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Biosynthetic Origin of [7.7]Paracyclophanes From Cyanobacteria

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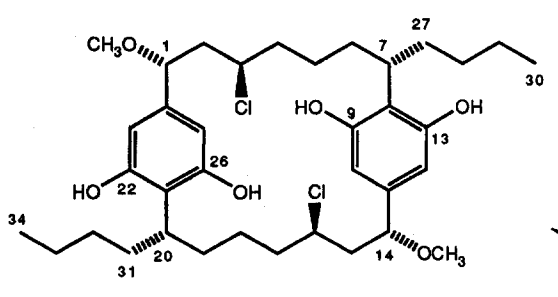
Abstract. The biosyntheses of nostocyclophane D (**1**) and cylindrocyclophane D (**2**) have been investigated by the administration of sodium [1,2-$^{13}C_2$]acetate, sodium [1-^{13}C,$^{2}H_3$]acetate, sodium [2-^{13}C,$^{2}H_3$]acetate, sodium [$^{2}H_3$]acetate, sodium [1-^{13}C,$^{18}O_2$]acetate, sodium [2-^{13}C,$^{18}O_2$]acetate, L-[methyl-^{13}C]methionine, and L-[methyl-^{13}C,$^{2}H_3$]methionine to cultures of the cyanobacteria *Nostoc linckia* and *Cylindrospermum licheniforme*, respectively. The isotopically labeled compounds produced by these feeding experiments indicate that the structural skeletons of these [7.7]paracyclophanes are polyketides that have been constructed by dimerization of acetate-derived nonaketides. Acetate has also been identified as the source of the branched methyl groups in cylindrocyclophane D.

INTRODUCTION

In the past decade chemical investigations of cyanobacteria (blue-green algae) have led to the isolation and identification of numerous novel secondary metabolites which include cyclic peptides such as the microcystins[1] and macrolides exemplified by the scytophycins.[2] More recently studies have been initiated on the biosynthesis of some of these natural products, e.g. microcystin-LR[3] and tolytoxin (6-hydroxy-7-*O*-methylscytophycin B).[4] In 1990 we reported the discovery of the first naturally-occurring [*m.n*]paracyclophanes. Cultures of *Nostoc linckia* (Roth) Bornet and *Cylindrospermum licheniforme* Kützing, two species of blue-green algae belonging to the family Nostocaceae, had been found to produce a number of related [7.7]paracyclophanes which exhibited moderate cytotoxicity against human tumor cell lines such as KB and LoVo.[5] Unfortunately none of the compounds showed any selective antitumor activity to mark them as candidates for drug development. [*m.n*]Paracyclophanes had already been extensively studied in the field of host-guest chemistry[6] and were well known through synthesis.

Nostocyclophane D (**1**) and cylindrocyclophane D (**2**) were identified as major [7.7]paracyclophanes in *N. linckia* UTEX B1932[7,8] and *C. licheniforme* ATCC 29204,[7,9] respectively. A perusal of their structures suggested several possible biosynthetic pathways to their formation utilizing polyketide and/or shikimic acid intermediates. To determine the origins of the various carbons, hydrogens, and oxygens, various stable isotope labeled precursors have now been fed to cultures of *N. linckia* and *C. licheniforme* and this has resulted in the isolation of isotopically enriched samples of **1** and **2**. Detailed NMR analysis of these labeled compounds has enabled us to determine the location of the enriched atoms in each metabolite. The results strongly suggest that these cyclophanes are produced by the dimerization and modification of acetate-derived nonaketides.



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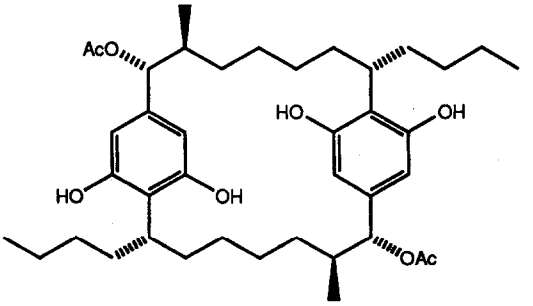

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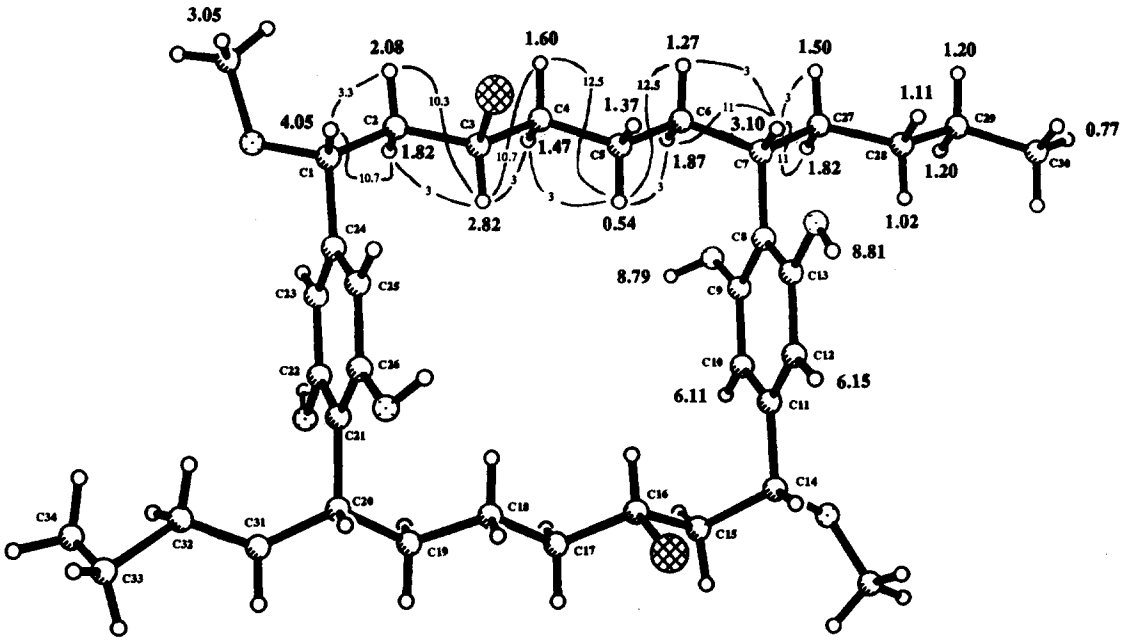

Fig. 1. Assignments of ^1H NMR chemical shifts for nostocyclophane D (1) based on correlations of coupling constants with a computer-generated X-ray crystallographic drawing.

