text{C}_2]$ -glycine resulted in labeling of C-8'' and C-9'' only, and the ^{13}C NMR data (Figure 5) indicated that the carbon skeleton of glycine was incorporated intact. In the second feeding experiment, incorporation of $[2-^{13}\text{C}, ^{15}\text{N}]$ glycine established that the glycine nitrogen was also retained during uptake and biosynthesis (Figure 6). As no ^{13}C – ^{13}C coupling occurred between C-8'' and C-9'' of $[1,2-^{13}\text{C}_2]$ -acetate-labeled **5** or **6**, we conclude that the incorporation of glycine into DTX-5 is not the result of relaxed substrate specificity of the PKS.

The manner in which glycine is incorporated into the sulfated side chain of **5** and **6** indicates that they are rare examples where an amino acid is used as an extender unit in a growing polyketide chain rather than as a starter or terminator unit. This strongly suggests that the DTX-5 synthetase is a hybrid of a PKS and nonribosomal peptide synthase (NRPS). Hybrid PKS/NRPS genes have been isolated from terrestrial cyanobacteria^{23,24} and actinomycetes¹⁸ and usually direct the incorporation of amino acids as starter or terminator units and, less frequently, as midchain extender units. To date, all hybrid PKS/NRPS systems characterized at the molecular level are modular,²⁵ which raises the likely possibility that DTX-5a and 5b are assembled on a modular PKS enzyme containing a module dedicated to the insertion of glycine into the growing PK.

The assembly of the sulfated diester is proposed to occur on a modular hybrid PKS/NRPS/PKS, though involvement of separate enzymes cannot be ruled out. Either way, it requires extension of a glycolate starter unit (based on labeling results with DTX-4), with six molecules of acetate followed by incorporation of a glycine molecule and subsequent chain extension with another three acetate units. The labeling data, which reveal the regiospecificity of the oxygen insertion step, indicate that the Favorskiase step precedes the BV step in the biosynthetic process. Sulfation is likely to be a post-PKS modification occurring after biosynthesis of the carbon chain is completed.

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Experimental Section

Culturing of *Prorocentrum maculosum*. For each feeding experiment, *P. maculosum* cultures (48 L) were grown in acid-washed Fernbach flasks (2.8 L capacity) each containing 1 L of an enriched filtered (0.3 μm) seawater medium.⁷ Tris buffer (0.5 g/L) prepared in distilled H₂O with pH adjusted to 7.4 before addition: 1.0 mM NaNO₃, 50 μM NaH₂PO₄, 10 μM Fe.EDTA. Trace metals: CuSO₄·5H₂O (0.04 μM), ZnSO₄·7H₂O (0.08 μM), CoCl₂·6H₂O (0.05 μM), MnCl₂·4H₂O (0.90 μM), Na₂·MoO₄·2H₂O (0.03 μM), Na₂EDTA (2.20 μM). Vitamins: cyanocobalamin (0.5 $\mu\text{g/L}$), biotin (0.3 $\mu\text{g/L}$), and thiamine·HCl (100 $\mu\text{g/L}$). Prior to inoculation, the prepared medium was autoclaved (20 min, 121 °C) and allowed to cool overnight. The Fernbach flasks containing medium were inoculated by adding a 60 mL aliquot of a *P. maculosum* stock culture to each flask under sterile conditions. Cultures were incubated at 21 °C (16 h light, 8 h dark cycle, irradiance approximately 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 30 d and shaken twice daily to facilitate nutrient and light distribution throughout the cultures. The cultures were supplemented with NaHCO₃ 15 d following inoculation as follows: NaHCO₃ (10 g) was prepared as a stock solution (120 mL), and aliquots (2 mL) were added to each *P. maculosum* culture under sterile conditions. A typical yield of dinoflagellate biomass was 310 g (wet weight). Labeled precursors [1,2-¹³C₂]glycine (99% ¹³C), [2-¹³C, ¹⁵N]glycine (99% ¹³C, >98% ¹⁵N), [2-¹³CD₃]acetate, and [1,2-¹³C₂]sodium acetate (99% ¹³C) were prepared as stock solutions (1.0 g of each glycine label in 53.5 mL distilled water, 3.6 g of each acetate label in 107 mL of distilled water) and autoclaved (121 °C, 15 min). Aliquots of each precursor stock solution (1.0 mL of glycine, 2.0 mL of acetate) were added to each Fernbach culture under sterile conditions 28 d after inoculation. All precursors were obtained commercially, except for [2-¹³CD₃]acetate, which was prepared from [2-¹³C]acetate (99% ¹³C) as described below.

Preparation of [2-¹³CD₃]Acetate from [2-¹³C]Acetate. [2-¹³CD₃]Acetate was prepared from [2-¹³C]acetate (3.0 g, 99% ¹³C) by successive exchanges in D₂O (22 mL, 160 °C) in the presence of solid sodium (approximately 130 mg). The mixture was evaporated to dryness following each exchange period, dissolved in D₂O (22 mL), and the process was repeated. The reaction mixture following eight exchange reactions was collected, and the pH was adjusted to 2.0 with 1 M HCl. The deuterated acetic acid was collected by distillation and adjusted to pH 8.0 with 1 M NaOH. The mixture was evaporated to dryness, and the sodium [2-¹³CD₃]acetate yield was recorded. The [2-¹³CD₃]acetate yield (99% ¹³C, >99% D as determined by NMR) obtained from two sets of eight exchange reactions was 5.0 g.

