Isolation and Structure of Halistatin 3 from the Western Pacific (Chuuk) Marine Sponge *Phakellia sp*

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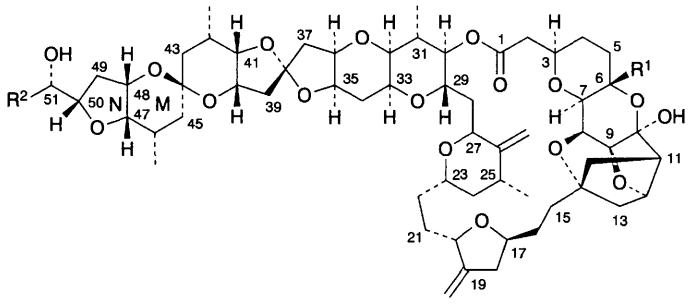
The Micronesian marine sponge *Phakellia sp.* has been found to contain key members of the extraordinarily potent (antineoplastic) halichondrin/halistatin polyether macrolide family.

Polyether macrolides of the Porifera halichondrin/halistatin (*cf.* **1a**)[1] series represent remarkably strong antineoplastic agents. Other interesting cell-growth inhibitory macrolides include the sea hare constituent aplyronine A **2**[2a] and from marine sponges lasonolide A[2b] and swinholide A.[2c] More broadly, certain of the marine dinoflagellate derived perhydropyran systems range in biological activity to some of the most extraordinarily toxic substances known such as the ciguotoxin/maitotoxin/polytoxin series.[3]

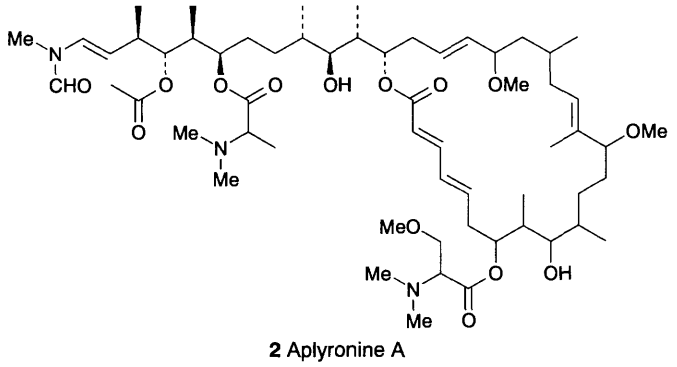
Our discovery of the clinically promising[4a] anticancer drug bryostatin 1 **3**[4b,5] and the selection of halichondrin B **1b**[1a–c] and halistatin 1[b–c] for preclinical development greatly stimulated our interest in such polyether macrolides and led to intensified efforts directed at increasing availability of the halistatins as well as uncovering related biosynthetic products. We now report discovery of halistatin 3 **1c** as a trace constituent of the orange *Phakellia sp.* (class Demospongiae, Order Axinellida, family Axinellidae) collected (1986–7) in the Federated State of Micronesia (Chuuk). Furthermore, this productive Porifera species was found to be a new source of halichondrin B (**1b**, 7.2 mg, 1.4×10^{-7}%), homohalichondrin B (2.5 mg, 5×10^{-8}%) and halistatin 1 (**1a**, 13.2 mg, 2.6×10^{-7}%).

A 500 kg (wet mass) collection of *Phakellia sp.* was extracted with methanol followed by dichloromethane–methanol. A murine P388 lymphocytic leukemia cell line (P388) active dichloromethane fraction prepared from these extracts was separated (P388 bioassay) by a sequence of size-exclusion and partition chromatographic steps employing Sephadex LH-20. Final isolation and purification was eventually realized by a combination of Sephadex LH-20 partition chromatography (hexane–dichloromethane–methanol, 5 : 1 : 1) and C-8 reversed-phase HPLC (propan-2-ol–methanol, 6.5 : 3) to afford the new cancer cell growth inhibiting macrolide designated halistatin 3 (**1c**, 1.9 mg, 3.8×10^{-8}%); mp 185–187 °C; $[\alpha]_D^{25}$ −62 (*c* 0.045, MeOH); UV (MeOH) λ_{max} 201.0 nm (ε 8000 $dm^3\ mol^{-1}\ cm^{-1}$).

