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ISOLATION AND STRUCTURE OF THE SYMMETRICAL DISTEROIDAL ALKALOIDS CEPHALOSTATIN 12 AND CEPHALOSTATIN 13[1a]

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Abstract: Re-examination of a Western Indian Ocean worm *Cephalodiscus gilchristi* has led to discovery of cephalostatins 12 and 13. Cephalostatin 12 (2) proved to be the first example of a symmetrical disteroidal alkaloid. Both new cephalostatins significantly inhibited growth of animal and human cancer cell lines.

While investigation of marine invertebrates (especially in the Phyla Porifera[2] and Coelenterata[3]) for cytotoxic terpene and/or steroid constituents has been increasing,[4] cephalostatins 1 (1)-4,[5] 7-9[6] and 10-11[7] which we discovered in the Western Indian Ocean marine worm *Cephalodiscus gilchristi* (Phylum Hemichordata, class Pterobranchia) continue to be the most potent cell growth inhibtitors of this type presently known. Interestingly, cephalostatins 5 and 6 bearing an aromatised C-ring in the right hand moiety were found to be least active (ED_{50} 0.01 to 0.001 μg/mL)[8], albeit still strong, as inhibitors of the murine P388 lymphocytic leukemia (PS system). The current need for a practical synthetic route to the potentially useful cephalostatins is an important objective and various synthetic approaches are currently under study.[9] We now report discovery of a symmetrical disteroidal alkaloid component of the tiny (~5 mm long) *C. gilchristi* designated cephalostatin 12 (2, PS ED_{50} 0.072 μg/mL) that will greatly simplify total synthesis of a cephalostatin. A new companion (differing by only a C-1' hydroxyl group) cephalostatin was also isolated from *C. gilchristi* and became the 13th (3, PS ED_{50} 0.046 μg/mL) member of this remarkable series of powerful human cancer cell line inhibitors.

The increasing need for cephalostatin 1[1] led us to complete a 450 kg (wet wt.) recollection of *C. gilchristi* off Southeast Africa in the summer of 1990. An initial dichloromethane fraction prepared by the 9:1 to 3:2 methanol-water solvent-partition sequence[10] successively using hexane → dichloromethane → *n*-butanol yielded a PS active (ED_{50} 0.25 μg/mL) *n*-butanol fraction. Previously, all research had been directed at the active (PS ED_{50} 0.044 μg/mL) dichloromethane fraction that led to cephalostatins 10 and 11.[7] A sequence (using PS guided bioassay) of steric exclusion and partition type

chromatographic separations of the active butyl alcohol fraction on Sephadex LH-20 followed by reversed-phase semi-preparative HPLC on Phenomenex Prepex C8 with methanol-water as mobile phase afforded 47.3 mg ($1.1x10^{-5}$% yield) of cephalostatin 12 (**2**) as an optically active amorphous solid, mp > 300°C, $[\alpha]_D^{20}$ + 157.5° (c 0.40 CH_3OH); *Rf* 0.25 (SiO_2 plate using 90:10:0.8 CH_2Cl_2-CH_3OH-H_2O); IR (KBr) ν_{max} 3416, 2977, 2924, 2882, 1645, 1448, 1400, 1219, 1117, 1049, 951, and 888 cm^{-1}. The UV (CH_3OH, log ϵ) spectrum showed absorptions at 288 (4.16) and 308 (3.89, shoulder) nm that suggested the presence of a pyrazine ring as in cephalostatins 1-11. The FABMS indicated a quasi-molecular ion $[M+H]^+$ at *m/z* 945. The high-resolution mass measurements established the molecular formula as $C_{54}H_{76}N_2O_{12}$ ($[M+H]^+$ *m/z* 945.5444). However, in the ^{13}C NMR spectrum only 27 signals were observed, suggesting a two-fold symmetrical axis. The APT spectrum exhibited the presence of four methyl, seven methylene, eight methine, and eight quaternary carbons, indicating a C_{27} steroidal subunit. Comparison of the NMR data with those of steroidal subunits of the known cephalostatins revealed the subunit to be identical to the right-side subunit of cephalostatin 1. Cephalostatin 12 (**2**) was therefore assigned to structure **2**.