RESULTS

Nostocyclophane D (1). Before analysis of samples from feeding experiments could be carried out, the ^1H and ^{13}C NMR spectra of nostocyclophane D (1) had to be completely and rigorously assigned. Much of this task had already been accomplished during the structure determination studies.[10] In the full paper on the structures of the nostocyclophanes,[8] however, some errors were inadvertently made in transposing these assignments from Dr. Chen's thesis. The correct assignments are presented here in Table 1 and Fig. 1. Assuming that the preferred solution-state conformation of 1 is identical with the solid-state conformation (Fig. 1), the *pro-R* and *pro-S* protons of the various methylenes could be distinguished on the basis of coupling constant data. For example, the ^1H signals at 1.37 and 0.54 ppm were assigned to the *pro-R* and *pro-S* hydrogens on C-5(18), respectively, using the following arguments: The H-7(20) signal at 3.10 ppm, a triplet (J = 11 Hz) of triplets (J = 3 Hz), was shown to be coupled by 11 Hz to the *pro-S* H-6(19) signal at 1.87 ppm and by 3 Hz to the *pro-R* H-6(19) signal at 1.27 ppm. In turn the *pro-S* H-6(19) signal at 1.87 ppm, a 1:3:3:1 quartet (J = 11 Hz) of doublets (J = 3.5 Hz), showed a 11 Hz coupling to the *pro-R* H-5(18) signal at 1.37 ppm and a 3.5 Hz coupling to the *pro-S* H-5(18) signal at 0.54 ppm, whereas the *pro-R* H-6(19) signal at 1.27 ppm, a broad triplet (J = 11 Hz) of triplets (J = 3.5 Hz), showed a 3.0 Hz coupling to the *pro-R* H-5(18) signal at 1.37 ppm and a 12.5 Hz coupling to the *pro-S* H-5(18) signal at 0.54 ppm. Similar arguments were used to assign the signals at 1.11 and 1.02 ppm to the *pro-R* and *pro-S* hydrogens on C-28(32), respectively. Large and small couplings between protons could be easily detected and distinguished by relative intensities of cross peaks in the COSY spectrum (Fig. 2). For example, the H-7(20) signal at 3.10 ppm showed intense cross peaks (large couplings) to the *pro-S* H-6(19) signal at 1.87 ppm and the *pro-R* H-27(31) signal at 1.82 ppm, but weak cross peaks (small couplings) to the *pro-R* H-6(19) signal at 1.27 ppm and the *pro-S* H-27(31) signal at 1.50 ppm. In turn the *pro-S* H-27(31) signal at 1.50 ppm showed intense cross peaks to the *pro-R* H-27(31) signal at 1.82 ppm and the *pro-S* H-28(32) signal at 1.02 ppm and a weak cross peak with the *pro-R* H-28(32) signal at 1.11 ppm, whereas the

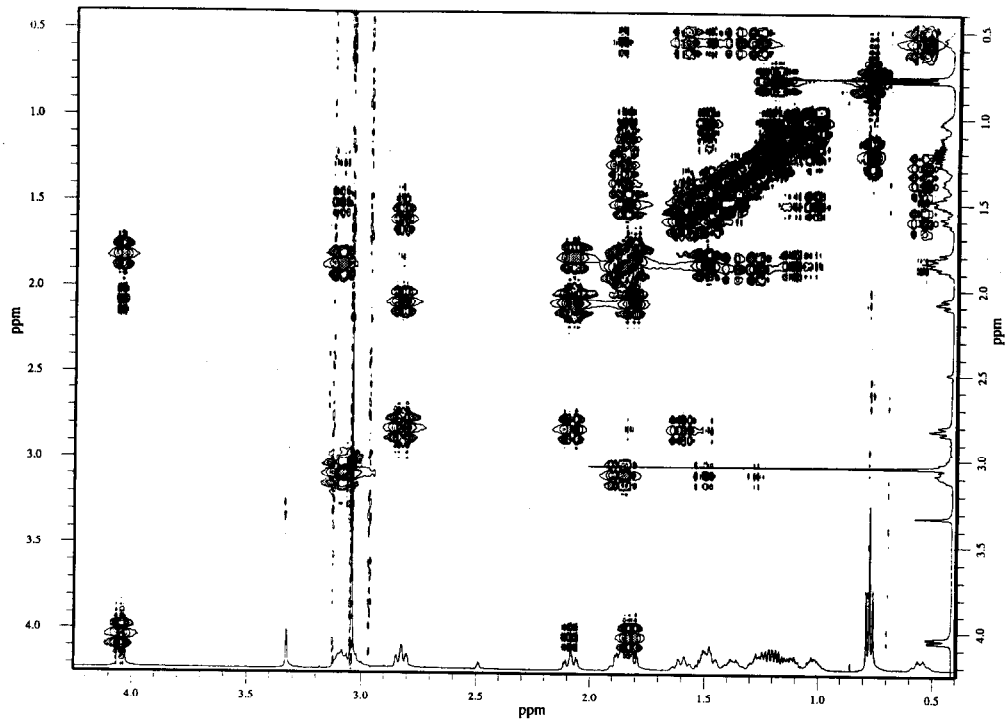

Fig. 2. 500 MHz ^{1}H double-quantum filtered (phase-sensitive) COSY spectrum of nostocyclophane D (1) in DMSO-d_6.

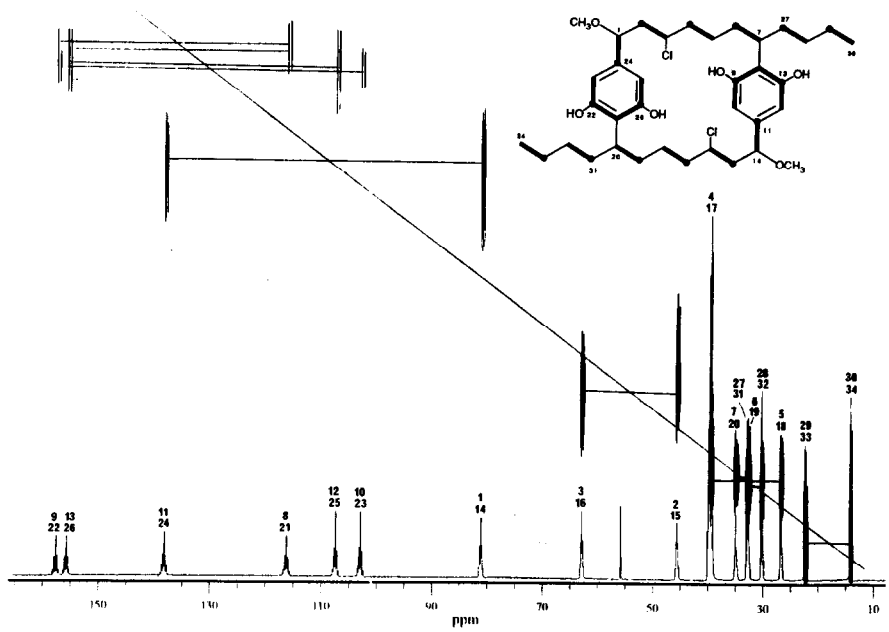

Fig. 3. 125 MHz ^{13}C INADEQUATE spectrum of ^{13}C-labelled nostocyclophane D (1) in DMSO-d_6. Sample obtained from *Nostoc linckia* UTEX B1932 grown on sodium [1,2-^{13}C]acetate.

pro-R H-27(31) signal at 1.82 ppm showed an intense cross peak with the *pro-R* H-28(32) signal at 1.11 ppm and a weaker cross peak with the *pro-S* H-28(32) signal at 1.02 ppm.

