Isolation and Structure of the Powerful Human Cancer Cell Growth Inhibitors Spongistatins 4 and 5 from an African *Spirastrella spinispirulifera* (*Porifera*)[1a]

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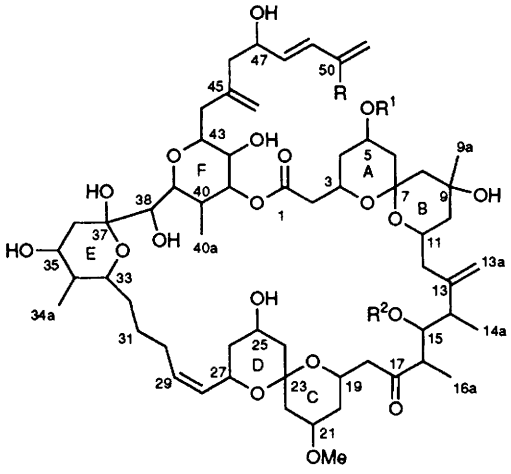
The Southwest Indian Ocean marine sponge *Spirastrella spinispirulifera* has been found to contain (in 10^{-7}% yields) two extraordinarily potent (GI_{50} *ca.* 10^{-8} μg ml^{-1}) human cancer cell line inhibitors designated spongistatins 4 **1d** and 5 **2**.

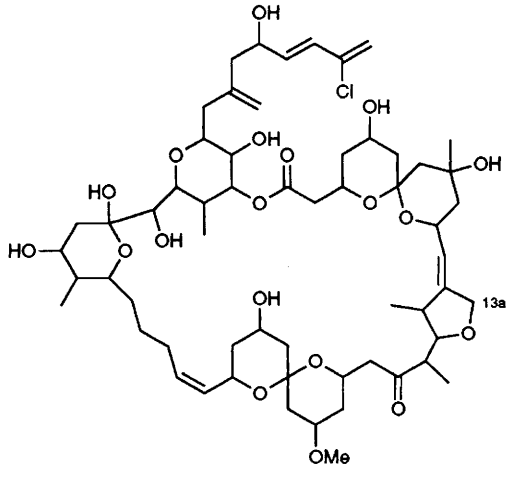
The typically bright coloured (reds, purples) marine sponges of the genus *Spirastrella* (class Demospongiae, order Hadromerida, family Spirastrellidae)[2] have not heretofore been examined for biologically active constituents except for the arsenic content of *S. insignis*.[3] In 1973, we began an investigation of antineoplastic constituents in *Spirastrella spinispirulifera* collected off the Southeast Coast of Africa. We report herein the isolation and structures of two remarkably potent antineoplastic substances from this sponge designated spongistatins 4 **1d** and 5 **2**. Previously, we discovered spongistatins 1–3 **1a–c**[4] in a Republic of Maldives *Spongia sp.* corresponding to a different order (Dictyoceratida) and family (Spongiidae). Because spongistatins 4 and 5 proved to be only trace (10^{-7}% yields) constituents their isolation and structural elucidation was especially difficult and protracted. A synopsis now follows.

Increasingly larger (to 360 kg) recollections of *Spirastrella spinispirulifera* and chemical/biological research over the period to 1980 proved inadequate. By that time all effort was focused on a 2409 kg sponge recollection preserved in ethanol that led to the discovery (the first few micrograms of spongistatin 4 were isolated in September, 1982) of macrocyclic lactones **1d** and **2**. A murine P388 lymphocytic leukaemia (PS system) active dichloromethane fraction prepared[5] from the alcohol extract was initially separated by HPLC employing a unique pilot-plant-scale HPLC system (silica gel, 3 m × 0.15 m column at 150 psi). Bioassay (PS) directed separation was continued using a series of Sephadex LH-20 (gel permeation and partition) and HPLC (Merck RP-2 silica gel with methanol–water gradients, Prepex RP-8 with acetonitrile–water and finally LiChrospher 100 RP-18 with acetonitrile–water) afford 10.7 mg (4.4 × 10^{-7}%, PS ED_{50} 4.9 × 10^{-5} μg ml^{-1}) of colourless spongistatin 4 **1d**; mp 153–154 °C; $[\alpha]^{22}_D$ + 23.0 (*c* 0.19, MeOH); UV (MeOH) λ_{max} 229 nm, ε 170 790; IR (film) 3435, 2938, 1736, 1643, 1593, 1385, 1258, 1177, 1086, 993 cm^{-1}, high resolution FABMS, *m/z* 1219.5546 $[M + K]^+$ corresponding to $C_{61}H_{93}ClO_{20}K$ (M_c 1219.5586) and 12.9 mg (5.4 × 10^{-7}%, PS ED_{50} 6.6 × 10^{-5} μg ml^{-1}) of spongistatin 5 **2**; mp 186–187 °C; $[\alpha]^{22}_D$ = −11.1 (*c* 0.23, MeOH); UV (MeOH) λ_{max} 228 nm, ε 14 840; IR (film) 3430, 2936, 1734, 1643, 1591, 1387, 1273, 1173, 1090, 982 cm^{-1}; high resolution FABMS, *m/z* 1175.5239 $[M + K]^+$ corresponding to $C_{59}H_{89}ClO_{19}K$ (*M*c 1175.5324).

