Antineoplastic Agents. 398. Isolation and Structure Elucidation of Cephalostatins 18 and 19[1]

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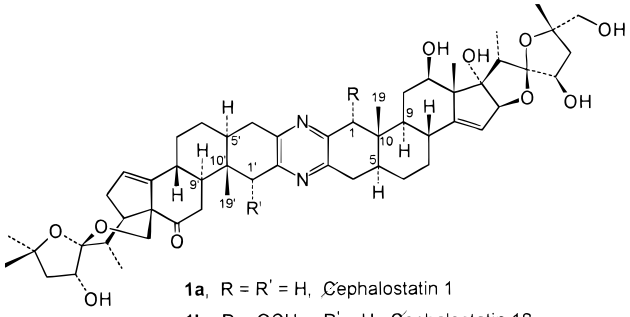
Continued investigation of murine leukemia (P-388) active fractions from the African marine worm *Cephalodiscus gilchristi* has resulted in the discovery of cephalostatins 18 (**1b**) and 19 (**1c**). The structures were determined by interpretation of their highfield (500 MHz) ^{1}H, ^{13}C, and 2D NMR and HRMS. Both of these new methoxy steroidal alkaloids exhibited strong activity against the murine P-388 lymphocytic leukemia cell line (ED_{50} ca. 10^{-3} μg/mL), a mini panel of human cancer cell lines (GI_{50} $<10^{-3}$ μg/mL), and the U.S. National Cancer Institute's 60 human cancer cell line panel (mean panel GI_{50} ca. 10^{-9} M).

The cephalostatins[2] represent a quite remarkable series of cancer cell growth inhibitors. We first detected the presence of these unique steroidal alkaloids in extracts of a 1972 collection of the small (several millimeters) South East African marine worm *Cephalodiscus gilchristi* and, in 1988, reported the isolation and structure determination (by X-ray crystallography) of the first member, cephalostatin 1 (**1a**).[3] Subsequently, we isolated and characterized another eight cephalostatins[4] from a 1981 (166 kg, wet wt) re-collection and the next eight[5] from 1990 specimens (450 kg, wet wt) in 10^{-6} to 10^{-7}% yields. More recently, the cephalostatin series has been considerably expanded by the addition of 26 members obtained from the Japanese marine tunicate *Ritterella tokioka*,[6] where only 8.2 kg of this invertebrate afforded greatly increased (0.7−34 mg) yields. Meanwhile, preclinical development, especially of cephalostatin 1 (**1a**), has been continuing and has necessitated (and stimulated) devising practical total synthetic routes. That objective has now been realized for cephalostatins 7 and 12,[7] for one of the tunicate cephalostatins (ritterazine K),[7] and in part for cephalostatin 1.[8] Interestingly, even a cursory understanding of the cephalostatin 1 biochemical mechanism of antineoplastic activity has remained elusive and is further accelerating research in this area. For the purpose of increasing our overall knowledge of this potentially important series of biologically potent steroids, we now report discovery of what we believe to be the last currently detectable cephalostatins contained in the 1990 recollection of *C. gilchristi*, namely, cephalostatins 18 (**1b**) and 19 (**1c**).

One of the murine P-388 lymphocytic leukemia cell line active fractions that led to cephalostatins 1, 2, 14, and 15[5b,9,10] was further separated as described in the Experimental Section to yield cephalostatin 18 (**1b**, 6.1 mg) and cephalostatin 19 (**1c**, 1.3 mg). HRFABMS indicated compositions of $C_{55}H_{76}N_2O_{11}$ for **1b** and **1c**.



