Isolation and Structure of the Remarkable Human Cancer Cell Growth Inhibitors Spongistatins 2 and 3 from an Eastern Indian Ocean *Spongia* sp.[1]

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A black *Spongia* sp. in the Porifera class Demospongiae had been found to contain two new and exceptionally potent cell (human cancer) growth inhibitors named spongistatins 2 (**1b**) and 3 (**1c**).

Marine animal constituents of the macrocyclic lactone type, and especially those bearing perhydropyran systems, are proving to be exceptionally important sources of new anti-cancer drug candidates.[2] Illustrative are current human clinical trials of bryostatin 1[3] and the advancing preclinical development of halichondrin B,[2,4a,4b] halistatin 1[2b] and ecteinascidin 729.[4c] Seven interesting (and cytotoxic) perhydropyrans of the onnamide series[5a] (from a *Theonella* sp. of marine sponge) and 13-deoxytedanolide,[5b] a cytotoxic macrocylic lactone, from *Mycale adhaerens* (Porifera) are representative of related advances.

Spongistatin 1 **1a**, which we discovered[2a] in an Indian Ocean *Spongia* species (family Spongiidae, class Demospongiae), represents the most extraordinarily potent substance presently known against a subset of highly chemoresistant tumour types in the US National Cancer Institute (NCI) panel of 60 human cancer cell lines. Intensive investigation of other active (P388 lymphocytic leukaemia cell line bioassay) fractions from the

same sponge species has revealed the presence of two new and exceptionally potent (NCI panel) macrocyclic lactones designated spongistatins 2 **1b** and 3 **1c**. A synopsis of the isolations, structural elucidation and human cancer cell line activities now follows.

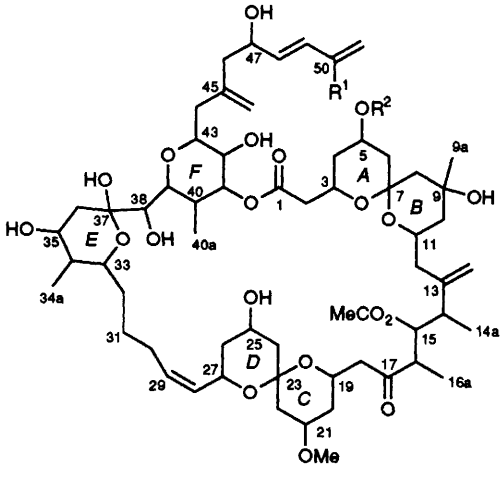
The cell growth inhibitory (P388 leukaemia) dichloromethane fraction prepared[2a] from a 400 kg (wet wt.) scale-up recollection (1988) of *Spongia* sp conducted in the Republic of Maldives was separated by a series of steric exclusion and partition chromatographic steps employing Sephadex LH-20 to obtain P388 active fractions reminiscent of spongistatin 1.[2a] A careful HPLC sequence using C-8 silica gel (Prepex and Ultremex) and 1:1 methanol–water to 5:5:8 methanol–acetonitrile–water afforded 4.34 mg (1×10^{-6}% yield) of amorphous spongistatin 2 **1b**: m.p. 140–141 °C; $[\alpha]_D$ + 24.5, (*c* 0.39, MeOH); UV(MeOH) λ/nm 220 (log ε 4.21) and 273 (2.95) nm; IR (film) ν_{max}/cm^{-1} 3426, 2937, 1736, 1651, 1603, 1381, 1234, 1177 and 1086; high resolution FAB MS, *m/z* 1227.6040 $[M + K]^+$ corresponding to $C_{63}H_{96}O_{21}K$ (calc. mass 1227.6081). In contrast to the spectrum of spongistatin 1, no $[M-35]^+$ ion was found. Spongistatin 3 **1c** was also obtained (2.69 mg, 6×10^{-8}% yield) as a colourless and noncrystalline powder; m.p. 148–149 °C; $[\alpha]_D$ + 28.1 (*c* 0.15, MeOH); UV (MeOH) λ/nm 226 (log ε 4.14) and 268 (3.24); IR (film) ν_{max}/cm^{-1} 3426, 2934, 1734, 1653, 1591, 1387, 1231, 1173 and 1090 cm^{-1}; high resolution FAB MS, *m/z* 1219.5556 $[M + K]^+$ corresponding to $C_{61}H_{93}ClO_{20}K$ (calc. mass 1219.5584).

