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STRUCTURES OF CYLINDROCYCLOPHANES A-F

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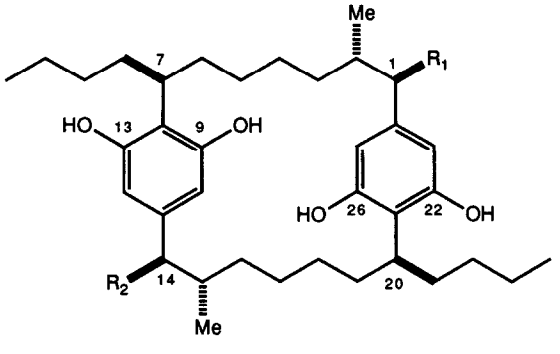
Abstract. Five new [7.7]paracyclophanes, cylindrocyclophanes B-F (**2-6**), have been isolated from three strains of the terrestrial blue-green alga *Cylindrospermum licheniforme* and their total structures elucidated. The absolute stereochemistry of cylindrocyclophane A (**1**) has been determined by Mosher's method.

The first [m.n]paracyclophanes to be found in Nature were isolated from two species of terrestrial blue-green algae belonging to the Nostocaceae, viz. cylindrocyclophane A (**1**) from *Cylindrospermum licheniforme* Kützing and nostocyclophane D from *Nostoc linckia* (Roth) Bornet.[1] Three additional chlorine-containing nostocyclophanes A, B, and C were subsequently isolated from *N. linckia* UTEX B1932 and their total structures determined.[2] In this paper we report the isolation and identification of five new [7.7]paracyclophanes from *C. licheniforme* that are related to cylindrocyclophane A.

The additional cylindrocyclophanes were found in three strains of *C. licheniforme*, viz. ATCC 29204, ATCC 29412 and UTEX 2014. Cylindrocyclophanes B (**2**), C (**3**) and D (**4**), along with A (**1**), were the major cyclophanes in ATCC 29204 whereas cylindrocyclophanes D, E (**5**) and F (**6**) were the major cyclophanes in ATCC 29412 and UTEX 2014. The cylindrocyclophanes were isolated by extracting the freeze-dried cells of the cultured cyanophytes with 70% aqueous ethanol and subjecting the resulting extract to successive silica gel chromatography, reversed-phase C-18 chromatography, and reversed-phase C-18 HPLC.



1 $R_1 = R_2 = OH$
2 $R_1 = OH$, $R_2 = OAc$
3 $R_1 = OH$, $R_2 = H$
4 $R_1 = R_2 = OAc$
5 $R_1 = OAc$, $R_2 = H$
6 $R_1 = R_2 = H$

Detailed spectral analysis, most notably NMR, had established the gross structure and relative stereochemistry of cylindrocyclophane A as **1**.[1] We had suggested that **1** probably possessed the same absolute configurations at C-1, C-7, C-14 and C-20 as nostocyclophane D, the major cyclophane from *N. linckia* UTEX B1932,[1,2] the structure of which had been determined by X-ray

crystallography. Using Mosher's method, which has been used recently to elucidate the absolute stereochemistry of several natural products,[3] we have confirmed the proposed[1] absolute stereochemistry for **1**. The four phenolic groups of **1** were first exhaustively methylated with excess diazomethane and the resulting 9,13,22,26-tetra-*O*-methyl derivative was converted to both the (*S*)- and (*R*)-methoxytrifluorophenylacetate (MTPA) diesters. The $\Delta\delta$ (δ_S-δ_R) values that were determined (Fig. 1) were only compatible with the absolute stereochemistry depicted in **1**.