It was apparent from both the ^{1}H and ^{13}C NMR spectra, which exhibited only 18 resonances instead of 36 due to the two-fold symmetry in the molecule, that nostocyclophane D was a dimer. Taking into account the dimeric nature of these carbocyclic compounds, we hypothesized that the carbon skeleton of nostocyclophane D had to be constructed from two identical subunits which were connected at C-7/C-8 and C-20/C-21. On first inspection the molecular subunit appeared to have a totally acetate-derived polyketide origin, but in the absence of feeding experiments one could not rule out the possibility that shikimic acid was the source of the aromatic portions of nostocyclophane D.

The acetate-derived origin of the contiguous carbons in nostocyclophane D (**1**) was immediately revealed from a feeding experiment with sodium [1,2-$^{13}C_2$]acetate. For this experiment the precursor was diluted with unlabeled acetate to reduce the probability of interconnected intact [1,2-$^{13}C_2$]acetate units in the **1** that was produced. The proton-decoupled ^{13}C NMR spectrum of the nostocyclophane D exhibited "triplet" patterns for all of the carbons except the OMe carbons. Each of these "triplets" was composed of a singlet for the natural abundance ^{13}C superimposed upon a doublet for the excess ^{13}C from incorporation of intact [1,2-$^{13}C_2$]acetate. The incorporation of intact [1,2-$^{13}C_2$]acetate units was verified by ^{13}C-^{13}C COSY and INADEQUATE (Fig. 3) experiments. Additional correlations could be seen in the aromatic "triplets" as a result of conformational isomerism for the aromatic rings. In one conformation C-9(22) and C-10(23) are *anti* to H-14(1) and H-7(20) whereas C-12(25) and C-13(26) are *syn* as shown in Fig. 1. In the second conformation, however, C-9(22) and C-10(23) are *syn* to H-14(1) and H-7(20) whereas C-12(25) and C-13(26) are *anti*. Even though [1,2-$^{13}C_2$] acetate is assimilated intact into only certain carbons of the aromatic rings, visualization of the labeling pattern is obscured by this transmutation (Fig. 3).

The manner in which sodium [1,2-$^{13}C_2$] acetate is incorporated into nostocyclophane D (**1**) suggests that the compound is constructed from two identical subunits (Table 1). Each subunit appears to be biosynthesized in a typical polyketide fashion whereby eight malonate units are condensed sequentially with a starter acetate unit to form a nonaketide. Carbons from the last four acetate units of the nonaketide are involved in the formation of the aromatic ring and the last carbon atom in the chain is apparently lost by decarboxylation. No significant ^{13}C-^{13}C coupling is observed between C-7(20) and C-8(21) to indicate that a more complex biosynthetic scheme is involved.

The incorporation of sodium [2-^{13}C,$^{2}H_3$]acetate and sodium [1-^{13}C,$^{2}H_3$]acetate into nostocyclophane D verified the conclusions drawn from the sodium [1,2-$^{13}C_2$] acetate labeling experiment. Carbon atoms which were expected to be derived from C-2 of acetate were enriched (4-6% specific incorporation) in the sodium [2-^{13}C,$^{2}H_3$]acetate feeding experiment (Table 2). α-Isotope-shifted carbon resonances[11] were observed in the $^{13}C\{^{1}H,^{2}H\}$ NMR spectrum[12] of this sample which indicated that a single deuterium was present on each of C-1(14), C-3(16), C-5(18), C-7(20), C-10(23), C-12(25), and C-28(32) and three deuteriums were present on C-30(34). It has been well-established that one deuterium atom is lost during the condensation of CO_2 and acetate to form malonate, while a second one is lost during the dehydration step in the reduction-dehydration-reduction cycle of the polyketide chain elongation process. Carbon atoms which were derived from C-1 of acetate were enriched (6-19% specific incorporation) in the sodium [1-^{13}C,$^{2}H_3$]acetate feeding experiments. The β-isotope-shifted carbon resonances[13] observed in the $^{13}C\{^{1}H,^{2}H\}$ NMR spectrum of this sample also indicated the presence of a single deuterium on each of C-1(14), C-3(16), C-5(18), C-7(20), C-10(23), C-12(25), and C-28(32) and three deuteriums on C-30(34), in full agreement with the results obtained from the sodium [2-^{13}C,$^{2}H_3$]acetate feeding experiment.

In the $^{13}C\{^{1}H,^{2}H\}$ NMR spectrum of [2-^{13}C,$^{2}H_3$]acetate-labeled **1** (Fig. 4), the four singlets at 30.10, 30.02, 29.68, and 29.60 ppm are assigned to C-28(32) found in four different molecular species having the $^{1}H/^{2}H$ labeling patterns C(28,32)H_2-CH_2-CH_3, C(28,32)H_2-CH_2-CD_3, C(28,32)HD-CH_2-CH_3, and C(28,32)HD-CH_2-CD_3, respectively. The two broad singlets at 26.61 and 26.18 ppm are assigned to

TABLE 1. ^{13}C NMR data [125 MHz, DMSO-d_6] for nostocyclophane D (1) labelled by sodium [1,2-$^{13}C_2$]acetate.[a]

Carbon position	δ_C (Number of attached protons; J_{CC} in Hz)	INADEQUATE correlations
30(34)	14.0 (3; 34.5)	29(33)
29(33)	22.2 (2; 34.5, 34.4)	30(34)
28(32)	30.1 (2; 34.9, 34.4)	27(31)
27(31)[b]	32.7 (2; 34.9, 34.6)	28(32)
7(20)	34.9 (1; 44.6, 34.6, 34.5)	6(19)
6(19)[b]	32.6 (2; 34.7, 34.5)	7(20)
5(18)	26.6 (2; 35.1, 34.7)	4(17)
4(17)	39.3 (2; 36.1, 35.1)	5(18)
3(16)	62.7 (1; 37.8, 36.1)	2(15)
2(15)	45.5 (2; 40.8, 37.8)	3(16)
1(14)	81.0 (1; 47.0, 40.8)	24(11)
24(11)	138.1 (0; 59.4, 59.0, 47.0)	1(14)
23(10)[c]	107.3 (1; 63.3, 59.4)	22(9)
22(9)[b,c]	155.6 (0; 69.3, 63.3)	21(8), 23(10)
21(8)	116.1 (0; 69.3, 69.3, 44.6)	22(9), 26(13)
26(13)[b,c]	157.5 (0; 69.3, 64.2)	21(8), 25(12)
25(12)[c]	102.9 (1; 64.2, 59.0)	26(13)
OCH_3	55.7 (3)	—

[a]Specific incorporation into each carbon is roughly 6.5% (see Experimental). [b]Signals reassigned from previous literature.[8] [c]Assignments made by correlating ^{13}C and ^1H chemical shifts (HMQC data) and then comparing ^1H chemical shifts of 1 with those of nostocyclophane B. In the cases of nostocyclophanes A and B, H-10(23) and H-12(25) were first assigned from NOE data;[8] C-9(22), C-10(23), C-12(25), and C-13(26) assignments then followed from HMQC and HMBC data.

