ISOLATION AND STRUCTURE OF AXINASTATIN 4 FROM THE WESTERN INDIAN OCEAN MARINE SPONGE *AXINELLA cf. CARTERI*[1a,1b]

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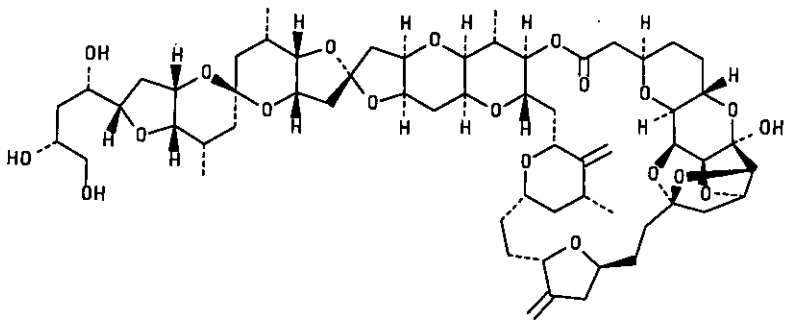
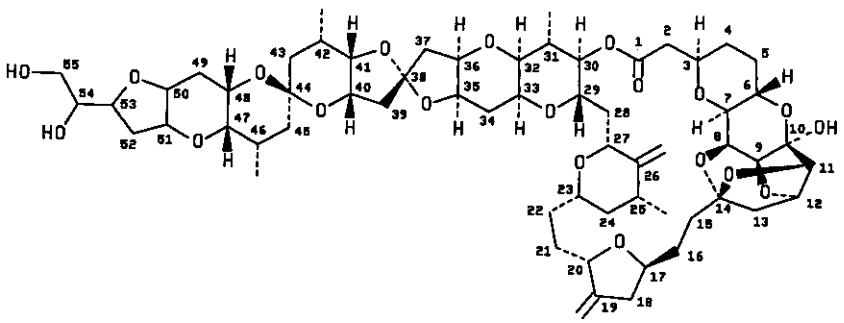
Abstract - The Republic of The Comoros marine sponge *Axinella cf. carteri* has been found to contain a cell growth inhibitory (P388 lymphocytic leukemia cell line ED_{50} 0.057 μg/ml, and comparable activity against a series of human cancer cell lines) *cyclo*-heptapeptide named axinastatin 4 (3). The new peptide was obtained in 1.0 x 10^{-6}% yield (6.1 mg) from 600 kg (wet wt) of the sponge. The structure of axinastatin 4 was deduced as *cyclo*-(Pro-Leu-Thr-Pro-Leu-Trp-Val) by a combination of high field (400 and 500 Mhz) 2D nmr (^1H-^1H, ^1H-^{13}C, HMBC and NOE) and high resolution mass spectral (principally by ms/ms) measurements and subsequent interpretations.

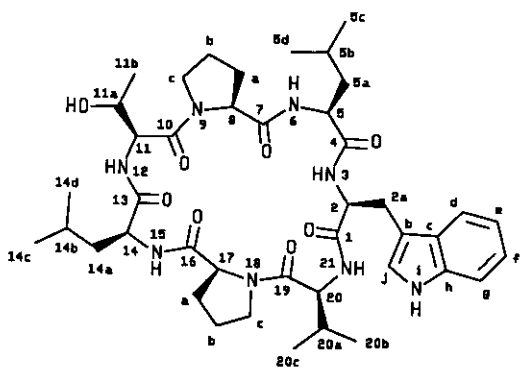
Subsequent to our early[2] bioassay (murine P388 lymphocytic leukemia) directed isolation of the antineoplastic geodiastatins[3] from a black marine sponge, a number of cytotoxic and/or antineoplastic compounds have been isolated from marine Porferia.[4,5] Recently such potentially useful compounds have been uncovered in a diverse series of sponges representing the *Axinella*,[1a] *Phakellia*,[6] *Stylotella*,[7] *Mycale*,[8] *Theonella*,[9,10] *Hippospongia*,[11] and *Aplysina*,[12] genera. In addition, antiviral constituents have been found in the *Stronglophora*,[13] *Aplysina*[14] and *xestospongia* [15] genera and antibacterials in a *Luffariella*[16] species. Our current intensive investigation of *Axinella cf carteri* from the Republic of the Comoros has already provided the very potent antineoplastic components, halichondrin B,[17,18] homohalichondrin B[17,18] and halistatins 1 (1) and 2 (2).[19-20] We now report the isolation and



1, HALISTATIN 1



2, HALISTATIN 2



3, AXINASTATIN 4

structural elucidation of axinastatin 4 (3), a new *cyclo*-heptapeptide and significantly active cell growth inhibitor, from this very useful sponge.

