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ISOLATION AND STRUCTURE OF AXINASTATIN 5 FROM A REPUBLIC OF COMOROS MARINE SPONGE[1]

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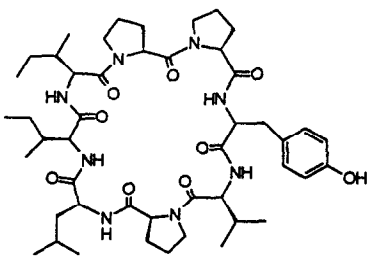
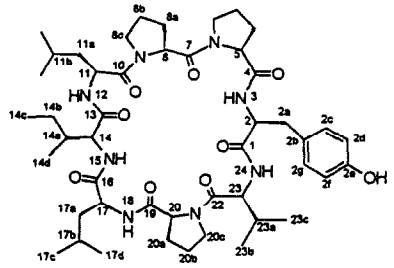
Abstract: The new cancer cell growth (human and murine) inhibitory (GI_{50} 0.3 to 3.3 μg/ml) *cyclo*-octapeptide axinastatin 5 (2) was isolated in 3.8 x 10^{-7}% yield from the Western Indian Ocean marine sponge *Axinella cf. carteri*. Structural elucidation was achieved by high field (400 and 500 MHz) 2 D-NMR techniques and the amino acid sequence was confirmed by results of NMR and high resolution mass spectral MS/MS interpretations. Other peptide cancer cell growth inhibitory constituents of this orange sponge were found to be the cyclic hepta- and octapeptides axinastatin 1 and hymenistatin 1 (1).

Marine animal peptide constituents offer increasingly attractive routes to new anticancer drugs. Illustrative are the remarkable sea hare peptides dolastatins 10 and 15 now undergoing preclinical development.[2] In a parallel long-term investigation employing marine Porifera, we have discovered a series of peptides with cancer cell growth inhibitory properties where the *cyclo*-heptapeptide phakellistatins,[3] stylostatins,[4] axinastatins[5] and the *cyclo*-octapeptide hymenistatin 1 (1)[6] provide an overall view of recent progress. Recently, the Okinawa marine sponge *Hymeniacidon sp.* has yielded five new cyclic heptapeptides[7] (hymenamides A-E) and a Fiji marine ascidian[8] has provided two more proline-derived *cyclo*-heptapeptides.[9] More broadly, the terrestrial plants *Rubia akane*[10a] and *R. Cordipolia*[10b] have produced the antineoplastic RA series of cyclic heptapeptides where RA-VII has been placed in preclinical development.[10c]

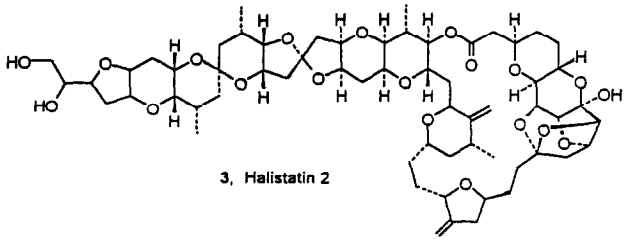
We now report that further bioassay (murine P388 lymphocytic leukemia cell line, PS system) guided separation of trace, albeit, PS active fractions prepared from a 600 kg (wet wt) recollection (1989, Republic of Comoros) of the orange marine sponge *Axinella cf. carteri*[5] has led to discovery of a new PS (ED_{50} 3.5 μg/ml) and human cancer inhibitory (*cf.* Table 2) cyclic octapeptide designated axinastatin 5 (2). PS active fractions remaining from isolation of halistatin 2 (3)[11] were further separated using a cyclohexane-2-propanol-methanol (8:1:1) partition chromatographic system on Sephadex LH-20 followed by reversed (Prepex RP-8) phase HPLC in acetonitrile-methanol-water (5:5:6). That sequence led to halistatin 2 (3, 4.0 mg),[11] hymenistatin 1 (1, 17.9 mg) and axinastatin 5 (2, 2.3 mg, 3.8 x 10^{-7}% yield): mp >300°C; $[\alpha]_D$ -141° (c, 0.11,CH_3OH); HRFABMS calcd. for $C_{47}H_{73}N_8O_9$ [M+H] 893.55052, found 893.5426. Interestingly, our antibiotic[7] and cancer cell growth inhibitory cyclic heptapeptide axinastatin 1[5] (51.1 mg) was isolated from a companion trace fraction. Each of the previously reported substances was found to be identical with authentic specimens.



