Isolation and Structure of the Hemichordate Cell Growth Inhibitors Cephalostatins 2, 3, and 4

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Three new cephalostatins possessing powerful cytostatic activity have been isolated from the Indian Ocean marine worm *Cephalodiscus gilchristi*; structures were assigned on the basis of detailed high-field n.m.r. and mass spectral analyses.

The Phylum Hemichordata is divided into three classes containing some 90 species.[1] Only the class Enteropneusta (acorn worms) appears to have received any prior chemical examination. Those studies involved isolation of 2,5-dibromophenol from *Balonoglossus biminiensis*[2,3] and deoxyribonucleosides from *Ptychodera flava*.[4] Our discovery[5] of the powerful murine P388 lymphocytic leukemia (PS system) cell growth inhibitor cephalostatin 1 (**1**) in the South African (Indian Ocean) Pterobranchia member *Cephalodiscus gilchristi* represented the first investigation of chemical constituents in this unusual class of two tube-living genera. The Pterobranchia display distinctive tentacled arms emanating from the collar dorsal side, and the colonial *Cephalodiscus* has up to nine pairs of such arms. Interestingly, *C. gilchristi* is not confined to the coenecium (worm tube) but is independent and can move in or out of the tube using a sucker-like (and secretion) proboscis of the buds. Perhaps such exposure to predators during food harvesting has necessitated, in part, biosynthetic development of the cephalostatins.

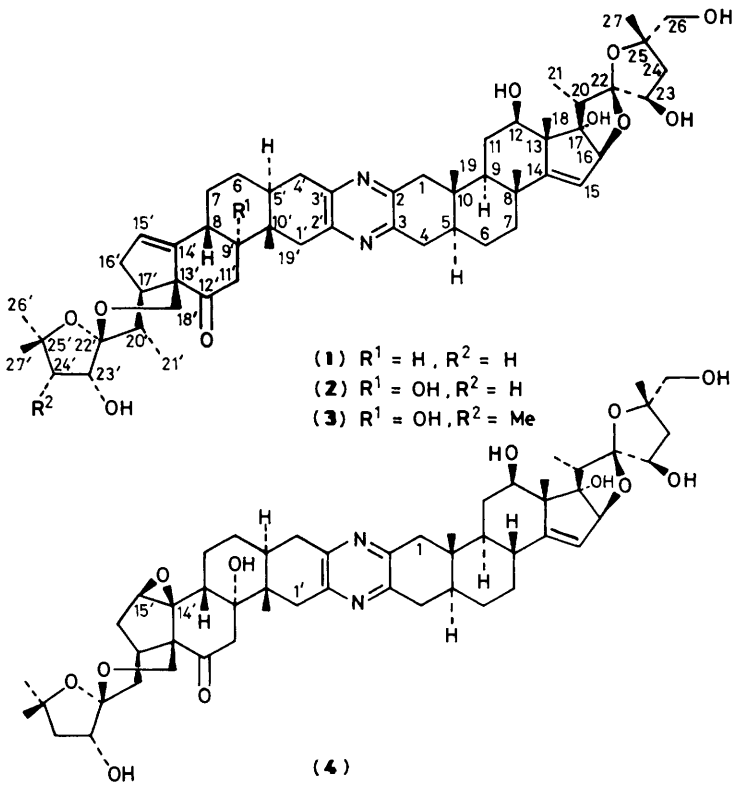
We now report that further bioassay(PS)-guided separation of *C. gilchristi* has led to the isolation of three new steroidal alkaloids, designated cephalostatins 2, 3, and 4, similar in cell growth inhibition (PS E.D.$_{50}$ 10^{-7}—10^{-9} μg ml^{-1}) to cephalostatin 1. A methylene chloride–methanol extract prepared from 166 kg of wet *C. gilchristi* was separated by an extensive series of solvent partition and gradient column chromatographic (size exclusion and partition on Sephadex, partition and adsorption on silica gel, and h.p.l.c.) techniques.

Cephalostatin 2 (**2**) crystallized from ethyl acetate–methanol as needles (242.8 mg, 14.6 × 10^{-4}% yield), m.p. >350 °C; R_f(SiO_2) 0.28 (90:10:0.8 methylene chloride–methanol–water); $[\alpha]_D$ +111° (*c* 0.07 in MeOH); secondary ion mass spectrometry (SP-HRSIMS)[6] (glycerol–CF_3SO_3H) *m/z* 927.5230 $(M + H)^+$ for $C_{54}H_{75}N_2O_{11}$ (calc. 927.5372); u.v. (EtOH) $\lambda_{max.}$ (ε 13 700) and 308sh nm; i.r. (KBr) $\nu_{max.}$ 3430, 3055, 2975, 2930, 2880, 1710, 1655—1625br, 1448, 1402, 1385, 1092, 1045, and 950 cm^{-1}. Cephalostatin 3 (**3**) also crystallized as needles (21.2 mg, 1.2 × 10^{-4} % yield) from

Table 1. ^{13}C N.m.r. assignments for cephalostatins 2—4 in [$^2H_5$]pyridine.