TABLE 2. $^{13}C\{^1H,^2H\}$ NMR data for nostocyclophane D (1) labelled With sodium [2-^{13}C,$^2H_3$] acetate.[a]

Carbon	δ_C	% SI[b] ^{13}C	% SI[b] ^2H	Isotope shifts $\Delta\delta_C$ (rel int)[c]	Isotope shift type[d]
30(34)	14.0	3.9	3.8	-0.61 (25)	2α
				-0.64 (10)	2α + γ
				-0.92 (142)	3α
				-0.94 (31)	3α + γ
29(33)	22.2	0	0		
28(32)	30.1	4.8	3.4	-0.09 (31)	3γ
				-0.42 (76)	α
				-0.51 (63)	α + 3γ
27(31)	32.7	0	0		
7(20)	34.9	4.2	0.5	-0.42 (12)	α
6(19)	32.5	0	0		
5(18)	26.6	5.8	5.0	-0.42 (188)	α
4(17)	39.3	0	0		
3(16)	62.7	5.3	4.2	-0.39 (151)	α
2(15)	45.5	0	0		
1(14)	81.0	4.2	3.5	-0.46 (144)	α
24(11)	138.1	0			
23(10)	102.9	3.9	1.1	-0.26 (27)	α
22(9)	157.5	0			
21(8)	116.1	3.9			
26(13)	155.6	0			
25(12)	107.3	3.5	1.1	-0.29 (29)	α
OCH_3	56.7	0	0		

[a]125 MHz, DMSO-d_6. [b]Rough estimates of specific incorporation. [c]In ppm relative to natural abundance ^{13}C signal (relative intensity = 100). [d]Isotope shifted peaks are due to the effect(s) of deuterium at the positions noted.

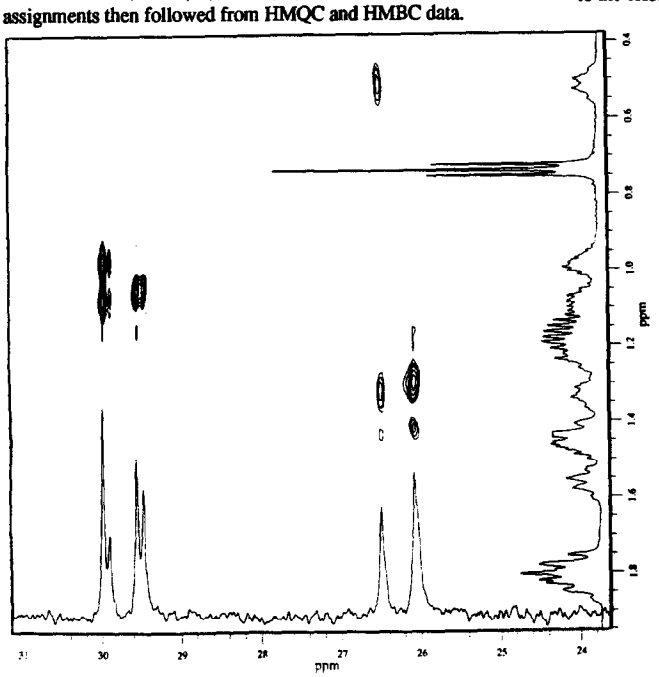

Fig. 4. $^{13}C\{^1H,^2H\}$ HETCOR spectrum for nostocyclophane D (1) labelled by sodium [2-^{13}C,$^2H_3$]acetate. Shown in the ^{13}C domain are the unshifted and ^2H isotope-shifted signals for C-5(18) in the region 26.0-26.7 ppm and C-28(32) in the region 29.5-30.2 ppm. The α peak for C-5(18) and the α and α+3γ peaks for C-28(32) show cross peaks with only the *pro-R* proton signals.

undeuterated and monodeuterated C-5(18), respectively. The high field shoulders on these latter peaks denote the presence of molecular species of **1** possessing C(5,18)H_2-CH_2-CD and C(5,18)HD-CH_2-CD units, respectively.

Analysis of the two-dimensional $^{13}C\{^1H,^2H\}$ HETCOR spectrum[14] of the [2-^{13}C,$^2H_3$]acetate-labeled **1** (Fig. 4) allowed us to determine where the deuteriums were located on C-5(18) and C-28(32). The signals for undeuterated C-5(18) (26.61 ppm) and C-28(32) (30.10/30.02 ppm) showed cross peaks to the two non-equivalent H_2-5(18) (0.54 and 1.37 ppm) and H_2-28(32) (1.02 and 1.11 ppm) signals, respectively. Each of the signals for monodeuterated C-5(18) (26.18 ppm) and C-28(32) (29.68/29.60 ppm), however, showed only one cross peak to a proton signal. The 26.18 ppm signal correlated with a *pro-R* H-5(18) signal at 1.34 ppm (upfield deuterium isotope shift from 1.37) whereas the 29.68/29.60 ppm signals correlated with a *pro-R* H-28(32) signal at 1.07 ppm (upfield isotope shift from 1.11 ppm). From these data we concluded that the acetate-derived hydrogens on C-5(18) and C-28(32) were in the *pro-S* positions.

Lower levels of deuterium incorporation were observed at C-10(23) and C-12(25) in both the [2-^{13}C,$^2H_3$] and [1-^{13}C,$^2H_3$]acetate-labeled compounds. Both of these carbons would have been in the middle of a 1,3-dione segment of the polyketide chain late in the biosynthetic process. Deuterium on a carbon in the middle of such a dione system is frequently washed out by enolization and exchange with the medium. A lower incorporation of deuterium was also observed at C-7(20) in duplicate experiments.

Administration of sodium [1-^{13}C,$^{18}O_2$]acetate to cultures of *Nostoc linckia* produced **1** which was enriched in ^{13}C (5-8% specific incorporation) at each of the carbons which had been proposed to be derived from C-1 of acetate. ^{18}O isotope-shifted peaks[15] were observed at C-22(9) ($\Delta\delta$ = -0.012, 3.3% specific incorporation) and C-26(13) ($\Delta\delta$ = -0.012, 3.6% specific incorporation), indicating, as expected, that the phenolic oxygens are acetate-derived. Curiously this sample of **1** exhibited a more broadened resonance for C-1(14) than usual and this prompted us to check the possibility that the oxygen on C-1(14) might also be acetate-derived, arising by the rearrangement of oxygen from C-24(11) to C-1(14) *via* an epoxide intermediate formed from an alkene chlorohydrin. This proposed pathway was investigated by preparing and feeding sodium [2-^{13}C,$^{18}O_2$]acetate to *N. linckia*. No isotope-shifted resonance for C-1(14), however, was observed in the ^{13}C NMR spectrum of nostocyclophane D isolated from this culture. Therefore the oxygen at C-1(14) appeared to be derived from molecular oxygen; however, this route was not rigorously established by experiment.