1a, R = R′ = H, Cephalostatin 1
1b, R = OCH_3, R′ = H, Cephalostatin 18
1c, R = H, R′ = OCH_3, Cephalostatin 19

Interpretation of the ^{1}H, ^{13}C, APT, and 2D (HMQC, HMBC, H−H COSY, TOCSY, and ROESY) NMR spectra of cephalostatin 18 (**1b**) gave definitive structural information. Preliminary comparison of ^{1}H and ^{13}C NMR signals with those displayed by cephalostatin 1 (**1a**) showed many similarities. A notable exception was evidence in that a methoxyl group at δ 57.37/3.53 (3H, s) and a methine at δ 83.34/4.14 (1H, s) had replaced a methylene group. Those observations corresponded with the molecular formula obtained by mass spectrometry. An HMBC[11] cross peak between the methoxyl protons (δ 3.53, 3H, s) and the methine carbon (δ 83.34) indicated their direct bonding (−CH−OCH_3). Detailed analysis of the 2D NMR spectra and HMBC correlations to the methine proton (δ 4.14) from C-2 (δ 149.64), C-3 (δ 147.81), C-5 (δ 35.14), and C-10 (δ 40.66) suggested the new methine unit (−*CH*−OCH_3) should be assigned to C-1. The assignment was confirmed by observing the significant α, β, and γ effects[12] (Table 2). The stereochemistry of cephalostatin 18 (**1b**) was ascertained by recognition of two sets of NOE effects in the ROESY spectrum: 1-H (δ 4.14, equatorial) with 4-H (δ 2.63, axial) and 19−3H (δ 0.70, axial); 1-OCH_3 (δ 3.53, axial) with 9-H (δ 1.86, axial). Thus, cephalostatin 18 (**1b**) proved to be 1-α-methoxy-cephalostatin 1.

Preliminary inspection of the physical data obtained from cephalostatin 19 (**1c**) also indicated a very close

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Table 1. The Highfield ^{1}H (500 MHz) and ^{13}C (100 MHz) NMR Results for Cephalostatins 18 (**1b**) and 19 (**1c**) in C_5D_5N