A 600 kg (wt wt) recollection of *A. carteri* completed in 1989 was used to obtain a fraction (0.80 g) rich in halichondrin-like compounds[20] and peptides. Further separation in cyclohexane-2-propanol-methanol (8:1:1) on a column of Sephadex LH-20 followed by semipreparative hplc using a silica gel Rp-8 column with acetonitrile-methanol-water afforded (6.1 mg, 1.0 x 10^{-6}%) axinastatin 4 (3, P388 cell line ED_{50} 0.057 μg/ml); amorphous, mp 201-206° (uncorrected); $[\alpha]_D^{25}$ -92.8° (c, 0.5, CH_3OH); R_f 0.86 (on silica gel using 95:5 methylene chloride-methanol); and ir NaCl ν_{max}: 3584 (NH), 3335 (OH), 1645 (C=O), 1524 (C=C) cm^{-1}.

Axinastatin 4 exhibited a parent ion in an HRFAB ms spectrum at m/z 807.4784 $[M+H]^+$ suggesting a molecular formula of $C_{42}H_{62}N_8O_8$ (calcd for $C_{42}H_{63}N_8O_8$ 807.4769). The ^1H and ^{13}C nmr spectra (Table 1) contained signals characteristic of peptides. The presence of two Leu, two Pro, one Val, one Thr and one Trp units was confirmed by detailed analysis of the ^1H- and ^{13}C-nmr spectra, the 2D COSY, and ^1H-^{13}C nmr spectra. Moreover, amino acid analyses agreed with the presence of Leu, Pro, Val, Thr and Trp in a ratio of 2:2:1:1:1.

Two sets of FAB ms fragmentations resulted from protonation of the two Pro units and were indicative of a cyclic peptide structure for axinastatin 4:

m/z	211	312	409	522	708							
Pro -	Leu -	Thr -	Pro -	Leu -	Trp -	Val						
		m/z		211	397	496	593	706				
			Pro -	Leu -	Trp -	Val -	Pro -	Leu -	Thr			

Evidence supporting a cyclic peptide was also obtained by counting the unsaturation numbers and reconciling that result with the nmr and ms data. The axinastatin 4 molecular formula required 16 unsaturation sites corresponding to two Pro, one Trp and seven carbonyls thereby accounting for 15. The remaining unsaturation site was attributed to the additional cyclic peptide ring. The amino acid sequence of axinastatin 4 was further substantiated by careful analysis of the HMBC and NOE (Tables 1 and 2) spectra. An HMBC experiment (in deuterioacetonitrile with a mixing time setting at 120 micro seconds) led to the following correlations: H-3 correlated with C-2 and C-4; H-6 with C-7 and C-5; H-12 with C-10 and C-13; H-15 with C-16; H-21 with C-1 and C-19; H-17c with C-19. When signals for the

exchangeable protons were assigned by 2D COSY nmr, it was possible to deduce the peptide sequence as *cyclo*-(Pro-Leu-Thr-Pro-Leu-Trp-Val).

Chiral GC analysis[21] based on *N*-pentafluoropropyl/isopropyl ester[22] derivatives of the propionic acid-hydrochloric acid[22] hydrolysate of axinastatin 4 (3) was employed to determine the absolute configuration of the constituent amino acids. Based on direct and indirect comparisons, L-Leu and L-Pro were detected. Due to the presumed decomposition of Trp and the close retention times for Val and Thr, the absolute configuration of these three amino acids were not assigned. Further experiments are underway in order to determine the absolute stereochemistry of all the amino acids in axinastatin 4.

Axinastatin 4 (3) proved to be quite active against a series of human cancer cell lines with GI_{50} values of 0.028 to 0.086 μg/ml against ovarian (OVCAR-3), CNS (SF-295), renal (A498), lung (NCI-H460), colon (KM 20L2) and melanoma (SK-MEL-5) where GI refers to growth inhibition.[18] The new *cyclo*-heptapeptide structure corresponding to axinastatin 4 represents a promising condidate for further antineoplastic evaluations and structure/activity studies. The presently rapidly evolving series of novel cell growth inhibitory cyclic peptides[6,7,18,23-25] provides an important insight into sponge biochemistry and design of future anticancer cyclic peptides.

ACKNOWLEDGMENTS

We received the very necessary financial assistance for this investigation from Outstanding Investigator Grant CA 44344-01A1-02-03 awarded by the Division of Cancer Treatment, National Cancer Institute, DHHS; the Fannie E. Rippel Foundation; the Arizona Disease Control Research Commission; the Robert B. Dalton Endowment Fund; Herbert and Diane Cummings (The Nathan Cummings Foundation, Inc.); Virginia Piper and Eleanor W. Libby. For other necessary assistance we thank the Republic of the Comoros (Damir Ben Ali, Mohammed Chaher, Karl Danga and Bill Carlson), Drs. Cherry L. Herald, Jean-Charles Chapuis, Jean M. Schmidt, and Mr. Larry Tackett, Ms. Denise Nielsen-Tackett, Mr. Lee Williams, Mrs. Kim M. Weiss, the U. S. National Science Foundation (Grants CHE-8409644 and BBS-88-04992) and the NSF Regional Instrumentation Facility in Nebraska (Grant CHE-8620177).