Cylindrocyclophane D (2). Using COSY and HMQC experiments, the signals in the ^1H and ^{13}C NMR spectra of **2** in MeOH-d_4 were completely assigned (see Experimental). Assuming that **2** has the same conformational structure as **1**, the *pro-R* and *pro-S* hydrogens of the various methylenes could be readily differentiated by determining the relative sizes of the couplings between these protons and adjacent protons. Large and small coupling constants were found to correlate nicely with strong and weak intensities of cross peaks in the COSY spectrum for **2**. The signal for H-7(20) of **2** (3.14 ppm) showed intense cross peaks (large couplings) to the *pro-S* H-6(19) signal at 2.02 ppm and the *pro-R* H-27(31) signal at 1.90 ppm and weak cross peaks (small couplings) to the *pro-R* H-6(19) signal at 1.32 ppm and the *pro-S* H-27(31) signal at 1.49 ppm. In turn the 1.32 ppm signal exhibited intense and weak cross peaks and the 2.02 ppm signal exhibited weak and intense cross peaks with the *pro-R* (0.71 ppm) and *pro-S* H-5(18) (0.94 ppm) signals, respectively, and the 1.49 ppm signal displayed intense and weak cross peaks and the 1.90 ppm signal displayed weak and intense peaks with the *pro-S* (1.06 ppm) and *pro-R* H-28(32) (1.17 ppm) signals, respectively. Similarly, the signal for H-2(15) of **2** (1.76 ppm) showed intense and weak cross peaks to the *pro-R* (0.69 ppm) and *pro-S* (0.78 ppm) H-3(16) signals. Unlike the *pro-R* H-5(18) and *pro-S* H-28(32) signals, which resonate at higher field than the *pro-S* H-5(18) and *pro-R* H-28(32) signals, possibly due to diamagnetic shielding of the *pro-R* H-5(18) and *pro-S* H-28(32) protons by the nearby aromatic rings, the *pro-S* H-3(16) signal resonates at lower field than the *pro-R* H-3(16) signal. The reason for this difference is unclear, but the *pro-R* H-5(18) and *pro-S* H-28(32) protons are oriented over the portions of the aromatic rings bearing the two phenolic hydroxy groups whereas the *pro-S* H-3(16) protons are not.

TABLE 3. $^{13}C\{^1H,^2H\}$ NMR data for cylindrocyclophane D (2) labelled with sodium [2-^{13}C,$^2H_3$] acetate.[a]

Carbon	δ_C	% SI[b] ^{13}C	% SI[b] ^2H	Isotope shifts $\Delta\delta_C$ (rel int)[c]	Isotope shift type[d]
30(34)	14.5	3.1	1.8	-0.30 (3)	α
				-0.61 (12)	2α
				-0.89 (42)	3α
29(33)	23.9	0	0		
28(32)	31.6	3.7	2.7	-0.42 (57)	α
27(31)	34.8	0	0		
7(20)	36.8	4.3	2.7	-0.42 (45)	α
6(19)	35.2	0	0		
5(18)	30.5	5.1	4.5	-0.41 (96)	α
4(17)	29.6	0	0		
3(16)	34.4	5.1	5.1	-0.36 (105)	α
2(15)	40.0	0	0		
1(14)	83.4	3.8	2.4	-0.42 (39)	α
24(11)	139.2	0			
23(10)	105.2	3.7	2.1	-0.25 (41)	α
22(9)	158.9	0			
21(8)	118.5	2.7			
26(13)	157.0	0			
25(12)	109.5	3.4	2.1	-0.28 (44)	α
CH_3CO_2	21.2	4.0	3.0	-0.21 (3)	α
				-0.44 (14)	2α
				-0.65 (65)	3α
CH_3CO_2	172.6				
CH_3	16.6	4.4	1.8	-0.28 (85)	α
				-0.56 (64)	2α

[a]125 MHz, MeOH-d_4. [b]Specific incorporation (see Experimental). [c]In ppm relative to natural abundance ^{13}C signal (relative intensity = 100). [d]Isotope shifted peaks are due to the effect(s) of attached deuterium(s).

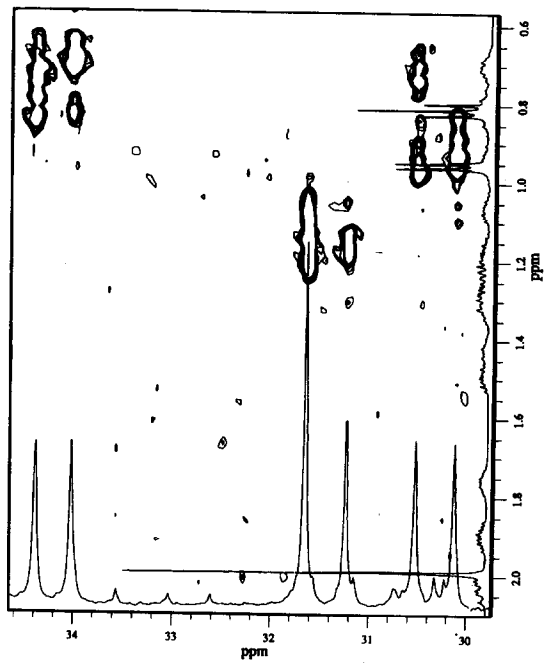

Fig. 5. $^{13}C\{^1H,^2H\}$ HETCOR spectrum for cylindrocyclophane D (2) labeled by sodium [2-^{13}C,$^2H_3$]acetate. Shown in the ^{13}C domain are the unshifted and ^2H isotope-shifted (to higher field) signals for C-5(18) in the region 30.0-30.5 ppm, C-28(32) in the region 31.2-31.7 ppm, and C-3(16) in the region 34.0-34.4 ppm.

The results from the feeding experiments with *Cylindrospermum licheniforme* were analogous to those described above with *N. linckia*. The NMR data for cylindrocyclophane D (**2**) isolated from a culture of *C. licheniforme* which had been administered sodium [1,2-$^{13}C_2$]acetate indicated that the carbon skeleton of cylindrocyclophane D is constructed in the same manner as in nostocyclophane D (**1**). Feeding experiments with sodium [2-^{13}C,$^2H_3$] acetate further verified this analogy (Table 3), although the loss of deuterium label at C-7 in cylindrocyclophane D was insignificant compared with the nostocyclophane D case. Again α-isotope-shifted carbon resonances in the $^{13}C\{^1H,^2H\}$ NMR spectrum of this sample indicated that one deuterium was present on C-1(14), C-3(16), C-5(18), C-7(20), C-10(23), C-12(25), and C-28(32) and three deuteriums were present on C-30(34). Analysis of the two-dimensional $^{13}C\{^1H,^2H\}$ HETCOR spectrum[14] of the [2-^{13}C,$^2H_3$]acetate-labeled **2** (Fig. 5) established that deuterium was located solely in the *pro-R* position on C-5(18) and the *pro-S* position on C-28(32); the deuterium on C-3(16), however, was distributed between the two methylene protons, but predominantly (75%) in the *pro-S* position.