	1b				**1c**		
no.	^{13}C NMR	^{1}H NMR (J in Hz)	HMBC	no.	^{13}C NMR	^{1}H NMR (J in Hz)	HMBC
right				right			
1	83.34n[a]	4.14 (1H, s)	1-OCH_3, 19-H	1	46.15p	2.62 (1H), 3.12 (1H)	19-H
OCH_3	57.37n	3.53 (3H, s)	1-H				
2	149.64p		1-H, 4-Ha, 4-Hb	2	151.35p		1-Ha, 1-Hb
3	147.81		1-H, 4-Ha	3	148.16p		1-Hb
4	35.92p	2.63 (1H), 3.02 (1H)		4	35.80p	2.70 (1H), 2.95 (1H)	
5	35.14n	2.34 (1H)	1-H, 19-H	5	41.76n	1.57 (1H)	19-H
6	40.66p		1-H, 4-Hb, 19-H	6	30.01p	1.28 (1H), 1.54 (1H)	
7	28.33p	1.32 (1H), 1.65 (1H)		7	28.50p	1.30 (1H), 1.69 (1H)	
8	33.67p	2.14 (1H)	15-H	8	33.77n	2.06 (1H)	
9	45.90n	1.86 (1H)	11-Ha	9	53.14n	0.87 (1H)	
10	40.66p		1-H, 4-Hb, 19-H	10	36.34p		1-Ha, 1-Hb, 4-Hb, 19-H
11	28.76p	1.85 (1H), 2.14 (1H)		11	28.92p	1.76 (1H), 2.06 (1H)	12-OH
12	75.71n	4.24 (1H)	18-H	12	75.55n	4.05 (1H, dd, J = 5, 9)	12-OH, 18-H
		4.41 (1H, OH, br)				4.69 (1H, OH, s)	
13	55.46p		15-H, 18-H	13	55.39p		15-H, 17-OH, 18-H
14	153.04p		16-H, 18-H	14	152.66p		15H, 16-H, 18-H
15	122.25n	5.64 (1H, s, br)		15	122.30n	5.64 (1H, s)	
16	93.24n	5.25 (1H, s, br)	15-H	16	93.16n	5.24 (1H, s)	15-H
17	91.71p	6.25 (1H, OH, s)	16-H, 18-H, 20-H	17	91.64p	6.21 (1H, OH, s)	15-H, 16-H, 17-OH, 20-H
18	12.67n	1.37 (3H, s)		18	12.60n	1.34 (3H, s)	
19	11.06n	0.70 (3H, s)	1-H	19	11.76n	0.77 (3H, s)	1-Ha, 1-Hb
20	44.52n	2.89 (1H)	21-H	20	44.51n	2.86 (1H)	21-H
21	9.04n	1.36 (3H, d, J = 7)	20-H	21	9.02n	1.35 (3H, d, J = 7)	20-H
22	117.16p		16-H, 20-H, 21-H, 24-Hb	22	117.17p		16-H, 20-H, 21-H, 24-Hb
23	71.57n	4.79 (1H, s)	20-H, 24-Ha	23	71.53n	4.79 (1H)	20-H, 24-Ha, 24-Hb
		8.12 (1H, OH, br)				8.06 (1H, OH, d, J = 7.5)	
24	39.44p	2.34 (1H), 2.72 (1H)	26-Hb, 27-H	24	39.57p	2.31(1H), 2.37 (1H)	27-H
25	82.82p		24-Ha, 26-Hb, 27-H	25	82.84p		24-Ha, 27-H
26	69.28p	3.72 (1H, d, J = 14)	24-Hb, 27-H	26	69.27p	3.71 (1H, dd, J = 5.5, 11.5)	24-Ha, 27-H
		3.81 (1H, d, J = 14)				3.81 (1H, dd, J = 5.5, 11)	
		6.59 (1H, OH, br)				6.52 (1H, OH, t, J = 6)	
27	26.42n	1.64 (3H, s)		27	26.44n	1.64 (3H, s)	24-Ha
left				left			
1′	46.00p	2.55 (1H)	19′-H	1′	82.92n	4.13 (1H, s)	1′-OCH_3, 19′-H
		3.08 (1H, d, J = 20)		OCH_3	57.55n	3.53 (3H, s)	1′-H
2′	150.75p		1′-Ha, 1′-Hb	2′	149.30p		1′-H, 4′-Ha, 4′-Hb
3′	147.81p		1′-Hb	3′	147.48p		1′-H
4′	35.73p	2.66 (1H), 2.93 (1H)		4′	35.80p	2.64 (1H), 3.06 (1H)	
5′	41.18n	1.58 (1H)	1′-Hb, 19′-H	5′	34.74n	2.33 (1H)	1′-H, 19′-H
6′	29.45p	1.24 (1H), 2.00 (1H)		6′	29.46p	1.62 (1H), 1.98 (1H)	
7′	30.02p	1.32 (1H), 1.52 (1H)	9′-H	7′	27.99p	1.25 (1H), 1.34 (1H)	
8′	35.61n	2.14 (1H)	11′-Ha	8′	35.47n	2.25 (1H)	
9′	52.14n	1.28 (1H)	11′-Hb, 19′-H	9′	44.38n	2.28 (1H)	19′-H
10′	36.30p		1′-Ha, 1′-Hb, 4′-Hb, 19′-H	10′	40.74p		1′-H, 4′-Hb, 19′-H
11′	38.80p	2.61 (1H), 2.78 (1H)		11′	38.75p	2.66 (1H), 2.83 (1H)	
12′	211.79p		17′-H, 18′-Ha, 18′-Hb	12′	212.04p		
13′	61.80p		15′-H, 16′-Ha, 17′-H	13′	61.87p		15′-H
14′	149.39p		16′-Ha	14′	150.17p		
15′	123.01n	5.44 (1H, s)		15′	123.30n	5.45 (1H, s)	
16′	32.37p	2.31 (1H), 2.87 (1H)		16′	32.38p	2.33 (1H), 2.87 (1H)	15′-H, 20′-H
17′	44.20n	2.75 (1H)	15′-H, 18′-Ha, 20′-H, 21′-H	17′	44.18n	2.79 (1H)	15′-H, 16′-Hb, 18′-Ha, 20′-H, 21′-H
18′	64.21p	4.03 (1H, d, J = 15)		18′	64.23p	4.07 (1H, d, J = 11)	
		4.08 (1H, d, J = 15)				4.11 (1H, d, J = 11)	
19′	11.37n	0.73 (3H, s)	1′-Ha, 1′-Hb	19′	10.52n	0.68 (3H, s)	1′-H
20′	32.90n	3.17 (1H, dq, J = 8, 8)	21′-H	20′	32.92n	3.18(1H, dq, J = 6.5, 6.5)	21′-H
21′	15.52n	1.46 (3H, d, J = 8)	20′-H	21′	15.52n	1.46 (3H, d, J = 6.5)	20′-H
22′	110.90p		17′-H, 18′-Ha, 18′-Hb, 20′-H 21′-H, 23′-H, 24′-Ha, 24′-Hb	22′	110.91p		17-H, 18′-Ha, 18′-Hb, 20′-H, 21′-H, 24′-H
23′	81.53n	4.81 (1H)	24′-Ha	23′	81.52n	4.79 (1H)	
		7.20 (1H, OH, br)				7.15 (1H, OH, d, J = 4.5)	
24′	47.33p	1.94 (1H, dd, J = 8, 15)	26′-H, 27′-H	24′	47.29p	1.94 (1H, dd, J = 6, 13.5)	26′-H, 27′-H
		2.36 (1H)				2.35 (1H)	
25′	81.12p		23′-H, 24′-Ha, 26′-H, 27′-H	25′	81.16p		26′-H, 27′-H
26′	29.76n	1.47 (3H, s)	24′-Ha, 27′-H	26′	29.77n	1.48 (3H, s)	24′-Ha, 27′-H
27′	29.45n	1.39 (3H, s)	24′-Ha, 26′-H	27′	29.46n	1.40 (3H, s)	24′-Ha, 24′-Hb, 26′-H