Cephalostatin 13 (**3**) was isolated (3.7 mg, $8.2x10^{-7}$% yield) as summarized above for the new steroidal alkaloid **2**. The amorphous solid with a mp > 300°C exhibited $[\alpha]_D^{20}$ + 108.1° (c 0.07, CH_3OH); *Rf* 0.18 (SiO_2 plate with 90:10:0.8 CH_2Cl_2-CH_3OH-H_2O); UV (CH_3OH log ϵ) λ_{max} 287.0 (3.89) and 308.0 (3.46, shoulder) nm; IR (KBr) ν_{max} 3426, 2978, 2926, 2859, 1643, 1449, 1398, 1385, 1221, 1119, 1105, 1044, and 953 cm^{-1}. The molecular formula, $C_{54}H_{76}N_2O_{13}$, was deduced from HRFABMS ($[M+H]^+$ *m/z* 961.5451). Comparison of the ^1H- and ^{13}C-NMR data for cephalostatin 13 (**3**) with those of cephalostatin 12 (**2**) uncovered their structural similarities. All of the right-side steroidal subunit and the D',E', and F' rings of the left-side subunit of cepahostatin 13 (**3**) were identical to those of cephalostatin 12 (**2**). The ^{13}C NMR data corresponding to the A', B', and C' rings of the left-side subunit compared with those of cephalostatin 12 (**2**) showed one fewer methylene carbons and instead the presence of one hydroxymethine carbon [^1H-NMR: δ 4.85 (d, 4.5 Hz); 7.35 (d, 4.5 Hz, OH); ^{13}C-NMR: δ 74.39 (d)], indicating an additional secondary hydroxyl group. And this proposal was consistent with the molecular formula for cephalostatin 13. Since the additional hydroxymethine proton was only coupled to the hydroxyl proton, the hydroxyl group was positioned at C-1'. Long-range ^1H-^{13}C correlations observed in the HMBC spectrum secured the C-1' position as shown in Figure 1. The remaining NMR signals were assigned by interpretation of ^1H-^1H COSY, 2D TOCSY, HMQC, and HMBC experiments (Table I).

The configuration of the hydroxyl group at C-1' was determined by a NOESY experiment

(mixing time = 600 msec). The NOESY spectrum of steroidal alkaloid 3 in pyridine-d_5 at 400 MHz showed only a small number of cross peaks. However, an NOE cross peak between H-1′ and H_{eq}-11′ (2.47 ppm) indicated the β-configuration for H-1′, and therefore the α-configuration for the hydroxyl group. Although the signals of H_{eq}-11′ and H-5′ were partially overlapped, which might lead to an incorrect conclusion, careful examination of cross sections clearly showed that H-1′ correlated to H_{eq}-11′. In the ^{13}C NMR spectrum, large high field shifts of C-5′ (-7.01 ppm) and C-9′ (-7.03 ppm), compared with those of cephalostatin 12 (2), was observed. Such large shieldings due to 1,3-diaxial γ-gauche type interactions between H-5′ and OH-1′and between H-9′ and OH-1′ supported the configurational assignment. To avoid any ambiguity, the configuration was re-examined by a ROESY[11] experiment (mixing time = 150 msec). The ROESY spectrum exhibited NOE's not only between H-1′ and H_{eq}-11′ but also between H-1′ and H_3-19′, H-5′ and H_{eq}-4′ (3.02 ppm), H-5′ and H_{ax}-7′ (1.41 ppm), and H-5′ and H-9′.

Cephalostatins 12 (2) and 13 (3) were comparatively evaluated, alongside cephalostatin 1 (1), in the U. S. National Cancer Institute's human tumor, disease-oriented in vitro primary screen.[12] Each compound was tested in quadruplicate at each of three different concentration ranges (10^{-6}, 10^{-7} and 10^{-8} M upper limits) against the entire panel of 60 human cancer cell lines comprising the screening panel. Cephalostatins 2 and 3 showed substantial growth inhibitory activity against many of the human tumor cell lines. Interestingly, the overall potencies were considerably lower than for most other members of the cephalostatin series studied to date. Whereas the benchmark cephalostatin 1 showed an expected[7] mean panel GI_{50} of approximately 1 nmolar, cephalostatins 12 (2) and 13 (3) yielded average GI_{50} of about 400 nmolar and >1000 nmolar, respectively.