An unusual feature of cylindrocyclophane D (**2**) is the presence of branched methyl groups at C-2(15). Experiments in which L-[methyl-^{13}C] methionine and L-[methyl-^{13}C,$^2H_3$] methionine were fed to cultures of *Cylindrospermum licheniforme* did not result in any enrichment of these methyls. The sodium [1,2-$^{13}C_2$]acetate feeding experiment, however, indicated that these methyl groups are also acetate-derived. This was verified by the sodium [2-^{13}C,$^2H_3$]acetate feeding experiment (Table 3). In addition to the normal ^{13}C signal (16.56 ppm) for Me on C-2(15), the $^{13}C\{^1H,^2H\}$ NMR spectrum showed isotope-shifted resonances [$\Delta\delta$ = -0.28ppm (1.1%

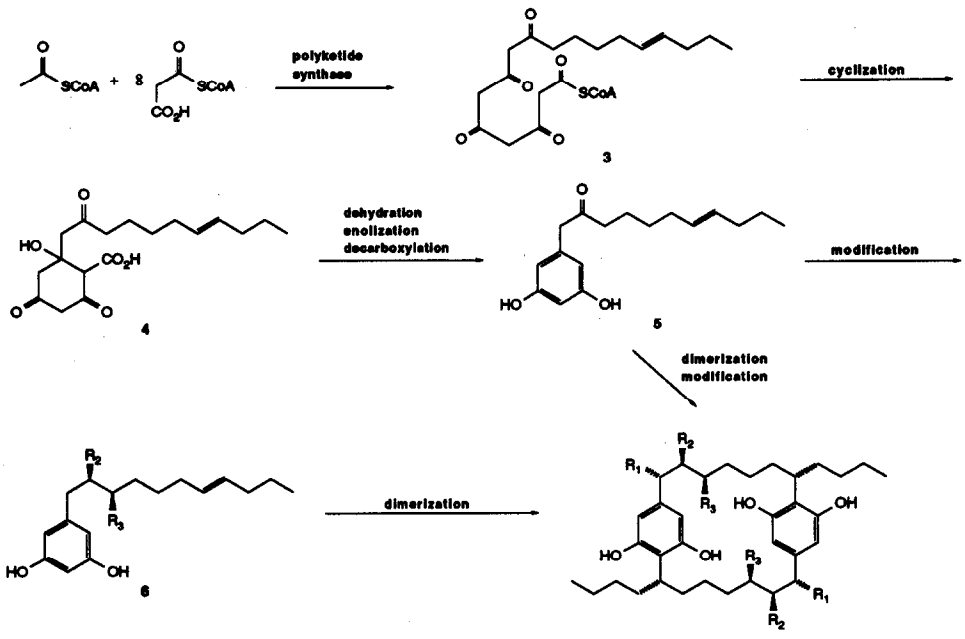

Fig. 6. Proposed biosynthetic pathways to nostocyclophane D (1: R_1=OMe, R_2=H, R_3=Cl) and cylindrocyclophane D (2: R_1=OAc, R_2=Me, R_3=H).

incorporation above natural abundance) and $\Delta\delta$ = -0.56ppm (0.7% incorporation above natural abundance)] due to the α-isotope effect of one and two deuterium atoms. This result indicates that acetate, presumably in the form of malonate, is added to the carbonyl group, the one that will become C-2(15) in **2**, of the polyketide precursor in an aldol-type condensation. The carboxylic acid group of the resulting acetate side-chain and the hydroxyl group are then lost by decarboxylation/dehydration and the exo-methylene that ensues is reduced to the methyl group. Methyl groups derived from C-2 of acetate have been found previously in antibiotics of the virginiamycin family,[16] aurantinins,[17] myxopyronin A,[18] pseudomonic acid (mupirocin),[19] and oncorhyncolide.[20]

DISCUSSION

Feeding experiments with stable isotope precursors indicate that nostocyclophane D (**1**) from *Nostoc linckia* and cylindrocyclophane D (**2**) from *Cylindrospermum licheniforme* are acetate-derived polyketides. A general biosynthetic scheme is presented in Fig. 6. In the first step of the biosynthesis, a polyketide synthetase (PKS) assembles a nonaketide, presumably **3**, by successive Claisen condensations of eight acetyl-CoA units (via malonyl-CoA units) with an acetyl-CoA starter unit. After each acetate unit is added to the growing polyketide chain, the PKS multienzyme complex processes the resulting intermediate, enzyme-bound β-ketoacylthioester.[21] The degree of processing varies with each acetate unit that is added. Full processing, which involves a series of reduction-dehydration-reduction reactions analogous to those carried out in cyanobacterial fatty acid biosynthesis,[21] occurs after the addition of acetate units 1, 3 and 4, resulting in methylenes at C-11, C-13 and C-17 in **3**. Partial processing (reduction-dehydration only) follows the addition of acetate unit 2, leading to a trans carbon-carbon double bond between C-14 and C-15 in **3**. No processing, however, occurs after the addition of each of the last four acetate units (units 5-8) and as a consequence keto groups remain on C-3, C-5, C-7 and C-9

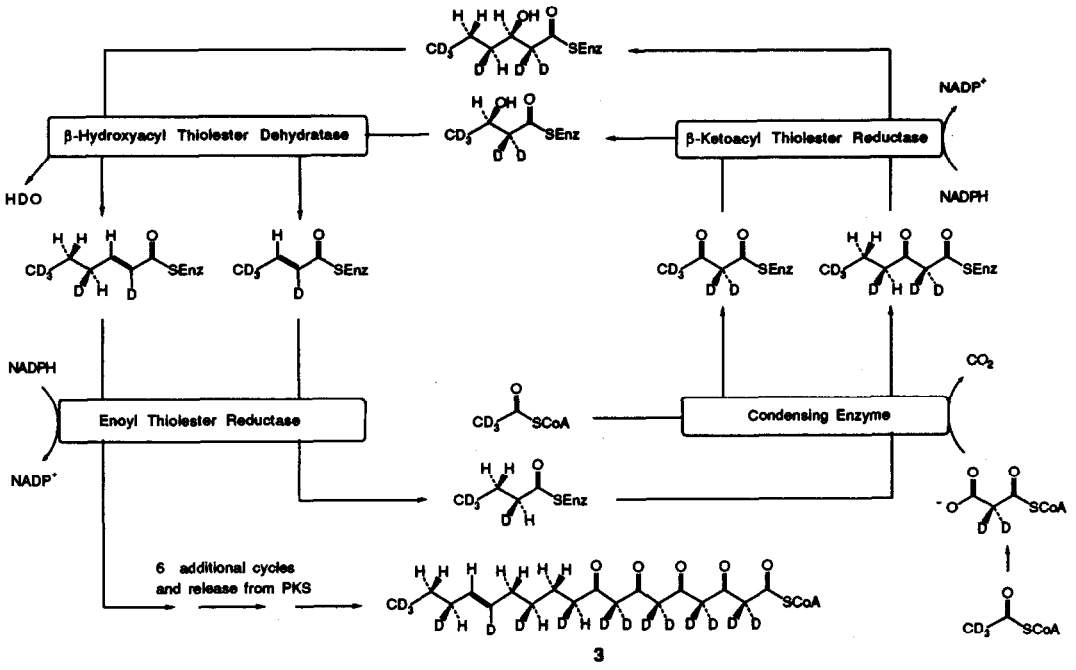

Fig. 7. Sequence of reactions mediated by the polyketide synthetase in constructing the proposed nonaketide **3**. The first two cycles of condensation-reduction-dehydration-reduction are shown. In the second cycle the reduction of the enoyl thioester is not carried out. The various reactions are illustrated with deuterated acetate and malonate to show the fate of the acetate-derived hydrogens. The enoyl thioester reductase is depicted introducing hydrogen into the *pro-R* position of the C-2 methylene group, as has been observed in fatty acid biosynthesis in the cyanobacterium *Anacystis nidulans*.[22] The labeling patterns found in **1** and **2** grown on [2-^{13}C,$^{2}H_3$]acetate are consistent with the labeled **3**.

of **3**. If polyketide and fatty acid processing in cyanobacteria have the same stereochemistry, full processing would be expected to result in retention of an acetate-derived proton in the *pro-S* position on C-10, C-12 and C-16 of **3**.[22] The same *pro-S* stereochemistry is observed in fungal polyketides, but curiously the opposite *pro-R* stereochemistry is found in fungal fatty acids.[22,23] Although only a very small number of cases have been examined, the results to date suggest that the stereochemistry of enoyl thiol ester reductases involved in polyketide and fatty acid biosynthesis in cyanobacteria may be the same. All of the protons on C-2, C-4, C-6, C-8, and C-14 of nonaketide **3** are acetate-derived as a consequence of partial processing in cycle 2 and no processing in cycles 5-8. The construction of deuterium-labeled nonaketide **3** from [$^{2}H_3$]acetate is shown in Fig. 7.