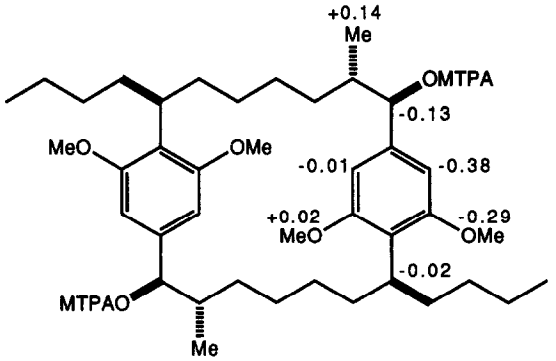


Fig. 1. $\Delta\delta$ values for MTPA diesters of cylindrocyclophane A

A field desorption mass spectrum established the molecular weight of cylindrocyclophane D (**4**) as 668 daltons, 84 mass units higher than that for **1**. Detailed NMR analysis clearly showed that **4** was cylindrocyclophane A 1,14-diacetate. The ^{13}C NMR spectrum of cylindrocyclophane D (**4**) (Table 1) exhibited the same 18 signals found for **1**, but two additional peaks were present at 21.2 and 172.7 ppm. Since only 20 carbon signals were visible, **4** like **1** also had to have a twofold axis of symmetry. DEPT experiments showed 15 signals for protonated carbons (3 methyls, 7 methylenes, and 5 methines) and comparison of the ^1H NMR spectra in DMSO-d_6 and MeOH-d_4 indicated the presence of two exchangable proton signals. Compound **4** therefore possessed 56 protons on carbon and four protons on oxygen. The ^1H NMR spectrum of **4** (Table 2) displayed signals for all the protons found in **1**, except for the one for the two OH groups on C-1 and C-14 and for chemical shift differences. A methyl singlet at 1.96 ppm, characteristic of an acetate methyl, was found instead. The molecular formula of **4** was therefore $C_{40}H_{60}O_8$. The H-1/H-14 signal was a doublet at 4.88 ppm and showed coupling (10.3 Hz) to the H-2/H15. For **1** the H1/H14 signal was a doublet of doublets at 3.95 ppm, the additional coupling being to the OH on C-1/C-14. As expected acid hydrolysis of **4** led to **1** and this transformation provided a rigorous proof of structure. Since the CD spectrum of the hydrolysis product was identical with natural **1**, **4** had to have the same absolute stereochemistry as **1**.

An EI mass spectrum established the molecular weight of cylindrocyclophane F (**6**) as 552 daltons, 32 mass units lower than the one for **1**, and a high resolution measurement was consistent with the elemental composition $C_{36}H_{56}O_4$. The NMR spectra

Table 1. ^{13}C NMR Chemical Shift Data for Cylindrocyclophanes A-F (**1**-**6**) in MeOH-d_4[a]

position	**1**	**2**		**4**	**6**
1,14	81.9 d	81.8	83.4	83.4	45.9 t
Me on 2,15	17.0 q	17.0	16.6	16.5	20.9
2,15	42.1 d	42.1	40.0	40.0	36.7
CH_3COO on 1,14			172.5 s	172.7	
CH_3COO on 1,14			21.2 q	21.2	
3,16	35.3 t	35.2	34.8	34.7	36.7
4,17	30.0 t	29.9	29.7	29.6	30.2
5,18	30.7 t	30.7	30.6	30.5	30.7
6,19	35.5 t	35.4	35.2	35.1	35.5
7,20	36.9 d	36.9	36.8	36.8	36.8
21,8	117.8 s	117.7	118.6	118.6	116.1
22,9	158.9 s	158.8	158.8	158.8	158.2
23,10	105.1 d	105.0	105.2	105.2	108.0
24,11	143.9 s	143.9	139.1	139.1	140.9
25,12	109.0 d	108.9	109.5	109.5	110.0
26,13	157.0 s	157.0	157.0	156.9	157.0
27,31	34.9 t	34.8	34.5	34.4	34.9
28,32	31.7 t	31.7	31.7	31.7	31.7
29,33	23.9 t	23.9	23.9	23.9	23.9
30,34	14.5 q	14.6	14.6	14.6	14.6

[a]The δ-values for C1-C7, C21-C30, and the carbons of the substituents on C1 and C2 for **3** and **5** are within ±0.1 ppm of the corresponding δ-values for **1** and **4**, respectively; similarly, the δ-values for C8-C20, C31-C34, and the carbons of the substituents on C14 and C15 are within ±0.1 ppm of the corresponding δ-values for **6**.