The complex structural determination of spongistatin 1 **1a** was accomplished using primarily high field (400 and 500 MHz) 2D NMR spectroscopy with extensive connectivity (HMBC, NOE) (HMBC = ^{1}H-detected multiple-bond heteronuclear quantum coherence) experiments. Once the general relationship of spongistatin 1 **1a** to spongistatins 2 **1b** and 3 **1c** had been revealed, the structure solutions were markedly accelerated. The data[2a] and spectral interpretation relied upon in arriving at the spongistastin 1 structure **1a** were utilized as follows. The ^{13}C NMR spectra of spongistatin 2 **1b** contained sixty-three carbon signals, while the ^{1}H NMR spectra exhibited four methyl doublet signals at δ 1.04, 1.21, 0.91 and 0.84, one methyl singlet at δ 1.14, two acetyl methyl singlets at δ 1.86 and 2.01 and one methoxy singlet at δ 3.33. The presence of three ester carbonyl groups was evident from the ^{13}C NMR signals at δ 173.54, 171.23 and 172.79 and the ^{1}H NMR signals at δ 2.64 (br. d., *J* 18 Hz), and 2.57 (dd, *J* 10 and 18 Hz). The presence of a ketone carbonyl and its connection were also suggested by the signals at δ 2.92 (dd, *J* 10 and 18 Hz), 2.74 (br. d, *J* 18 Hz) and 1.21 (3H, d, *J* 7.0 Hz), and the ^{13}C NMR signals at δ 213.27, 51.41 and 14.26. Five double bonds were obvious from the ^{1}H NMR signals at δ 4.92 (br. s), 4.85 (br. s), 5.40 (t, *J* 10 Hz), 5.47 (m), 4.95 (br. s), 4.93 (br. s), 5.71 (dd, *J* 7 and 15 Hz), 6.23 (br. dd, *J* 10 and 15 Hz), 6.34 (ddd, *J* 10, 10 and 16 Hz), 5.18 (br. d, *J* 16 Hz) and 5.05 (br. d, *J* 10 Hz), and the ^{13}C NMR signals at δ 148.71, 114.86, 131.49, 134.25, 143.99, 116.17, 137.66, 132.06, 138.02 and 117.52. Three hemiacetal or ketal signals appeared at δ 99.59, 100.31 and 99.32.

The preceeding NMR data suggested that spongistatin 2 (**1b**) had a structure similar to that of spongistatin 1 (**1a**). Detailed analysis of 2D COSY, ^{1}H–^{13}C correlation and HMBC spectra completed the assignment of the proton and the carbon-13 signals. Indeed, direct comparison of the NMR data from spongistatin 1 **1a** and spongistatin 2 **1b** suggested that the difference between the two compounds was at C-50. The presence of an ABX spin system in the ^{1}H NMR spectra of spongistatin 2 at δ 5.05 (br.d., *J* 10 Hz), 5.18 (br. d, *J* 16 Hz) and 6.34 (ddd, *J* 10, 10 and 16 Hz) verified this assumption. Also in accord with this structural difference was the observation that signals for C-51, C-50, C-49, C-48 and C-47 in spongistatin 2 had shifts Δδ of 1.32, −1.59, 4.17, −1.22 and 0.63 ppm compared to their positions for spongistatin 1. Other signals were essentially the same as those of spongistatin 1. Thus, it became clear that a hydrogen was attached to C-50 in spongistatin 2 rather than a chlorine atom as in spongistatin 1. Extensive HMBC studies of spongistatin 2 in CD_3OD and CD_3CN strongly supported structure **1b**.

The ^{13}C NMR spectra of spongistatin 3 **1c** pointed to sixty-one carbon atoms that included two ester carbonyl signals at δ 174.00 and 171.19 and a ketone carbonyl at δ 213.11. Three hemiacetal or ketal signals were found at δ 101.64, 100.29 and 99.22. Seven methyl signals appeared at δ 11.93, 14.25, 11.83, 12.85, 30.10, 20.67 and 55.90. One of these corresponded to an acetyl group and one to a methoxy group. Ten sp^2 signals arose at δ 150.29, 114.27, 131.47, 134.34, 143.85, 116.33, 138.83, 127.93, 139.64 and 116.25. In the ^{1}H NMR spectra of spongistatin 3, the seven methyl signals were assigned to C-9a (δ 1.15, s), CH_3CO (1.85, s), C-14a (1.03, d, *J* 6.7 Hz), C-16a (1.21, d, *J* 7.1 Hz), C-34a (0.91, d, *J* 7.2 Hz), C-40a (0.84, d, *J* 6.7 Hz) and OMe (δ 3.33). All were in accord with seven signals in the ^{13}C NMR spectrum. These interpretations suggested that the structures of spongistatin 3 **1c** and spongistatin 1 **1a** were closely related except that spongistatin 3 **1c** contained one fewer acetyl group.