[a] Notation reflects the APT, "n" for CH or CH_3, "p" for C or CH_2.

Table 2. Selected Comparison of the ^{13}C NMR Data Corresponding to C-1 and C-1′ in Cephalostatins **1a**−**c**

carbon	no.	Δ (in ppm)	**1b**	**1a**	**1c**	Δ (in ppm)
1	α	+37.36	83.34	45.98		
10	β	+4.34	40.66	36.32		
5	γ	−6.64	35.14	41.78		
9	γ	−7.30	45.90	53.20		
19	γ	−0.66	11.06	11.72		
1′	α			45.82	82.92	+37.10
10′	β			36.28	40.74	+4.46
5′	γ			41.20	34.74	−6.46
9′	γ			52.20	44.38	−7.82
19′	γ			11.31	10.52	−0.79

resemblance to cephalostatin 1 (**1a**). However, detailed comparison of ^{13}C NMR data pointed to significant changes arising from the A′ and B′ rings near C-1′. The ^{13}C NMR results led to assignment of another methoxyl group in structure **1c** at C-1′. The α (C-1′), β (C-10′), and γ (C-5′, C-9′, and C-19′) effects (Table 2) remained close to those found for steroid **1b**. A complete 2D NMR spectral interpretation confirmed the assignment of structure **1c** to cephalostatin 19. The distinctive positive optical rotation values at the sodium D line shown by the new cephalostatins suggested the same overall absolute configuration as already deduced for cephalostatin 1 by X-ray crystallographic techniques.

Both cephalostatin 18 and 19 exhibited strong activity against the P-388 lymphocytic leukemia cells corresponding to ED_{50} 4.3×10^{-3} μg/mL and 7.4×10^{-3} μg/mL, respectively, and against a selection (OVCAR-3, SF-295, A498, NCI-H460, KM20L2, and SK-MEL-5) of human cancer cell lines (GI_{50} $<10^{-3}$ μg/mL).