Table 2. ^1H NMR Chemical Shift Data for Cylindrocyclophanes A-F

position	A[a,b]	D[a,c]	F[d]
1a,14a	3.54 dd (9.5, 4)	4.88 d (10.3)	2.58 dd (-13.2, 3.8)
1b,14b			1.82 dd (-13.2, 11.8)
OH on 1,14	4.87 d		
CH_3COO on 1,14		1.96 s	
2,15	1.31	1.62	1.56
Me on 2,15	0.96 d (6.2)	0.87 d (6.2)	0.93 d (6.6)
3a,16a	0.56	0.69	1.00
3b,16b	0.56	0.56	0.64
4a,17a	1.36	1.37	1.42
4b,17b	0.68	0.76	0.77
5a,18a	0.88	0.86	0.97
5b,18b	0.61	0.57	0.73
6a,19a	1.89	1.90	1.96
6b,19b	1.28	1.28	1.32
7,20	3.05 br tt	3.05 br tt	3.11 br tt
10,23	6.16 s	6.13 s	6.08 s
12,25	5.94 s	6.07 s	5.92 s
27a,31a	1.82	1.75	1.92
27b,31b	1.43	1.46	1.49
28a,32a	1.08	1.13	1.17
28b,32b	1.00	1.01	1.08
29a,33a	1.21	1.22	1.29
29b,33b	1.16	1.22	1.20
30,34	0.76 t	0.77 t	0.80 t

[a]Determined in DMSO-d_6, 500 MHz. [b]Phenolic OH signals are sharp singlets at 8.56 and 8.59 ppm. [c]Phenolic OH signals are sharp singlets at 8.85 and 8.87 ppm. [d]Determined in MeOH-d_4, 500 MHz.

(Tables 1-2) lacked signals for OH or OAc substituents on C-1 and C-14. Instead of being a doublet for an oxygen-bearing methine, the C-1/C-14 signal was a triplet at 45.9 ppm which meant C-1 and C-14 were methylenes. This was further substantiated by the ^{1}H NMR spectrum which showed a doublet of doublets at 2.578 ppm and a triplet at 1.818 ppm for the two C-1/C-14 methylene protons. The latter protons were coupled to H-2/H-15 by 3.8 and 11.8 Hz, respectively, and to each other by -13.2 Hz. Other than the differences mentioned above, the ^{1}H and ^{13}C NMR spectra of **6** were comparable with those of **1** and **4**. Cylindrocyclophane F was therefore 1,14-dideoxycylindrocyclophane A. Finally the CD spectra of **6** and **1** showed a negative peak in the 220-235 nm region, strongly suggesting that their absolute stereochemistries were identical.

The structures of cylindrocyclophanes B (**2**), C (**3**) and E (**5**) were deduced by comparing their ^{1}H and ^{13}C NMR spectra which those of **1**, **4** and **6**. Both the ^{1}H and ^{13}C NMR spectra of **2**, **3** and **5** showed a doubling of peaks, i.e. two sets of peaks with each set having chemical shifts corresponding (within ±0.1 ppm) with those for either **1**, **4** or **6**. In other words the ^{13}C NMR spectra of **2**, **3** and **5** were virtually identical with the ^{13}C spectra of 1:1 mixtures of **1** and **4**, **1** and **6**, and **4** and **6**, respectively.

Cylindrocyclophanes B (**2**), C (**3**), and E (**5**) appear to have the same absolute stereochemistry on the basis of the following data. Hydrolysis of **2** gave **1** which exhibited a CD spectrum that was identical with that of natural **1**. The CD spectrum of **3** showed a negative peak in the 220-235 nm region, similar to the one observed for **1** and **6**. Cylindrocyclophanes B, D, and E displayed significantly different CD spectra than A, C, and F with each showing a positive peak at 231 nm. Removal of the acetate group(s), however, resulted in the appearance of a negative peak in the CD spectrum of the hydrolysis product.

All of the cylindrocyclophanes were found to be moderately cytotoxic (IC_{50}s 0.5-5 μg/mL), but none of the compounds showed any selective cytotoxicity against murine or human solid tumor cell lines in the Corbett assay.[4]

Experimental Section

Spectral Analysis. NMR spectra were determined on 11.75 and 7.05 tesla instruments operating at 500 and 300 MHz for ^{1}H and 125 and 75 MHz for ^{13}C, respectively. ^{1}H 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).