1, Hymenistatin 1



2, Axinastatin 5



3, Halistatin 2

The structure (2) of axinastatin 5 was initially determined by high field 2D-NMR studies (Table 1) and confirmed by results of MS/MS sequence determination. The ^{1}H-NMR spectrum of peptide 2 showed eight proton signals at δ 4.22 (1H, multiplet), 4.32 (1H, doublet, J = 7.2 Hz), 4.17 (1H, doublet of doublets, J = 3.5, 9.3 Hz), 4.64 (1H, multiplet), 4.09 (1H, triplet, J = 8.7 Hz), 3.71 (1H, multiplet), 3.91 (1H, triplet, J = 7.8 Hz) and 4.49 (1H, triplet, J = 10 Hz) all alpha to carbonyl groups. Furthermore, these eight proton signals correlated with eight carbon signals at δ 58.88, 61.68, 60.59, 50.64, 60.67, 57.37, 63.26 and 56.95 in the ^{1}H-^{13}C spectra. Eight carbonyl signals were observed in the ^{13}C-NMR spectrum recorded in CD_3CN. In sum this evidence indicated the presence of eight amino acid units in axinastatin 5. Detailed analysis of the ^{1}H-^{1}H COSY and ^{1}H-^{13}C correlated spectra completed assignment of the ^{1}H- and ^{13}C-NMR signals (except for the carbonyl signals assigned by HMBC) and established that the amino acid units were tyrosine, proline, leucine, isoleucine and valine in a ratio of 1:3:2:1:1 (see Table 1 for details). The amino acid analyses also agreed with the same composition and relative ratio.

Table 1. The NMR Assignments for Axinastatin 5 in Deuteroacetonitrile with Tetramethylsilane as Internal Standard (the mixing time was set at 60 ms for HMBC).