Cephalostatin 18 (**1b**) and 19 (**1c**) were evaluated comparatively, alongside cephalostatin 1 (**1a**) in the U.S. National Cancer Institute's 60 cell-line in vitro screen.[13,14] Each compound was tested in triplicate at three different concentration ranges (10^{-6}, 10^{-7}, and 10^{-8} M upper limits; five log-spaced concentrations in each range) against the entire 60 cell-line panel. The cephalostatin 1 (**1a**) standard yielded a mean panel GI_{50} concentration of 2.20 (±1.21) $\times 10^{-9}$ M. Steroidal alkaloids **1b** and **1c** yielded mean panel GI_{50} concentrations of 21.7 (±9.9) $\times 10^{-9}$ M and 16.6 (±9.5) $\times 10^{-9}$ M, respectively. Furthermore, as expected, the benchmark compound **1a** produced the distinctive 60-cell mean-graph "fingerprint", which is typical of all members of the cephalostatin series heretofore studied (e.g., see Pettit et al.[4a]). In the present investigation, cephalostatins 18 (**1b**) and 19 (**1c**) likewise produced this characteristic cytotoxicity profile, as confirmed by Compare[14] pattern-recognition analyses; the corresponding GI_{50}-Compare correlation coefficients were 0.94 and 0.92, for **1b** and **1c**, respectively, in reference to **1a**. The latter analyses implied that the cytotoxic mechanism of these newest cephalostatins does not diverge substantially from that of the known series.

The current good possibility that the cephalostatins inhibit cancer cell growth by affecting a novel molecular target(s), the ongoing total synthetic and SAR challenges, the possibility of locating a marine microorganism source actually responsible for their biosynthesis, and clinical development prospects suggest the cephalostatin field will become increasingly productive and useful.

Experimental Section

Isolation of Cephalostatins 18 and 19. One of the murine P-388 lymphocytic leukemia cell line active fractions (3.03 g) that led to cephalostatins 1, 2, 14, and 15[5b,9,10] was further separated on Sephadex LH-20 in hexane−toluene−MeOH (3:1:1), hexane−CH_2Cl_2−MeOH (5:1:1), and hexane−iProH−MeOH (8:1:1), followed by high-speed countercurrent chromatography using the solvent system hexane−EtOAc−MeOH−H_2O (3:7:5:5) to afford seven fractions. The fifth was subjected to Sephadex LH-20 column chromatography using hexane−CH_2Cl_2−MeOH (5:1:1) as the mobile phase. Purification of two resulting P-388 cell line active fractions, utilizing reversed-phase HPLC (C8, 5−20 μ, 10 × 250 mm) with MeOH−H_2O (4:1) as eluent, provided cephalostatins 18 (**1b**) and 19 (**1c**). Cephalostatin 18 (**1b**) was isolated as an amorphous solid (6.1 mg, 1.3×10^{-6}% yield); mp >320 °C; $[\alpha]^{25}_D$ +95° (*c*, 0.06, CH_3OH); UV (CH_3OH) λ 288 nm (log ε 4.06) and 308 nm (shoulder); IR (film) ν_{max} 3426, 2925, 1707, 1398, and 1088 cm^{-1}; HRFABMS *m/z* 947.5632 $[M + Li]^+$, calcd for $C_{55}H_{76}N_2O_{11}Li$ 947.5609; and FABMS *m/z* 947.6 $[M + Li]^+$ 550, 377, 313, and 160. Cephalostatin 19 (**1c**) was obtained as an amorphous solid (1.3 mg, 2.89×10^{-7}% yield); mp >320 °C; $[\alpha]^{25}_D$ +67° (*c*, 0.055, CH_3OH); UV (CH_3OH) λ 288 nm (log ε 3.99) and 308 nm (shoulder); IR (film) ν_{max} 3426, 2925, 1711, 1398, and 1088 cm^{-1}; HRFABMS *m/z* 941.5545 $[M + H]^+$, calcd for $C_{55}H_{77}N_2O_{11}$ 941.5527. The proton and carbon NMR data for cephalostatins 18 (**1b**) and 19 (**1c**) have been summarized in Table 1.