In the next step of the proposed biosynthesis, C-2 and C-7 of nonaketide **3** undergo an intramolecular aldol condensation to give alcohol **4** which aromatizes to resorcinol **5** by either successive dehydration, enolization and decarboxylation (Pathway A) or concerted dehydration-decarboxylation followed by enolization (Pathway B). Pathway A, which is analogous to the biosynthesis route for orcinol from 3,5,7-triketooctanoyl thiolester via orsellinic acid, requires that only one of the aromatic protons on C-4 and C-6 in resorcinol **5** be acetate-derived. Pathway B, however, requires that both of these protons be acetate-derived. Advanced intermediate **5** appears to possess the necessary functionality for dimerization. In the dimerization process, we propose that the alkenyl group of one molecule of the 5-(undec-7'-enyl)resorcinol (**5** or **6**) electrophilically substitutes at C-2 of the resorcinol of a second molecule and vice versa to form the C-7/C-8 and C-20/C-21 bonds of the [7.7]paracyclophane.

It is presently unclear whether chlorination at C-3(16) in **1**, which appears to proceed with retention of configuration, occurs before or after dimerization. It is possible that *E*-5-(3'-chloroundec-7'-enyl)resorcinol (**6** where R_2 = H, R_3 = Cl) is the modified nonaketide that dimerizes to the cyclophane that is then converted by oxidation and methylation at C-1(14) to **1**. Curiously [7.7]paracyclophanes such as the dibromo analog of **1** could not be detected when *N. linckia* was grown on a chloride-free, bromide-rich medium (S. C. Bobzin and G.M.L. Patterson, unpublished results). Whether this means that chlorination occurs before dimerization and is necessary for the cyclization to proceed is unknown. Further experiments are needed. Similarly, it is not known when the methylation at C-2(15) in **2** takes place. (2'*S*)-*E*-5-(2'-Methylundec-7'-enyl)resorcinol (**6** where R_2 = Me and R_3 = H) could be the nonaketide that dimerizes to the cyclophane leading to **2**. In this case the known cylindrocyclophane F,[9] with possibly two acetate-derived protons on C-1(14) if a 2-methylene intermediate is involved (see next paragraph), would result from the dimerization and all that would remain to complete the biosynthesis of **2** is oxidation and acetylation at C-1(14). H-1(14) in **2** (also **1**) are acetate-derived.

The [1,2-$^{13}C_2$]acetate and [2-^{13}C,$^2H_3$]acetate feeding experiments clearly show that the methyl groups on C-2(15) of **2** are derived from C-2 of acetate. Two deuteriums are retained on the methyl carbons and one deuterium is left on C-1(14) and C-3(16). The deuterium on C-3(16), however, is curiously distributed 3:1 between the *pro-S* and *pro-R* positions, possibly due to a small amount of enolization of the keto group at either C-2(15) in a cyclophane intermediate (e.g. **5**) or C-2' in a 5-(undec-7'-enyl)resorcinol intermediate prior to condensation of acetate with the keto group. Acetate is probably added to the keto group as malonate. The resulting β-hydroxyacyl acid is then apparently decarboxylated and dehydrated to an alkene possessing presumably an exo-methylene group[17] on C-2(15) of the cyclophane or C-2' of the undecenylresorcinol. Reduction leads to the methyl group. Decarboxylation and dehydration could proceed in a manner analogous to loss of carbon dioxide and water from mevalonic acid in terpenoid biosynthesis.

The lower incorporation of deuterium into H-7(20) of **1** from the [2-^{13}C,$^2H_3$]-feeding experiment is puzzling. Extensive (and reproducible) washout of deuterium label at this one position is not satisfactorily explained by post malonate exchange,[22,24] i.e. stereospecific loss of *pro-S* deuterium from enzyme bound malonyl unit by reaction with β-hydroxyacyl thioester dehydratase prior to condensation with acylthioester,[25] during the biosynthesis of nonaketide **3**. It is difficult to rationalize the loss of hydrogen from this position during the dimerization if the C-7(20)/C-8(21) coupling proceeds entirely by an electrophilic aromatic substitution process. An oxidative coupling between the phenolic and alkene systems is being considered as a possible alternative. Efforts are currently underway to synthesize proposed isotopically-labeled nonaketide precursors for cell-free experiments which may provide insight into the mechanism by which the dimerization reaction occurs.

EXPERIMENTAL

Materials. Sodium [1,2-$^{13}C_2$]acetate (99 %), sodium [1-^{13}C,$^2H_3$]acetate (99 % ^{13}C, 98 % ^2H), sodium [2-^{13}C,$^2H_3$]acetate (99 % ^{13}C, 98 % ^2H), sodium [1-^{13}C,$^{18}O_2$]acetate (99 atom % ^{13}C, 96% ^{18}O), and L-[methyl-^{13}C]methionine (96% ^{13}C) were obtained from Cambridge Isotope Laboratories (CIL). L-[methyl-^{13}C,$^2H_3$] methionine (99% ^{13}C, 99% ^2H) was obtained from Sigma Chemical Company. Sodium [2-^{13}C,$^{18}O_2$] acetate was prepared by basic hydrolysis of [2-^{13}C]acetonitrile (99% ^{13}C, CIL) with [^{18}O] water (96% ^{18}O, CIL) in 1.23 *M* potassium *tert*-butoxide in *tert*-butanol.[26] Sodium [$^2H_3$]acetate was prepared from [$^2H_4$]acetic acid (99.9% ^2H, CIL, 1.5 mL) by adjusting the pH to 9 with 10% NaOH. The resulting solution was lyophilized to yield sodium [$^2H_3$]acetate (2.05 g).

Spectral Analysis. NMR spectra were determined on 11.75 and 7.05 tesla instruments operating at 500 and 300 MHz for ^1H and 125 and 75 MHz for ^{13}C, respectively. ^1H chemical shifts are referenced in DMSO-d_6 to residual DMSO-d_5 (2.49 ppm) and in MeOH-d_4 to residual [CH_3-d_2] MeOD (3.30 ppm); ^{13}C chemical shifts are referenced in MeOH-d_4 to the solvent (49.0 ppm). Homonuclear ^1H connectivities were determined by using the double-quantum filtered COSY experiment.[27] Homonuclear ^1H NOEs were obtained by difference NOE experiments using a 3s irradiation period. One bond heteronuclear ^1H-^{13}C connectivities were determined by proton-detected HMQC[28] and carbon-detected HETCOR experiments.