Structural elucidation of halistatin 3 was achieved by a combination of 2D NMR, FABMS and HRFABMS analyses. The FAB mass spectrum showed a quasi-molecular ion [M + Na]$^+$ at *m/z* 1147.6. The high resolution mass measurements established the molecular formula as $C_{61}H_{88}O_{19}$ from the molecular ion at *m/z* 1147.5771 (Δ −4.1 ppm) for [M + Na]$^+$. This suggested the presence of one additional methylene group than is found in halichondrin B **1b** and was confirmed by the similarities between the ^{1}H NMR and ^{13}C NMR spectra of halistatin 3 and halichondrin B. The ^{1}H NMR spectrum of pyran **1c** displayed four secondary methyls at δ 0.95 (d, *J* 7.0 Hz), 0.99 (d, *J* 7.0 Hz), 1.05 (d, *J* 7.5 Hz), and 1.09 (d, *J* 6.5 Hz) characteristic of halichondrins/halistatins. The APT and HMQC spectra established the presence of four methyl, twenty two methylene (two exocyclic), twenty nine methine and six quaternary carbons, again indicating one more methylene than

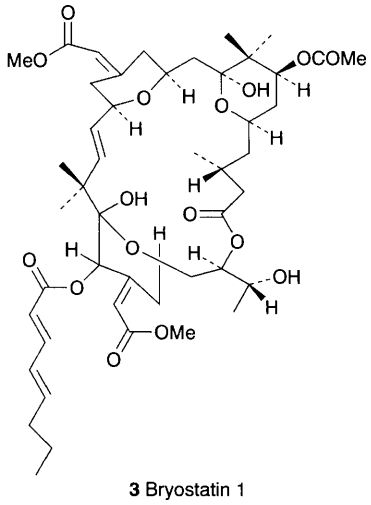


1a R^1 = OH, R^2 = $CH_2CH(OH)CH_2OH$, Halistatin 1
1b R^1 = H, R^2 = $CH_2CH(OH)CH_2OH$, Halichondrin B
1c R^1 = H, R^2 = $CH_2CH_2CH(OH)CH_2OH$, Halistatin 3



2 Aplyronine A

halichondrin B. Importantly, the NMR data established that halistatin 3, from rings A to M (the left terminal tetrahydropyran ring), was identical to that of halichondrin B **1b**, including the unique tricyclic halipyran ring system. The only differences observed were in the left terminal ring (N) and side-chain of the molecule. Thus, we focused on the structure beyond the M ring of halistatin 3. Since unsaturation equivalents from the molecular formula required only one more ring in the system (*i.e.* N), the additional methylene group would have to be inserted either in the N ring or in the side-chain. Analyses of COSY, TOCSY and HMQC spectra revealed the partial structure –CH(O)CH(O)CH_2CH(O)CH(O)CH_2CH_2CH(O)-CH_2OH. The N ring proton chemical shifts suggested a five- rather than a six-membered ring. A deuterium-induced shift experiment also supported the five-membered N ring ($\Delta\delta$ CD_3OH–CD_3OD: C-50, 0.19; C-51, 0.104). Therefore, the new methylene group was placed between the two hydroxymethines at C-51 and C-53 of halichondrin B.



3 Bryostatin 1

Hirata and Uemura[6] determined the halichondrin B stereochemistry at C-50 and C-51 based on its biosynthetic relationship to norhalichondrin A (structure by X-ray crystal structure analysis) isolated from the same sponge (*Halichondrin okadai*) and the configuration assignments were later confirmed by total synthesis.[7] Since we did not isolate any nor-series representatives from the Micronesian *Phakellia sp.*, the stereochemistry of the halistatin 3 side-chain was further examined by high field ^1H NMR analysis. The coupling constants corresponding to H-47 (t, *J* 2.5 Hz) indicated *cis*-fused M and N rings as in halichondrin B. Examination of results from decoupling experiments, in combination with ^1H–^1H COSY and ^1H *J*-resolved spectral analyses revealed the coupling patterns and coupling constants shown in Table 1. The absence of coupling between H-48 and H-49α suggested the dihedral angle to be 90°. The coupling constant between H-50 and H-49α proved to be 3.0 Hz, while the coupling constant between H-50 and H-49β was 9.2 Hz. A study of molecular models using these coupling constants revealed that the configuration of H-50 was β. Since a plausible biosynthetic route to the halichondrin/halistatin family may involve *cis*-epoxides as intermediates in forming five- and six-membered cyclic ether rings typical of polyether natural products,[8] the configuration of the hydroxyl group at C-51 is most likely α in relationship to H-50. Configuration of the hydroxyl group at C-54 will have to await an eventual X-ray crystal structure determination.