In summary, cephalostatin 12 (2) represents the first cephalostatin to possess a symmetrical structure, and cephalostatin 13 (3) is the unusual C-1′ hydroxyl biosynthetic product. The diminishment in human cancer cell growth inhibition displayed by cephalostatins 12 (2) and 13 (3) compared to cephalostatin 1 (1) may be related to their increased level of hydroxylation. Relative hydrophobicity may play an important role in the very strong biological activity of the cephalostatins. The discovery of cephalostatins 12 (2) and 13 (3) has provided some important new insights into cephalostatin structure/activity requirements and strategies for total syntheses.[13]

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Table I. ^{1}H and ^{13}C NMR Data for Cephalostatin 13 (3, in Pyridine-d_5).[a]

Left-side			Right-side		
Carbon no.	^{13}C (mult)	^{1}H (mult, J (Hz))	Carbon no.	^{13}C (mult)	^{1}H (mult, J (Hz))
1'	74.39 (d)	4.85 (d, 4.5)	1	46.12 (d)	2.54 (d, 17.4) 3.08 (d, 17.4)
2'	151.15 (s)		2	150.55 (s)	
3'	149.06 (s)		3	148.86 (s)	
4'	35.97 (t)	2.65 3.02 (dd, 18.0, 5.3)	4	35.71 (t)	2.62 2.86
5'	34.77 (d)	2.50	5	41.82 (d)	1.49
6'	28.50 (t)	1.37 1.61	6	28.19 (t)	1.23 1.47
7'	28.76 (t)	1.41 1.70	7	28.66 (t)	1.26 1.64
8'	33.75 (d)	2.18 (m)	8	33.75 (d)	2.07
9'	46.17 (d)	2.09	9	53.18 (d)	0.85 (m)
10'	40.38 (s)		10	36.33 (s)	
11'	28.41 (t)	1.86 (q, 11.9) 2.47	11	28.91 (t)	1.76 (q, 11.4) 2.03
12'	75.86 (d)	4.24 (dd, 11.9, 5.0)	12	75.55 (d)	4.03 (dd, 11.4, 4.8)
13'	55.52 (s)		13	55.38 (s)	
14'	153.38 (s)		14	152.70 (s)	
15'	122.06 (d)	5.66 (s)	15	122.25 (d)	5.62 (s)
16'	93.26 (d)	5.24 (s)	16	93.15 (d)	5.23 (s)
17'	91.73 (s)		17	91.66 (s)	
18'	12.64 (q)	1.38 (s)	18	12.58 (q)	1.33 (s)
19'	11.17 (q)	0.77 (s)	19	11.73 (q)	0.73 (s)
20'	44.51 (d)	2.87	20	44.51 (d)	2.87
21'	9.01 (q)	1.35 (d, 6.9)	21	9.01 (q)	1.35 (d, 6.9)
22'	117.16 (s)		22	117.16 (s)	
23'	71.52 (d)	4.79 (m)	23	71.52 (d)	4.79 (m)
24'	39.52 (t)	2.34 (t, 11.0) [b] 2.71	24	39.52 (t)	2.33 (t, 11.0) [b] 2.71
25'	82.75 (s)		25	82.80 (s)	
26'	69.29 (t)	3.71 (br d, 12.2) 3.81 (br d, 12.2)	26	69.29 (t)	3.71 (br d, 12.2) 3.81 (br d, 12.2)
27'	26.41 (q)	1.64 (s) [c]	27	26.41 (q)	1.63 (s) [c]
OH					
-1'		7.34 (d, 4.5)			
-12'		4.72 (s)	-12		4.70 (s)
-17'		6.22 (s)	-17		6.21 (s)
-23'		8.07 (d, 6.8)	-23		8.07 (d, 6.8)
-26'		6.55 (br s)	-26		6.55 (br s)

[a] ^{1}H NMR 400 MHz; ^{13}C NMR 100.6 MHz.