Carbon no.	(2)	(3)	(4)	Carbon no.	(2)	(3)	(4)
1	45.98	45.98	45.99	1′	39.52	39.52	39.03
2	148.56[a]	148.67[b]	148.68[c]	2′	148.69[a]	148.67[b]	149.20[c]
3	148.56[a]	148.67[b]	148.60[c]	3′	148.56[a]	148.51[b]	148.14[c]
4	35.77	35.77	35.77	4′	36.20	36.20	36.07
5	41.82	41.80	41.78	5′	34.21	34.19	33.85
6	28.16	28.16	28.21	6′	28.25	28.25	27.56
7	28.70	28.68	28.65	7′	24.58	24.60	20.64
8	33.80	33.79	33.78	8′	38.98	39.03	34.62
9	53.22	53.20	53.18	9′	78.74	78.73	80.97
10	36.35	36.33	36.32	10′	41.23	41.22	41.54
11	28.96	28.95	28.94	11′	45.60	45.46	45.55
12	75.61	75.59	75.58	12′	211.06	211.14	209.36
13	55.41	55.40	55.38	13′	61.57	61.25	56.33
14	152.74	152.73	152.71	14′	148.28[a]	148.27[b]	72.83
15	122.28	122.27	122.26	15′	124.48	124.51	54.10
16	93.17	93.16	93.15	16′	32.56	32.42	27.70
17	91.68	91.67	91.65	17′	44.20	43.92	33.15
18	12.60	12.59	12.57	18′	64.06	64.97	62.42
19	11.76	11.74	11.74	19′	15.06	15.03	14.87
20	44.52	44.51	44.50	20′	32.89	32.58	31.99
21	9.03	9.01	9.00	21′	15.49	15.20	15.06
22	117.18	117.18	117.16	22′	110.95	109.06	110.25
23	71.53	71.52	71.52	23′	81.60	87.17	81.48
24	39.51	39.52	39.52	24′	47.35	51.65	47.31
25	82.83	82.82	82.81	25′	81.14	81.32	81.10
26	69.30	69.28	69.28	26′	29.81	28.02	29.64
27	26.45	26.43	26.42	27′	29.53	23.33	29.32
28				28′		12.71	

[a,b,c] Signals may be interchanged.



ethyl acetate–methanol; m.p. >350 °C; R_f (SiO_2) 0.30 [conditions as for (**2**)]; $[\alpha]_D$ +99° (*c* 0.15 in MeOH); SP-HRSIMS (glycerol–CF_3SO_3H) *m/z* 941.5546 $(M + H)^+$ for $C_{55}H_{77}N_2O_{11}$ (calc. 941.5528); u.v. (EtOH) $\lambda_{max.}$ 290 (ε 12 900) and 308sh nm; i.r. (KBr) $\nu_{max.}$ 3430, 3050, 2967, 2928, 2872, 1707, 1645—1615br., 1446, 1383, 1040, 977, 952, and 935 cm^{-1}. Cephalostatin 4 (**4**) was isolated as a colourless solid (8.0 mg, 4.5 × 10^{-5} % yield); m.p. >350 °C; $[\alpha]_D$ +89° (*c* 0.11 in MeOH); SP-HRSIMS (glycerol–CF_3SO_3H) *m/z* 943.5309 $(M + H)^+$ for $C_{54}H_{75}N_2O_{12}$ (calc. 943.5343); u.v. (EtOH) $\lambda_{max.}$ 290 (ε 10 500) and 308sh nm; i.r. (KBr) $\nu_{max.}$ 3430, 2970, 2928, 2875, 1711, 1660—1600br, 1447, 1383, 1089, 1042, 948, and 904 cm^{-1}. With the *X*-ray crystal structure assigned cephalostatin 1 (**1**) as a reference point, it became possible by detailed analysis of the high-field (400 MHz) ^1H and ^{13}C n.m.r. spectra, mass spectra, and results of elemental analyses, to assign unequivocally structures (**2**), (**3**), and (**4**), respectively, to cephalostatins 2, 3, and 4.

Both cephalostatins 2 and 3 contain one more active hydrogen than cephalostatin 1 (**1**), as evidenced by SP-SIMS hydrogen–deuterium exchange.[7] However, both substances gave only tetra-acetate derivatives, like cephalostatin 1. Cephalostatin 2 displayed ^1H and ^{13}C n.m.r. spectra very similar to those of cephalostatin 1. Cephalostatin 2 was found to possess (by mass analyses) one more oxygen atom than cephalostatin 1. Evidence for this additional oxygen atom was also observed in the ^{13}C n.m.r. spectrum (Table 1) with the appearance of a quaternary carbon signal at 78.74 p.p.m. and the disappearance of the C-9′ methine carbon signal at 52.20 p.p.m.; this was indicative of a hydroxy group at C-9′.† The spectra of cephalostatins 2 and 3 proved almost identical, except for the signals assigned to carbon atoms 22′ to 27′. An additional methyl group in cephalostatin 3 was evidenced by a new three-proton methyl doublet at 1.10 p.p.m. and by its mass spectrum. The C-23′ methine proton exhibited a doublet (11.4 Hz) and showed a correlation peak with the new methyl doublet in a homonuclear relayed coherence transfer experiment (H,H-Relayed COSY[8]). Thus a 24′-methyl group was identified. The spectra of cephalostatin 4 lacked the signals attributed to the 14′,15′-double bond. The mass spectrum indicated the presence of an additional oxygen atom. By following the coupling network from C-21′ (methyl doublet at 1.30 p.p.m.) using COSY and H,H-Relayed COSY experiments, we found that C-15′ must share an epoxy group with C-14′, as shown in structure (**4**). The β-z configuration of the epoxide was deduced by comparison of the data with those of model compounds.[9] Interestingly, such steroid 14,15-epoxides are rare in nature; they are best known in the clinically useful bufadienolide resibufogenin from the toad venom preparation Sen So.[9,10]

The thirteen fused or otherwise connected rings of the cephalostatins may constitute the largest such systems known in marine animals. The isolation and identification of cephalostatins 2—4 will now allow an initial assessment of structure–activity relationships in this remarkable new series of vigorous cell growth inhibitors. Antineoplastic and other biological evaluations are in progress.

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† The steroid numbering system is used for both units of each cephalostatin.

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