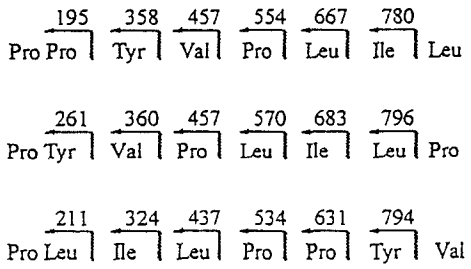
Posn. No.	^{13}C(100 MHz)	^{1}H(400 MHz)	HMBC(500 MHz, C to H)
Tyr 1	172.84p		H-2,H-2a,H-24,H-23
2	58.88n	4.22 m	H-3,H-2a
2a	37.73p	2.98 t(13;	H-3,H-2c
		3.17 dd(4.4,13)	
2b	128.76p		H-2a,H-2d
2c	130.85n	7.04 d(8.9)	H-2a,H-2g
2g	130.85n	7.04 d(8.9)	H-2c,H-2f
2d	116.58n	6.77 d(8.9)	H-2c,H-2f
2f	116.58n	6.77 d(8.9)	H-2d,H-2g
2e	157.77p		H-2c,H-2g,H-2d,H-2f
3	NH	7.76 brd(7.8	
	OH	3.57 br	
Pro 4	170.92p		H-3,H-5,H-5a
5	61.68n	4.32 d(7.2)	H-5a,H-5b,H-5c
5a	31.75p	2.15 m; 1.90 m	H-5,H-5b,H-5c
5b	22.14p	1.60 m; 0.75 m	H-5,H-5a,H-5c
5c	47.51p	3.25 ddd(4,11,11);	H-5,H-5a
		3.04 brt(11)	
Pro 7	172.16p		H-5,H-5c,H-8a
8	60.59n	4.17 dd(3.5,9.3)	H-8a,H-8b,H-8c
8a	29.09p	2.23 *; 1.80 *	H-8,H-8b,H-8c
8b	25.97p	2.02 *; 1.95 *	H-8,H-8c
8c	48.17p	3.55 *;	H-8b
		3.38 dt(11,11)	
Leu 10	171.40p		H-11,H-11a
11	50.64n	4.64 m	H-11a,H-11b,H-12
11a	42.25p	1.85 m; 1.24 m	H-11c,H-11d,H-11,H-11b
11b	26.06n	1.58 m	H-11c,H-11d,H-11,H-11a
11c	23.70n	0.84 d(6.5)	H-11d,H-11a,H-11b
11d	22.25n	0.79 d(6.5)	H-11c,H-11a,H-11b
12	NH	7.91 d(7.6)	
Ile 13	171.63p		H-12,H-14
14	60.67n	4.09 t(8.7)	H-14d,H-15,H-14a,H-14b
14a	38.85n	1.50 m	H-14c,H-14d,H-14,H-14b
14b	25.89p	1.11 m; 1.48 m	H-14d,H-14c,H-14
14c	10.88n	0.83 t(7.1)	H-14b
14d	15.79n	0.87 d(6.5)	H-14,H-14b
15	NH	7.86 brd(8.9)	
Leu 16	173.30p		H-17,H-15,H-14,H-17a
17	57.37n	3.71 m	H-17a
17a	39.34p	2.16 m; 1.59 m	H-17c,H-17d,H-17b,H-17
17b	25.97n	1.55 m	H-17c,H-17d,H-17a
17c	23.34n	0.91 d(6.3)	H-17d,H-17a
17d	21.38n	0.88 d(6.5)	H-17c,H-17a
18	NH	7.10 br	
Pro 19	173.86p		H-20,H-17,H-20a
20	63.26n	3.91 t(7.8)	H-20c,H-20a,H-20b
20a	30.52p	2.20 m; 1.85 m	H-20,H-20c,H-20b
20b	25.78p	2.05 m; 1.90 m	H-20c,H-20a
20c	49.24p	3.68 *; 3.68 *	H-20a
Val 22	172.97p		H-23,H-20,H-23a
23	56.95n	4.49 t(10)	H-23b,H-23c,H-24,H-23a
23a	33.23n	1.86 m	H-23b,H-23c,H-23
23b	19.76n	0.97 d(6.7)	H-23c,H-23,H-23a
23c	18.82n	0.94 d(6.7)	H-23b,H-23,H-23a
24	NH	7.41 d(10)	

* The coupling pattern and coupling constants for these signals were not measured due to overlapping

Connectivity of the amino acid units was solved by HMBC and NOE studies. The ^{1}H signals at δ 7.41 (H-24), 4.49 (H-23), 4.22 (H-2) and 2.98 (H-2a) showed correlations with the ^{13}C signal at δ172.84 (C-1) suggesting that the Val-NH was connected to the Tyr-CO. Hydrogen atom signals at δ 7.76 (H-3), 4.32 (H-5) and 2.15 (and 1.90, H-5a) showed cross peaks with the ^{13}C signal at δ 170.92 (C-4) establishing the Tyr-NH to Pro (1)-CO linkage. The ^{1}H signals at δ 4.32 (H-5), 3.25 (H-5c), 2.23 and 1.80 (H-8a) showed cross peaks with the ^{13}C signal at δ 172.16 (C-7) which allowed connection of the Pro (1)-N to the Pro (2)-CO. Similarly, HMBC correlation between the NH signal at δ 7.91 (H-12) and ^{13}C signals at δ 50.64 (C-11) and 171.63 (C-13) led to joining the Leu (1)-NH to the Ile-CO. The next HMBC viewed between the NH signal at δ 7.86 (H-15) and the ^{13}C signals at 60.67 (C-14) and 173.30 (C-16) allowed connection of the Ile-NH to the Leu (2)-CO. The exchangeable proton at the Leu (2) H-18 was spread from δ 5.4 to 7.3 ppm and prevented an observable HMBC cross peak. Fortunately, the H-17 signal at δ 3.71 was found correlated (HMBC) with the Pro (3) carbonyl signal at δ 173.86. In turn that provided the Leu (2)-NH to Pro (3)-CO linkage. The H-20 (δ 3.91) was analogously found correlated with the C-22 signal at δ 172.97 showing the connection between Pro (3)-N and the Val-CO.