Direct comparison of spongistatin 1 and 3 showed that the ^{13}C and the ^{1}H NMR signals in the C-47 to C-51 region were similar and suggested (confirmed by mass spectrometry) the presence of a chlorine atom in spongistatin 3 **1c**. Analysis of the 2D COSY spectra of spongistatin 3 **1c** allowed assignment



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1a, R^1 = Cl, R^2 = Ac Spongistatin 1
b, R^1 = H, R^2 = Ac Spongistatin 2
c, R^1 = Cl, R^2 = H Spongistatin 3

Table 1 Results of comparative antitumour evaluations of spongistatins 1–3 in the NCI *in vitro* primary screen[a]

Spongistatin	Mean panel GI_{50}[b] /10^{-10} mol l^{-1}	*Compare* correlation coefficient[c]
1	1.48	1.00
2	8.51	0.83
3	8.32	0.90

[a] All compounds were tested in quadruplicate at each of three different concentration ranges (10^{-7}, 10^{-8} and 10^{-9} mol l^{-1} upper limits; $\log_{10}$ dilutions × 5) against the entire panel of 60 human tumour cell lines comprising the NCI screen.[6,7] [b] Standard errors averaged less than 15% of the respective means. [c] Correlation coefficients from the *Compare* pattern-recognition algorithm were calculated by computer using the TGI-centred mean graph profiles of differential cellular sensitivities to 1–3. The TGI mean graph profile of **1** was used as the benchmark or 'seed' for all the comparisons.[6,7]

of the ^{13}C and the ^{1}H NMR signals. The diamagnetic shift (while the coupling pattern remained the same) of the 5-H signal from δ 5.03 in spongistatin 1 **1a** to δ 4.01 in spongistatin 3 established the hydroxy group at C-5 and assignment of structure **1c** to spongistatin 3.

Comparative testing of spongistatins **1a–c** in the NCI 60 cell line *in vitro* screening panel revealed somewhat diminished overall potency of **1b** and **1c** compared to **1a** (Table 1). However, all three compounds remain among the most potent of substances tested to date in the NCI screen. Furthermore, results of pattern-recognition analyses revealed that the highly distinctive mean-graph 'fingerprint' (pattern of relative cellular sensitivity) produced in common by **1a–c** (Table 1) is closely correlated in turn (data not shown) with that shared by the important general class of microtubule-interactive antimitotics.[8]

The structural variations thus far observed in this intriguing new family of antineoplastic substances do not result in a substantial loss of their critical *in vitro* activity. The advantageous or disadvantageous effects of these structural variations upon their potential for *in vivo* activity is unknown, but will be addressed in further biological evaluations of all of the available compounds so remarkably active *in vitro*.

Financial support was provided by Outstanding Investigator Grant CA44344-01-04 and PHS grants CA-16049-07-12 awarded by the Division of Cancer Treatment, NCI, DHHS, the Fannie E. Rippel Foundation, the Arizona Disease Control Research Commission, the Robert B. Dalton Endowment Fund, Virginia Piper, Eleanor W. Libby, Polly Trautman and the Fraternal Order of Eagles Art Ehrmann Cancer Fund. We also thank the Government of Republic of Maldives (Maizan Hassan Maniku, A. Naseer and M. Shiham), Drs Dennis L. Doubek, Fiona Hogan-Pierson, Ronald A. Nieman, Jean M. Schmidt and Michael D. Williams, Mr Larry P. Tackett, Ms Denise Nielsen-Tackett, Mr Lee Williams, the US National Science Foundation (Grant CHE-8409644), and the NSF Regional Instrumentation Facility in Nebraska (Grant CHE-8620177).

Received, 13th April 1993; Com. 3/02119J

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