Table 1. Axinastatin 4 (3) ^{13}C, ^{1}H and HMBC nmr assignments (recorded in CD_3CN).

	^{13}C(100 MHz)	^{1}H(400 MHz)	HMBC(500 MHz)
Trp 1	173.44		H-2,H-3,H-2a,H-20,H-21
2	53.84	4.65 m	H-2a,H-3
2a	26.68	3.26 dd(9.4,14);2.82 dd(4.4,14)	H-2
2b	112.26		H-2,H-2a,H-2j,H-2f
2c	128.64		H-2a,H-2j,H-2e,H-2g,H-2d
2d	119.59	7.73 d(7.5)	H-2e
2e	119.76	7.06 brt(7.2)	H-2f,H-2d
2f	122.35	7.12 brt(7.3)	H-2e
2g	112.22	7.37 d(7.7)	H-2d,H-2e
2h	137.25		H-2d,H-2j,H-2f,H-2e
2i(NH)		9.10 brs	
2j	124.28	6.96 d(2.3)	H-2a
3(NH)		7.35 d(8.7)	
Leu 4	172.52		H-3,H-5,H-5a
5	52.61	4.31 ddd(4.5,9.6,11)	H-5a,H-6
5a	41.60	1.57 m; 1.42 dd(8.4,11)	H-5,H-5c,H-5d
5b	25.75	1.54 m	H-5,H-5c,H-5d
5c	21.55	0.83 d(6.0)	H-5d,H-5a
5d	23.24	0.90 d(6.0)	H-5c,H-5a
6(NH)		6.70 d(9.6)	
Pro 7	172.04		H-6,H-8a,H-8
8	63.39	4.16 dd(7.9,9.7)	H-8a
8a	30.40	2.32 m; 1.93 m	H-8
8b	26.34	1.98 m; 1.85 m	H-8c
8c	48.87	3.81 brt(8.4);3.64 ddd(4.6,10.2,10.2)	H-8a
Thr10	171.71		H-11,H-12
11	56.69	4.79 dd(3,8.0)	H-11b
11a	69.21	4.56 brm	H-11,H-11b
11b	20.31	1.23 d(6.2)	
12(NH)		7.65 d(8.1)	
11a(OH)		3.60 brs	
Leu13	173.65		H-14,H-12,H-11,H-14a
14	56.40	4.17 m	H-14a
14a	41.60	2.11 m;1.72 m	H-14,H-14c,H-14d
14b	26.09	1.73 m	H-14,H-14a,H-14c,H-14d
14c	21.75	0.91 d(6.2)	H-14a,H-14d
14d	23.29	0.97 d(6.2)	H-14a,H-14c
15(NH)		8.33 d(7.9)	
Pro16	171.71		H-17,H-15,H-17a
17	61.91	4.39 brd(7.3)	H-17a,H-17b
17a	31.75	2.41 m; 1.90 m	H-17,H-17c
17b	22.60	1.89 m; 1.60 m	H-17,H-17c,H-17a
17c	47.03	3.50 ddd(7.5,11,11);3.35 brt(8.6)	H-17,H-17a,H-17b
Val19	171.26		H-20,H-17,H-20a,H-17c,H-21
20	59.67	4.12 dd(4.3,6.5)	H-21,H-20a,H-20b,H-20c
20a	30.40	1.90 m	H-20,H-21,H-20b,H-20c
20b	18.48	0.92 d(6.7)	H-20,H-20a,H-20c
20c	19.62	0.92 d(6.7)	H-20,H-20a,H-20b
21(NH)		7.05 d(6.5)	

Table 2. Axinastatin 4 (3) NOE difference spectroscopy (recorded in CD_3CN at 400 MHz).

Signals Irradiated	Signals enhanced
H-2(δ 4.65)	H-2d(δ 7.73); H-21(δ 7.05)
H-2a'(δ 2.82)	H-2d
H-21(δ 9.10)	H-2g(δ 7.37); H-2j(δ 6.96)
H-3(δ 7.35)	H-6(δ 6.70)
H-5(δ 4.31)	H-6
H-6(δ 6.70)	H-3; H-5
H-11(δ 4.79)	H-11a(δ 4.56); H-8c(δ 3.81)
H-11b(δ 1.23)	H-11;H-11a
H-12(δ 7.65)	H-15
H-15(δ 8.33)	H-12
H-17(δ 4.39)	H-20
H-20(δ 4.12)	H-17
H-21(δ 7.05)	H-2

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Received, 11th January, 1993