No HMBC effects were observed between Pro (2) and Leu (1). However, their union was detected by NOE difference spectroscopy: irradiation of the H-11 signal at δ 4.64 enhanced dramatically one of the H-8C signals at δ 3.55. Reciprocal irradiation of the signal at δ 3.55 enhanced greatly the H-11 signal. These NOE results could only be explained by the Pro (2)-N to Leu (1)-CO bonding. The HMBC and NOE results established the amino acid units and sequence of axinastatin 5 (2). Assignment of the amino acid units as all (S) was achieved by chiral gas chromatographic analyses[12] of N-pentafluoropropyl isopropyl esters[13] prepared from propionic acid-hydrochloric acid hydrolysis[13] of peptide 2.

Confirmation of the cyclic octapeptide structural proposal (2) was achieved as follows. Collisional activation of the $[M + H]^{+}$ ions of axinastatin 5 produced immonium ions characteristic of Pro (m/z 70), Val (m/z 72), Leu/Ile (m/z 86), and Tyr (m/z 136). When combined with the determined exact mass of $[M + H]^{+}$ this data provides an amino acid composition of 3 x Pro, 3 x Leu (Ile), 1 x Val, and 1 x Tyr. Protonation of the amide nitrogen of any of the three proline units upon FAB resulted in ring opening and formation of three different acylium ions.[14] When these acylium ions were collisionally activated they decomposed, principally by cleavage of the amide bonds, to form three series of fragment ions that define the sequence as *cyclo*-(Pro-Pro-Tyr-Val-Pro-Leu-Ile-Leu) (see the following fragmentation scheme). Other ions observed in the CAD spectrum provided additional



confirmation for the sequence. These internal fragments, resulting from two backbone cleavages, helped to define the coupling of the amino acid units. An important group of the internal fragments was dipeptide species. Ions for the series of possible dipeptides present in a proposed sequence should be observed if the proposed sequence is valid and for axinastatin 5, these ions were found at; m/z 195 (Pro-Pro), 197 (Pro-Val), 211 (Leu/Ile-Pro or Pro-Leu/Ile), 227 (Leu-Ile or Ile-Leu), 261 (Pro-Tyr) and 263 (Tyr-Val).

The NMR results indicated the presence of two Leu and one Ile units. Their location was ascertained by careful examination of the MS/MS spectrum. Since acylium fragment ions formed upon collisional activation can eliminate CO from the C-terminus, ions that have either Leu or Ile at the C-terminus can undergo further degradation to eliminate C_3H_6 (-42 μ) in the case of Leu or C_2H_4 (-28 u) if the C-terminus is Ile.[15] Observation of an ion at m/z 724 [780-CO(28-28] indicated the presence of Ile. Conversely, the ion observed arising from m/z 667 namely m/z 597 [667-CO(28)-42] placed Leu at this position.

The *cyclo*-octapeptides axinastatins 5 (2) and hymenistatin 1 (1) both exhibited significant activity against a mini-panel (Table 2) of human cancer cell lines. Results of the present research suggest that more extensive antineoplastic evaluation of such promising marine sponge cyclic peptides will be a useful avenue to pursue.

Table 2. Inhibitory Activity of Hymenistatin 1, and Axinastatin 5 Against Selected Human Cancer Cell Lines

Cell Type	Cell Line	Hymenistatin 1 GI-50* (μg/ml)	Axinastatin 5 GI-50* (μg/ml)
Ovarian	OVCAR-3	0.04	0.3
CNS	SF-295	0.3	1.6
Renal	A498	0.7	2.3
Lung-NSC	NCI-H460	0.3	0.5
Colon	KM20L2	0.1	0.7
Melanoma	SK-MEL-5	0.06	0.5

* GI = Growth Inhibition

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