Culture Conditions. *Cylindrospermum licheniforme* strains ATCC 29204 and ATCC 29412 were purchased from the American Type Culture Collection and strain UTEX 2014 was obtained from the University of Texas. The algae were grown in 25-L glass bottles containing an aqueous inorganic medium (A_3M_7) using a previously published procedure.[5] Following incubation at 24 ±1 °C for periods of 14-17, 19-22, and 12-14 days for ATCC 29204, ATCC 29412 and UTEX 2014, respectively, the algae were harvested by filtration and freeze-dried. Yields averaged 0.20, 0.33 and 0.15 g/L.

Isolation of Cylindrocyclophanes A, B, C, and D. Freeze-dried ATCC 29204 (6.36 g) was extracted with 500 mL CH_2Cl_2 in a Soxhlet apparatus for 15 h to give 495 mg of extract (IC_{50} 2 μg/mL against KB cell line).

The extract was divided into seven portions and each portion was applied to a 500 mg column of BondElut Si. Each column was eluted with 5 mL amounts of CH_2Cl_2, CH_2Cl_2/acetone (1:1), and MeOH. Aliquots of each fraction were assayed for cytotoxicity which showed that all of the activity was in the green semisolid residue (250 mg) eluted with 1:1 CH_2Cl_2/acetone. To remove the pigments, this material was dissolved in MeOH and chromatographed on six 500 mg columns of BondElut C18. Each column was eluted with 5 mL MeOH/H_2O (7:3) followed by MeOH wash. The MeOH/H_2O (7:3) fraction was evaporated to give 71 mg of a white solid (KB IC_{50} 100 ng/mL). Final purification was achieved by reverse-phase HPLC on a 22x250 mm C-18 column with 7:3 MeCN/H_2O (3.5 mL/min flow rate) to give after lyophilization, cylindrocyclophanes A (6.9 mg), B (28.6 mg), C (2.8 mg), and D (14.4 mg). The cyclophanes eluted at 28 min. (A), 39 min. (B), 55 min. (C), and 59 min. (D).

Cylindrocyclophane A (1): mp 276-278 °C; FDMS m/z 584; FABMS m/z 567 $(M - H_2O + H)^+$; EIMS m/z 549 $(M - 2H_2O + H)^+$; high resolution EIMS m/z 549.3870 ($C_{36}H_{56}O_4$, Δ-0.4 mmu); $[\alpha]_D$ -20° (MeOH, c 0.5); CD (MeOH) λ nm (θ) 208 (6400), 222 (-6100), 248 (200), 278 (-2300).

Cylindrocyclophane B (2): FDMS, m/z 626 ($C_{38}H_{58}O_7$, M^+); FABMS, m/z 549 $(M - AcOH - H_2O + H)^+$, m/z 609 $(M - H_2O + H)^+$; $[\alpha]_D$ +25° (MeOH, c 0.6); CD (MeOH) λ nm (θ) 215 (6800), 231 (4700), sh 250 (0), 279 (-1600).

Cylindrocyclophane C (3): FABMS, m/z 551 $(M - H_2O + H)^+$, m/z 569 $(M + H)^+$; $[\alpha]_D$ -40° (MeOH, c 0.1); CD (MeOH) λ nm (θ) 208 (6700), 231 (-4300), 250 (200), 279 (-1300).

Cylindrocyclophane D (4): FDMS, m/z 668 ($C_{40}H_{60}O_8$, M^+); FABMS, m/z 551 $(M - AcOH - C_4H_9)^+$, m/z 549 $(MH - 2AcOH)^+$, m/z 491 $(M - 2AcOH - C_4H_9)^+$; $[\alpha]_D$ +83° (MeOH, c 0.2); CD (MeOH) λ nm (θ) 218 (17600), 228 (9700), 231 (10600), sh 250-255 (0), 281 (-1500).