Halistatin 3 was found to strongly (ED_{50} 3.5×10^{-5} μg ml^{-1}) inhibit the P388 leukemia cell line and a 'mini' panel of human cancer cell lines (GI_{50}, μg ml^{-1}): brain (SF-295, 3.5×10^{-5}), lung (NCI-460, 2.5×10^{-5}), colon (KM 2062, 5.1×10^{-6}), ovary (OVCAR-3, 1.3×10^{-5}) renal (A498, 5.6×10^{-5}) and melonoma (SK-MEL-5, 2.5×10^{-5}). In summary, halistatin 3 **1c** is an important new member of the halistatin

Table 1 The 500 MHz ^1H and 126 MHz ^{13}C NMR chemical shift assignment for halistatin 3 **1c** determined in CD_3OD

Position	^{13}C (multiplicity)	^1H (multiplicity, *J*/Hz)	Position	^{13}C (multiplicity)	^1H (multiplicity, *J*/Hz)
1	172.86 (s)		29	73.80 (d)	4.23 (m)
2	41.26 (t)	2.44 (dd, 17.5, 2.0)	30	77.43 (d)	4.62 (dd, 7.5, 5.0)
		2.55 (dd, 17.5, 8.0)	31	37.54 (d)	2.05
3	74.94 (d)	3.87	31–Me	15.86 (q)	1.05 (d, 7.5)
4	31.88 (t)	1.34, 1.72	32	78.06 (d)	3.21 (dd, 6.5, 4.5)
5	31.33 (t)	1.40, 2.02	33	65.75 (d)	3.86
6	69.64 (d)	4.32	34	30.88 (t)	1.83, 2.06
7	79.15 (d)	2.97 (dd, 9.5, 2.0)	35	77.34 (d)	4.06
8	75.89 (d)	4.29	36	78.01 (d)	4.09
9	75.11 (d)	4.11	37	45.60 (t)	2.02, 2.39 (dd, 13.5, 6.5)
10	77.94 (d)	4.17 (t, 4.5)	38	114.86 (s)	
11	83.88 (d)	4.59 (t, 4.5)	39	45.02 (t)	2.33
12	82.48 (d)	4.69 (t, 4.5)	40	73.00 (d)	4.03
13	49.42 (t)	1.98, 2.08	41	80.83 (d)	3.68
14	111.31 (s)		42	27.19 (d)	2.26
15	35.82 (t)	1.59, 2.17	42–Me	18.18 (q)	0.95 (d, 7.0)
16	29.46 (t)	1.43, 2.18	43	38.14 (t)	1.27, 1.48
17	76.36 (d)	4.09	44	98.31 (s)	
18	39.75 (t)	2.31, 2.79 (m)	45	38.06 (t)	1.38, 1.51
19	153.22 (s)		46	27.11 (d)	2.32
19=CH_2	44.51 (d)	5.01, 5.06	46–Me	18.37 (q)	0.99 (d, 7.0)
20	76.15 (d)	4.44 (br d, 10.5)	47	81.24 (d)	3.55 (t, 2.5)
21	31.10 (t)	1.36, 1.97	48	73.46 (d)	4.07
22	33.06 (t)	1.49, 1.67	49	35.36 (t)	2.00, 2.17
23	75.42 (d)	3.70	50	82.19 (d)	3.84
24	44.95 (t)	1.01, 1.72	51	74.55 (d)	3.60
25	37.21 (d)	2.29	52	31.13 (t)	1.46, 1.70
25–Me	18.42 (q)	1.09 (d, 6.5)	53	30.65 (t)	1.57
26	153.35 (s)		54	73.31 (d)	3.59
26=CH_2	104.77 (t)	4.80, 4.87	55	67.48 (t)	3.43 (dd, 11.0, 6.5)
27	75.11 (d)	3.60			3.48 (dd, 11.0, 4.5)
28	37.85 (t)	1.82, 2.25			

series of antineoplastic polyether macrolides and has provided new structure/activity information.

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