Once the structure of spongistatin 1 **1a** was established[4] and its relationship to spongistatin 4 **1d** and 5 **2** revealed, structure solutions for the *Spirastrella* antineoplastic constituents were accelerated. In the ^1H NMR spectrum of spongistatin 4, one acetate, one methoxy, and another five methyl groups were obvious by the signals at δ 2.03 (3H, singlet), 3.33 (3H, singlet), 1.13 (3H, singlet), 0.97 (3H, doublet, *J* 6.9 Hz), 1.12



1a; R = Cl, $R^1 = R^2$ = COMe Spongistatin 1
b; R = H, $R^1 = R^2$ = COMe Spongistatin 2
c; R = Cl, R^1 = H, R^2 = COMe Spongistatin 3
d; R = Cl, R^1 = COMe, R^2 = H Spongistatin 4



2, Spongistatin 5

(3H, doublet, *J* 7.3 Hz), 0.91 (3H, doublet, *J* 7.2 Hz) and 0.83 (3H, doublet, *J* 6.6 Hz). The chemical shift of the acetyl methyl singlet was indicative of its attachment at the C-5 position rather than at C-15 as in the case of spongistatin 3 **1c**. Detailed analysis of the 2D COSY spectrum of spongistatin 4 and the difference in the chemical shifts of H-5 and H-15 readily confirmed a C-5 acetyl group. Both the ^{13}C and ^{1}H NMR signals from C-44 to C-51 were basically the same as those of spongistatins 1 and 3 and were in agreement with the presence of a chlorine atom (supported by the HRFABMS) at C-50. The NMR signals arising from the remaining structure were essentially the same as those of spongistatin 3. Thus, structure **1d** was assigned to spongistatin 4.

Superficially the ^{13}C and ^{1}H NMR spectra of spongistatin 5 **2** were similar to those of spongistatins **1a–d** suggesting a similar skeleton. The four methyl doublet signals at δ 1.01 (*J* 6.7 Hz), 1.14 (*J* 7.0 Hz), 0.90 (*J* 7.1 Hz), and 0.85 (*J* 6.7 Hz), one methyl singlet at δ 1.14, one methoxy signal at δ 3.31 and the sp^2 proton signals at δ 5.38 (doublet of doublets, *J* 10,11 Hz), 5.47 (doublet of triplets, *J* 7,11 Hz), 4.97 (broad singlet), 4.95 (broad singlet), 6.13 (broad doublet of doublets, *J* 6,15 Hz), 6.41 (broad doublet, *J* 15 Hz), 5.42 (broad singlet) and 5.33 (broad singlet) were consistent with this assumption. Furthermore, the coupling pattern of the signals at δ 5.42, 5.33 and 6.41 and the lack of an H-50 signal suggested a chlorine atom at C-50. But further analyses of 1D and 2D NMR spectra

Table 1. Results of comparative antitumour evaluations of spongistatins 1, 4 and 5 in the NCI *in vitro* primary screen[a]

Spongistatin	Mean panel GI_{50} $\times 10^{-10}$ mol dm^{-3} [b]	Compare correlation coefficient[c]
1 **1a**	1.17	1.00
4 **1d**	1.02	0.93
5 **2**	1.23	0.92