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References and Notes

(1) Pettit, G. R.; Tan, R.; Melody, N.; Cichacz, Z. A.; Herald, D. L.; Hoard, M. S.; Pettit, R. K. *Bioorg. Med. Chem. Lett.*, manuscript in preparation.
(2) Ganesan, A. *Angew. Chem., Int. Ed.* **1996**, *35*, 611−615.
(3) Pettit, G. R.; Inoue, M.; Kamano, Y.; Herald, D. L.; Arm, C.; Dufresne, C.; Christie, N. D.; Schmidt, J. M.; Doubek, D. L.; Krupa, T. S. *J. Am. Chem. Soc.* **1988**, *110*, 2006−2007.
(4) (a) Pettit, G. R.; Kamano, Y.; Inoue, M.; Dufresne, C.; Boyd, M. R.; Herald, C. L.; Schmidt, J. M.; Doubek, D. L.; Christie, N. D. *J. Org. Chem.* **1992**, *57*, 429−431. (b) Pettit, G. R.; Kamano, Y.; Dufresne, C.; Inoue, M.; Christie, N.; Schmidt, J. M.; Doubek, D. L. *Can. J. Chem.* **1989**, *67*, 1509−1513. (c) Pettit, G. R.; Inoue, M.; Kamano, Y.; Dufresne, C.; Christie, N.; Niven, M. L.; Herald, D. L. *J. Chem. Soc., Chem. Commun.* **1988**, 865−867.
(5) (a) Pettit, G. R.; Xu, J.-P.; Schmidt, J. M.; Boyd, M. R. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2027−2032. (b) Pettit, G. R.; Xu, J.-P.; Ichihara, Y.; Williams, M. D.; Boyd, M. R. *Can. J. Chem.* **1994**, *72*, 2260−2267. (c) Pettit, G. R.; Ichihara, Y.; Xu, J.-P.; Boyd, M. R.; Williams, M. D. *Bioorg. Med. Chem. Lett.* **1994**, *4*, 1507−1512. (d) Pettit, G. R.; Xu, J.-P.; Williams, M. D.; Christie, N. D.; Doubek, D. L.; Schmidt, J. M.; Boyd, M. R. *J. Nat. Prod.* **1994**, *57*, 52−63.

(6) (a) Fukuzawa, S.; Matsunaga, S.; Fusetani, N. *Tetrahedron*, **1995**, *51*, 6707- 6716. (b) Fukuzawa, S.; Matsunaga, S.; Fusetani *J. Org. Chem*., **1997**, *62*, 4484−4491.
(7) Jeong, J. U.; Sutton, S. C.; Kim, S.; Fuchs, P. L. *J. Am. Chem. Soc.* **1995**, *117*, 10157−10158.
(8) (a) Bhandaru, S.; Fuchs, P. L. *Tetrahedron Lett.* **1995**, *36*, 8351−8354. (b) Heathcock, C. H.; Smith, S. C. *J. Org. Chem*. **1994**, *59*, 6828−6839.
(9) Arene, E. O.; Pettit, G. R.; Ode, R. H. *Lloydia* **1978**, *41*, 186−189.
(10) Pettit, G. R.; Kamano, Y.; Aoyagi, R.; Herald, C. L.; Doubek, D. L.; Schmidt, J. M.; Rudloe, J. J. *Tetrahedron* **1985**, *41*, 985−994.
(11) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(12) (a) Hsiu, S.-L.; Takai, H.; Sasaki, Y. *Chem. Pharm. Bull.* **1984**, *32*, 2091−2099. (b) Whitesell, J. K.; Minton, M. A. *J. Am. Chem. Soc.* **1987**, *109*, 225−228.
(13) Boyd, M. R. In *Anticancer Drug Development Guide*; Teicher, B., Ed.; Humana Press: Totowa, NJ, 1997; pp 23−42.
(14) Boyd, M. R.; Paull, K. *Drug Dev. Res.* **1995**, *34*, 91−109.

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