Harvesting and Extraction of *Prorocentrum maculosum* Biomass. Cells of *P. maculosum* biomass were harvested by centrifugation, 30 d following inoculation, and immersed in boiling water (10 min) to inactivate esterases or lipases that hydrolyze either of the two ester linkages in DTX-5a and 5b. The dinoflagellate cells (weight not recorded) were extracted by sonication (10 min) in methanol (approximately 3 L), and the extract was evaporated to dryness (Büchi RE III Rotovapor). The yield of methanol-extracted residue varied between experiments, ranging from 8.78 g ([2-¹³CD₃]acetate feeding experiment) to 20.93 g ([1,2-¹³C₂]glycine feeding experiment). For each experiment, the methanol-soluble residue was dissolved in 70% methanol/30% water and partitioned successively against hexane (3 × 150 mL), diethyl ether (3 × 150 mL), and butanol (200 mL, 150 mL, 110 mL). The aqueous phase following hexane extraction was adjusted to 75% water prior to subsequent partitioning steps.

Purification of DTX-5a and DTX-5b. After each feeding experiment, DTX-5a (**5**) and -5b (**6**) were purified from the butanol-extracted residue obtained from the partitioning step. This began with chromatography of the methanol-soluble residue using LH-20 (2 cm × 72 cm, 100% methanol mobile phase). Fractions (76 × 100 drops) were collected and analyzed by thin-layer chromatography. The mobile phase was prepared

by partitioning a 40% butanol, 50% H₂O, 10% acetic acid mixture and collecting the upper butanol layer. Fractions containing DSPs appeared as pink spots on the vanillin-sprayed TLC plates. DSP-containing fractions were combined, evaporated to dryness, and dissolved in a mixture of 60% dichloromethane/40% methanol (1 mL) and further purified by normal phase flash chromatography (1.0 cm × 47.0 cm column; 60% dichloromethane/40% methanol mobile phase) under N₂ pressure. Fractions (72 × 50 drops) were collected and analyzed by TLC in the usual way. Once again, DSP-containing fractions were combined, evaporated to dryness, and dissolved in methanol for LH-20 chromatography (0.9 cm × 53.4 cm; 100% methanol mobile phase). Fractions (46 × 40 drops) were analyzed by TLC, and those containing DSP toxins were once again combined and evaporated to dryness. The combined fractions were analyzed by analytical HPLC (4.6 mm × 2.5 cm column; 31% acetonitrile/69% water, 1 mM ammonium acetate mobile phase; 1 mL/min flow rate, UV detection at 235 nm) using our own DTX-5a and 5b standards. Final purification of **5** and **6** was achieved by semipreparative HPLC (9.4 mm × 25 cm column; 31% acetonitrile/69% water, 2 mM ammonium acetate mobile phase; 2.0 mL/min. flow rate; UV detection at 210 nm). Peaks corresponding to **5** and **6** were collected following multiple injections (50 μL) and fractions containing pure DTX-5a and 5b evaporated to dryness and stored at -20 °C.

NMR Spectroscopy. Samples (2–10 mg) of purified **5** and **6** were dissolved in CD₃OD (0.6 mL in 5 mm tubes) for NMR spectroscopy at 500.13 MHz (¹H) or 125.7 MHz (¹³C) at 20 °C. ¹H and ¹³C resonances of **5** and **6** in CD₃OD have been assigned previously.¹² Procedures used for recording spectra under quantitative conditions for measurement of absolute ¹³C enrichment at each position and calculation methods were similar to those previously described.^{13,15,26} Quantitative ¹³C spectra with {¹H}-Waltz, or {¹H, D}-Waltz/Garp, decoupling were recorded with a 5 s delay and with no ¹H irradiation between acquisitions, to suppress NOE. A few microliters of CH₂Cl₂ at natural ¹³C abundance (assumed 1.108% ¹³C) was added to the solution as an NA “standard”, the molar ratio *r* of **5** (or **6**) to CH₂Cl₂ being measured from the ¹H spectrum. The percent ¹³C at position *i* of **5** or **6** is 1.108 *I_i*/*rI*_{CH₂Cl₂}, where *I_i* and *I*_{CH₂Cl₂} are the respective ¹³C integrated intensities of position *i* and of the ¹³CH₂Cl₂ resonance. Patterns of intact acetate unit incorporation were determined from the intensities of ¹³C singlet, doublet, and multiplet resonances and from matching of ¹³C–¹³C coupling constants. Two-dimensional ¹³C INADEQUATE spectra were also recorded from samples enriched from [1,2-¹³C₂]acetate to confirm the positions of incorporation of intact ¹³C–¹³C units. Acquisition conditions were: f2 spectral width (SW2) 198.8 ppm, 1K data points; f1 SW1 25 kHz, 512 increments; 320 scans/increment (**5**); 192 scans/increment (**6**). Some isotopically-shifted resonances in **5** and **6** derived from [2-¹³CD₃]acetate were located by comparison with spectra of **4** derived from the same precursor, in areas where the structures coincided and incorporation was low.

Acknowledgment. The authors thank Ms Cheryl Craft and Dr. Anthony Windust for assistance and advice. G.R.M. was supported by a Natural Sciences and Engineering Research Council of Canada grant to J.L.C.W. Issued as NRCC publication # 42372.

Supporting Information Available: ¹³C NMR Spectra, ¹³C INADEQUATE spectra, and chemical shifts and coupling constant of DTX-5a, DTX-5b, and DTX-4. This information is available free of charge via the Internet at <http://pubs.acs.org>.

JO0204754

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