[b,c] Assignments with the same superscript may be interchanged.

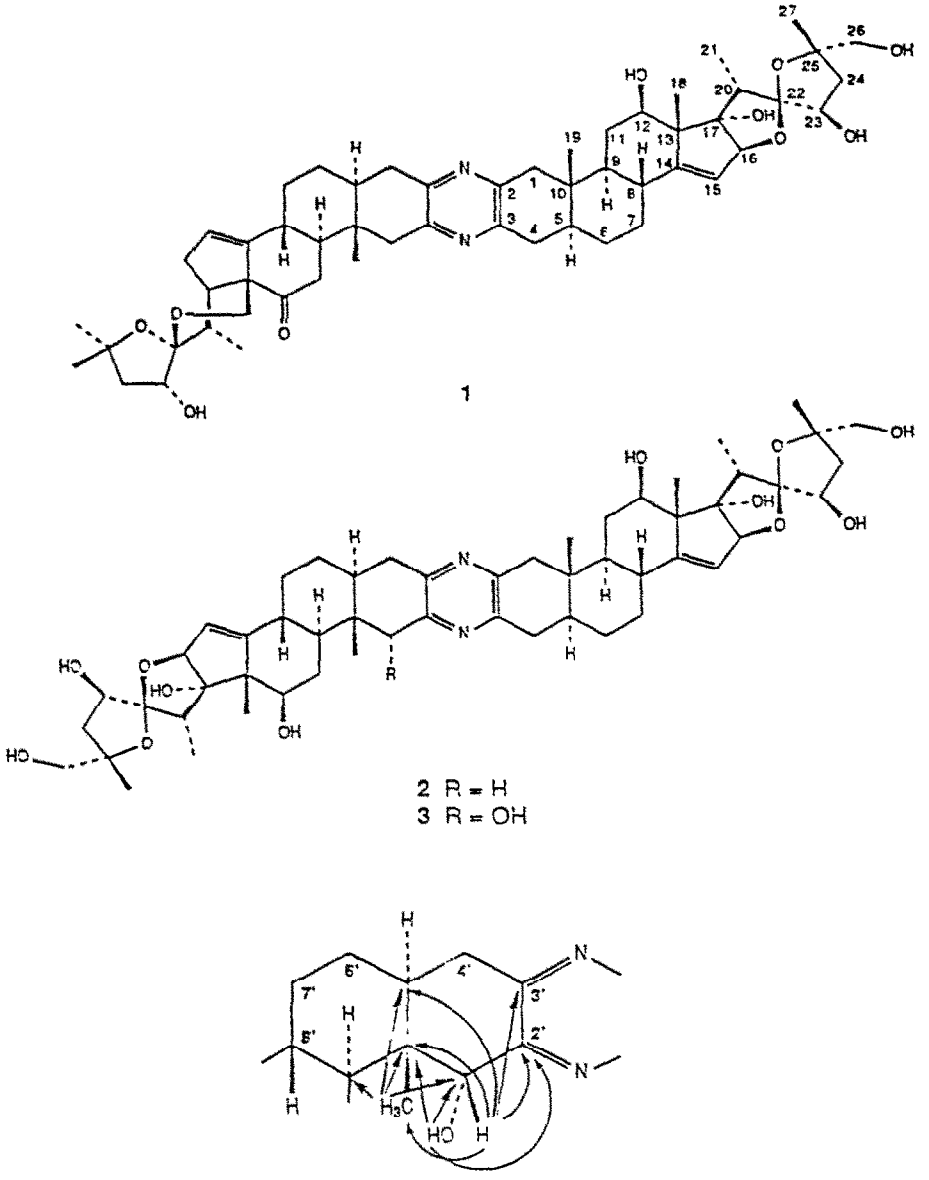

FIGURE 1. HMBC Correlations for 3.

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