Enrichment, i.e. specific incorporation (SI), of ^{13}C into a specific carbon was estimated by determining the NMR spectra of the enriched and unenriched (natural abundance) compounds with identical instrument parameters and in the same solvent at the same concentrations. SI was then calculated from (a-b)/b where a = the integrated NMR signal of the enriched carbon and b = the integrated NMR signal of the same carbon in the unenriched compound.

Culture Conditions for *Nostoc linckia*. *N. linckia* (Roth) Bornet (UTEX B1932) was purchased from the University of Texas Culture Collection. A 1 L flask culture of alga was used to inoculate a 20 L glass carboy containing an inorganic medium, designated A_3M_7.[29] Cultures were continuously illuminated at an incident intensity of 300 μeinstein/m^2s from banks of cool-white fluorescent tubes and aerated at a rate of 5 L/min with a mixture of 0.5% CO_2 in air at a temperature of 24 ± 1 °C. Typically, the culture was harvested by centrifugation and filtration after 18-20 days.

Isotopically labeled precursors were administered to cultures of *Nostoc linckia* 14 days after innoculation. Labeled sodium acetate (~250 mg) was usually diluted with unlabeled sodium acetate (~500 mg) to reduce the complexity of the spectra due to interunit couplings or isotope shifts. This dilution procedure was not followed when sodium [$^2H_3$]acetate was administered to the culture. Alga was harvested by centrifugation and filtration 72-78 h after the precursor had been fed and immediately freeze-dried.

Culture Conditions for *Cylindrospermum licheniforme*. *C. licheniforme* Kützing (ATCC 29204) was purchased from the American Type Culture Collection. A 1 L flask culture of alga was used to inoculate a 20 L glass carboy containing an inorganic medium, designated A_3M_7.[29] Cultures were grown under the same conditions described above. Typically, the culture was harvested by filtration after 24-26 days.

Isotopically labeled precursors were administered to cultures of *Cylindrospermum licheniforme* 20 days after inoculation. Similar labeling experiments were carried out with sodium acetates as described above. Labeled methionines (~250 mg) were administered without dilution with unlabeled material. Alga was harvested and freeze-dried as described above.

Isolation of Nostocyclophane D (1). In a typical run lyophilized alga (6.5 g) from a 20 L culture of *Nostoc linckia* (UTEX B1932) was extracted with 70% EtOH (3 x 500 mL) to yield a brown residue (1.5 g). This extract was separated by flash chromatography[30] on a 29 x 2.3 cm column of C_{18} silica (YMC, ODS-120A, T63) using a 20% stepwise gradient from H_2O to MeOH (250 mL each portion) to elute material from the column The fractions which eluted with MeOH/H_2O (4:1) and the first portion of the 100% MeOH effluent exhibited KB cytotoxicity (MIC<5μg/ml). These fractions were combined and filtered through a C_{18} solid phase extraction column (Alltech, 500 mg) using MeOH/H_2O (4:1, 15 mL). The solvents were removed and the material eluted (127 mg) redissolved in MeOH (1 mL) and the solution filtered through a 0.2 μm filter. The material in this solution was separated by reversed-phase HPLC on C_{18} silica (YMC 120A, 25x2.5 cm column) using MeOH/H_2O/TFA (80/20/0.05) as the eluant at a flow rate of 5 ml/min. The UV active peak which eluted at 64 min was further purified by reversed-phase HPLC on Econosil C_{18} (10μ, 25x1.0 cm column) using MeOH/H_2O/TFA (80/20/0.05) as the eluant (flow rate 1.5 ml/min). Pure nostocyclophane D (24 mg, 0.37% dry weight) eluted after 55.5 min. The yield of nostocyclophane D varied appreciably (±40%) with each experiment.

Isolation of Cylindrocyclophane D (2). Lyophilized alga (3.8 g) from a 20 L culture of *Cylindrospermum licheniforme* (ATCC 29204) was extracted with 70% EtOH (2 x 400 mL) to yield a brown-green residue (1.5 g). This extract was triturated with MeOH (100 mL) and filtered through a scintered glass funnel. The soluble material (522 mg) was separated by vacuum flash chromatography (column size 6.5x3.1 cm) on silica (Davisil, 200-425 mesh) using CH_2Cl_2, CH_2Cl_2/acetone (1:1), and MeOH (150 mL portions) to elute fractions from the column. The orange and green material (90 mg) which eluted with CH_2Cl_2/acetone (1:1) exhibited KB cytotoxicity (MIC<5μg/ml). The cytotoxic material was separated by vacuum flash chromatography on a 6.5x3.4 cm column of C_{18} silica (YMC, ODS-120A, T63) using 125 mL portions of MeOH/H_2O (7:3), MeOH, MeOH/CH_2Cl_2 (2:1), and CH_2Cl_2 to elute fractions. The fractions which eluted with MeOH and MeOH/CH_2Cl_2 (2:1) were combined and the solvents removed to yield a yellow oil (52 mg). This material was resuspended in MeOH (1 mL), filtered through a 0.2 μm filter, and separated by reversed-phase HPLC on C_{18} silica (YMC 120A, 25x2.5 cm column) using CH_3CN/H_2O (7:3) as eluant at a flow rate of 4.5 ml/min. Cylindrocyclophane D (14 mg, 0.37% dry weight) eluted after 98 min. The yield of cylindrocyclophane D was very consistent (±10%) in all of the experiments. ^1H NMR (500 MHz, MeOH-d_4) δ 1.99 [s, OMe], 4.98 [d, J = 10.5 Hz, H-1], 1.76 [tqd, J = 10.5, 6.5 and 3 Hz, H-2(15)], 0.96 [d, J = 6.5 Hz, Me on C-2(15)], 0.78 (qt, J = 13 and 3 Hz, *pro-S* H-3(16)], 0.69 (m, *pro-R* H-3(16)], 1.43 [qt, J = 12 and 3 Hz, *pro-S* H-4(17)], 0.84 [qt, J = 12 and 4 Hz, *pro-R* H-4(17)], 0.94 [qt, J = 12 and 4 Hz, *pro-S* H-5(18)], 0.71 [m, *pro-R* H-5(18)], 2.02 [qd, J = 12 and 4 Hz, *pro-S* H-6(19)], 1.32 [tt, J = 12 and 4-5 Hz, *pro-R* H-6(19)], 3.14 [qdd, J = 10-12, 5.5 and 4.5 Hz, H-7(20)], 6.15 [s, H-12(25)], 6.11 [s, H-10(23)], 1.90 [dtdd, J = 13, 10, 5.5 and 4.5 Hz, *pro-R* H-27(31)], 1.49

[ddt, J = 13, 10 and 5.5 Hz, *pro-S* H-27(31)], 1.17 [m, *pro-R* H-28(32)], 1.06 [m, *pro-S* H-28(32)], 1.25 [m, H-29(33)], 1.20 (m, H-29(33)], 0.82 (t, J = 7.1 Hz, H_3-30(34)].

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