Isolation of Cylindrocyclophanes E and F. Ten grams of freeze-dried ATCC 29412 was extracted twice in 1.5 L 7:3 EtOH/H_2O for 24 h to yield 2.92 g of extract (KB IC_{50} 10 μg/mL) which was chromatographed on a 5x8.5 cm Si flash column. Fractions were eluted with CH_2Cl_2 followed by 9:1, 8:2, and 5:5 mixtures of CH_2Cl_2/acetone and finally MeOH. The 9:1 and 8:2 CH_2Cl_2/acetone fractions were evaporated, and the residue, in a minimal ammount of MeOH, was further fractionated on a flash, reverse-phase 4x4.5 cm C-18 column. The cyclophanes eluted with 9:1 MeOH/H_2O and were purified by HPLC on a 22x250 mm C-18 column with 8:2 MeCN/H_2O (4 mL/min flow rate) to give cylindrocyclophanes D (4.4 mg, t_R 29 min), E (3.6 mg, t_R 36 min) and F (61.8 mg, t_R 43 min). Similar amounts of cylindrocyclophanes D, E and F were obtained from UTEX 2014.

Cylindrocyclophane E (5): FDMS, m/z 610 ($C_{38}H_{58}O_6$, M^+); $[\alpha]_D$ +10° (MeOH, c 0.1); CD (MeOH) λ nm (θ) 216 (10000), sh 231 (4100), sh 255-260 (0), 279 (-700).

Cylindrocyclophane F (6): EIMS m/z (rel intensity) 552 (85) M^+, 495 (100) $[M-C_4H_9]^+$; high resolution EIMS, m/z 552.4164 ($C_{36}H_{56}O_4$, Δ1.5 mmu); $[\alpha]_D$ -72° (MeOH, c 0.9); CD (MeOH) λ nm (θ) 209 (9200), 234 (-6900), 251 (500), 256 (200), 275 (600).

Hydrolysis of Cylindrocyclophanes B and D. Compound **2** (2 mg) was treated with 0.5 mL of 2% KOH in MeOH for 0.5 h at room temperature. The reaction mixture was diluted with water and acidified with acetic acid and the hydrolysis product was absorbed onto a 500 mg C-18 BondElut column. After washing the column with water (10 mL), **1** (1.5 mg) was eluted with 10 mL of MeOH. The ^{1}H NMR and CD spectra of the hydrolysis product were identical to those of cylindrocyclophane A. Using a similar procedure, **4** was also hydrolyzed to **1**.

MTPA Esters of Cylindrocyclophane A. Compound **1** (4 mg) was treated with excess ethereal diazomethane for 15-18 h, i.e. until NMR and TLC analyses indicated that a single product had been formed. After chromatography on a silica BondElut column with ether/hexane, pure 9,13,22,26-tetra-*O*-methylcylindrocyclophane A (1.5 mg) was obtained: ^{1}H NMR ($CDCl_3$) δ 6.55 and 6.27 (s, H-10,12,23,25), 3.74 and 3.72 (s, OMe on C-9,13,22,26), 1.09 (d, Me on C-2,15).

The tetramethyl ether (0.7 mg) in 0.5 mL $CDCl_3$ was treated with 0.5 mg of (*S*)-(-)-methoxytrifluorophenylacetic acid (MTPA), 0.5 mg of DCC, and 50 μg of DMAP. The mixture was shaken and allowed to stand overnight at room temperature. ^{1}H NMR analysis indicated that the esterification had been completed. The reaction mixture was filtered and purified by HPLC on a 250 x 10 mm Econosil silica column (6% THF in hexane, flow rate 2.5 mL/min) to give 0.5 mg of the (*S*)-MTPA diester (t_R 12 min); ^{1}H NMR ($CDCl_3$) δ 6.44 and 6.01 (s, H-10,12,23,25), 5.22 (d, J = 10.3 Hz, H-1,14), 3.77 and 3.35 (s, OMe on C-9,13,22,26), 3.23 (m, H-7,20), 1.03 (d, Me on C-2,15). Using a similar procedure 0.7 mg of the tetramethyl ether was converted to 0.5 mg of (*R*)-MTPA diester (t_R 24 min); ^{1}H NMR ($CDCl_3$) δ 6.45 and 6.39 (s, H-10,12,23,25), 5.35 (d, J = 10.5 Hz, H-1,14), 3.75 and 3.64 (s, OMe on C-9,13,22,26), 3.25 (m, H-7,20), 1.84 (m, H-2,15), 0.89 (d, Me on C-2,15), 0.81 (t, H_3-30,34).

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