[a] All compounds were tested in quadruplicate at five different concentrations (10^{-8}, 10^{-9}, 10^{-10}, 11^{-11} and 10^{-12} mol dm^{-3}) against the entire panel of 60 human tumour cell lines comprising the NCI screen.[7-9] [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, 4 and 5. The TGI mean graph profile of 1 was used as the benchmark or 'seed' for all of the comparisons.[7,8]

revealed significant differences between spongistatin 5 and the other spongistatins in two respects. First, the absence of an acetyl signal. Secondly, the pair of sp^2 methylene signals for H-13a common to spongistatins 1–4 were not present. In a ^{1}H–^{13}C correlated spectrum, a ^{13}C signal at δ 70.72 was found correlated with two ^{1}H signals at δ 4.47 (broad doublet, *J* 13 Hz) and 4.09 (broad doublet, *J* 13 Hz). The ^{1}H–^{1}H COSY spectrum of spongistatin 5 displayed two signals at δ 4.47 and 4.09 with long-range couplings to a signal at δ 5.24 (broad doublet, *J* 11 Hz). In turn, the signal at δ 5.24 was found coupled with a signal at δ 5.28 (broad doublet of doublets, *J* 9,11 Hz). In the ^{13}C NMR spectrum of spongistatin 5, signals for sp^2 carbon atoms at C-13, C-28, C-29, C-45, C-45a, C-48, C-49, C-50, C-51 were observed with chemical shifts essentially the same as found for spongistatins 1, 3 and 4. The remaining one sp^2 carbon signal at δ 120.13 showed a correlation only with the one proton signal at δ 5.24 in the ^{1}H–^{13}C spectrum. Such evidence indicated that a C-12,13 double bond allylic to a C-13a atom bonded to oxygen that resulted in the AB pattern at δ 4.47 and 4.09 in the ^{1}H NMR spectrum was present in spongistatin 5 **2**. The dramatic downfield shift of the C-15 signal at δ 84.46 (δ 73.75 in spongistatin 4) suggested that a tetrahydrofuran ring comprising C-15, C-14, C-13 and C-13a was present. The molecular formula suggested by FABMS also favoured this conclusion. The presence of a tetrahydrofuran ring was further confirmed by an HMBC spectrum in which the ^{13}C signal at δ 84.46 (C-15) was strongly correlated with one of the two H-13a signals at δ 4.47. All of the ^{1}H and ^{13}C NMR data as well as the HMBC correlations strongly supported assignment of structure **2** to spongistatin 5. Clearly, the C-50 chlorine atom is an important and common[6] structural feature of the spongistatins. Only spongistatin 2 lacks chlorine.

Evaluation[7,8,9] of spongistatins 4 and 5 against the US National Cancer Institute panel of 60 human cancer cell lines gave dramatic results. Comparative testing of spongistatins **1a**, **1d** and **2** in the NCI 60 cell line *in vitro* screening panel[7-9] revealed an overall potency of spongistatins **1d** and **2** comparable with **1a** (*e.g.* panel mean GI_{50} 10^{-10} mol dm^{-3}; Table 1). The three compounds are among the most potent of all substances tested to date in the NCI screen. Interestingly, several of the human breast cancer cell lines recently incorporated into the NCI screening panel were among the most sensitive (*e.g.* GI_{50} 10^{-11}–10^{-12} mol dm^{-3}). Furthermore, results of pattern-recognition analyses revealed that the highly distinctive mean-graph 'fingerprint' (pattern of relative cellular sensitivity) produced in common by spongistatins **1a** and **2** (Table 1) is closely correlated in turn (data not shown) with that shared by the important general class of microtubule-interactive antimitotics.[9] The structural variations thus far observed in this intriguing new family of antineoplastic substances do not result in substantial loss of the critical *in*

vitro activity attributes. The advantageous or disadvantageous effects of these structural variations upon the *in vivo* activity potential is unknown, but will be addressed in further biological evaluations of all of the available compounds so remarkably active *in vitro*.

Discovery of the spongistatins in quite distant (in respect to taxonomy and geography) *Porifera* species suggests that this very important new series of remarkable antineoplastic agents may prove to be widely distributed in such marine invertebrates and/or associated marine microorganisms. Interestingly, a recent first-study of *Porifera* found adjoining Easter Island, the most remote South Pacific Island, uncovered both *Spirastrella cunctatrix* and *Spongia virgultosa* in the same general area.[10] A future examination of these two sponges for spongistatins should prove useful. Presently, we are pursuing extended *in vivo* human cancer xenograft evaluations of spongistatins 4 **1d** and 5 **2** and research directed at completing the absolute configurational assignments for the spongistatins by X-ray crystal structure determinations.

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