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Alkaloids from the Antarctic Sponge *Kirkpatrickia varialosa*. Part 1: Variolin B, A New Antitumour and Antiviral Compound

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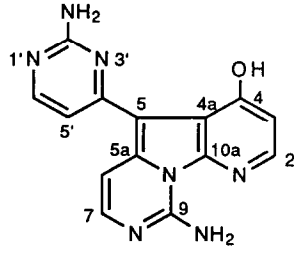
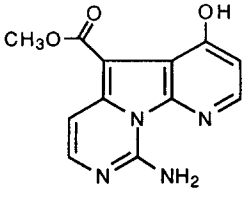
Abstract: Variolin B (**1**), a new type of pyridopyrrolopyrimidine alkaloid with antitumour and antiviral properties, has been isolated from the Antarctic sponge *Kirkpatrickia varialosa*, and its structure determined by X-ray crystallography. A degradation product, variolin D (**2**), has been identified from its spectroscopic data.

The ecology of the benthic community in McMurdo Sound, Antarctica, has been extensively studied by Dayton.[1] In a follow-up study, some of the sponges in this community were found to be toxic to fish, but no toxins were identified.[2] Apart from sterols, the only biologically active compounds which have been reported from Antarctic sponges are from the bright yellow sponge *Dendrilla membranosa* (family Aplysillidae, order Dendroceratida).[3,4] In an independent study on the ecology of the invertebrate community in McMurdo Sound, we have assayed invertebrate extracts for antiviral, antileukemic and antimicrobial activity. All collections of the sponge *Kirkpatrickia varialosa* Kirkpatrick (family Myxillidae, order Poecilosclerida) gave extracts active against P388 leukemia cells. One of these extracts was also antiviral.

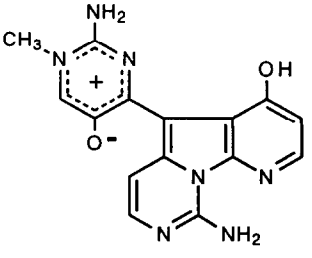
A routine P388 bioassay guided the isolation of several bioactive components which were named variolins. Several features of the ^{1}H and ^{13}C NMR spectra of these compounds were reminiscent of polycyclic aromatic alkaloids. HMQC, HMBC and NOE NMR experiments were performed, but these did not allow unambiguous solutions of the structures of these compounds.



variolin B (**1**)



variolin D (**2**)



variolin A (**3**)

The structure of variolin B (**1**) was established by an X-ray diffraction study of a crystal obtained from a TFA/H_2O solution. The structure of variolin D (**2**) was deduced by comparison of its spectral data with those of **1** and by chemical transformation. The elucidation of the structure of variolin A (**3**) is described in the following paper.[5]

Variolin B (**1**) crystallized as yellow prisms, mp 45°C (dec.) from a TFA/H_2O solution. HREIMS established the molecular formula as $C_{14}H_{11}N_7O$. The UV spectrum displayed absorbances in the 221-422 nm region which changed significantly upon addition of HCl, suggesting protonation of a heteroaromatic system. The IR spectrum (KBr disc) exhibited very broad absorption between 2500 and 3600 cm^{-1} (amine and hydroxyl functionalities).

As the initial approaches to the solution of the variolin structure using NMR techniques were unsuccessful, an x-ray crystallographic approach was used. Very small crystals, obtained from TFA/H_2O solutions, were too small to be used on a diffractometer with a sealed-tube x-ray source, but were suitable for a rotating-anode source. Data were collected at 120 K, using a Cu anode operated at 15 kW, yielding 6304 independent reflections; 4719 of these had Fo > 4.σ(Fo). Variolin B (**1**) crystallizes in the triclinic space group $P\bar{1}$ with cell parameters a = 7.914(1), b = 7.914(1), c = 29.839(3) Å, α = 87.58(1)°, β = 79.62(1)°, γ = 89.65(1)°. The unit cell contains 4 variolin moieties, 12 trifluoroacetates, and six waters. The structure was solved with direct methods (SHELXS-90[6]) and refined with SHELXL-93[7], using all reflections. Some disorder in trifluoroacetates and waters was accounted for in the refinement. The standard R index is 0.0529 for Fo > 4.σ(Fo) and 0.0661 for all data; wR2 = 0.1737, GooF = 1.092 for all data.[8] The structure of variolin B (**1**) is thereby firmly established, and the NMR data could be readily interpreted in terms of this structure. The trifluoroacetate molecules are involved in hydrogen bonds with water at 2.78 Å and make closest O-N approaches to N1' and N2' in the range 2.65 - 2.90 Å. The molecules contain intramolecular hydrogen bonds, firstly between OH4 on the pyridopyrrolopyrimidine ring system and N3' of the 2-aminopyrimidine ring at 2.687 Å and secondly between HN9 and N1, both of the tricyclic moiety, at 2.063 Å. As a consequence of this hydrogen bond the 2-aminopyrimidine ring is 23.8° out of the pyridopyrrolopyrimidine mean plane.

With the structure of variolin B (**1**) in hand, the extensive NMR data collected were now amenable to analysis. Most NMR spectral data were obtained in a mixture of deuterated DMSO and TFA (salt) or DMSO alone (free base) despite the very limited solubility of the free base form; these data are summarized in Table 1.

The proton NMR spectrum (Table 1) displayed three aromatic AB pairs and five exchangeable protons. The two broad signals at δ 8.6 and 9.9 ppm were assigned to the protons of a primary amine group on C9 based on the observation that selective irradiation of either of these signals resulted in the disappearance of the other, and from the coalescence of these two signals at increased temperature. The difference in the chemical shift was interpreted as a result of restricted rotation about C9-N due to hydrogen bonding between HN9 and N1. This hydrogen bonding was also observed in the X-ray data. The broad signal at δ 16.1 ppm was assigned to the hydroxyl on C4. This very deshielded signal is probably due to the hydrogen bonding with N3', which favoured the pyridol form, rather than the more normal pyridone form. An intense NOE between H6 and H5' indicated that the pyrimidine ring should be close to the plane of the tricyclic part of the compound. A 24° angle between these two parts has been found from the crystal diffraction analysis.

Both the ^{13}C data, which are comparable to those found in the literature for a pyrrolopyridine and pyrimidine rings,[9,10] and ^{1}H-^{13}C long range correlations, obtained from HMBC experiments (initially optimised

for J = 8.3 and 4 Hz), are in agreement with the proposed structure. A definitive structure could not be proposed from these data. Subsequent to the crystal structure determination, the HMBC experiment was repeated for J = 2 Hz. This is a very low sensitivity experiment because of the long delay time required for optimising on J = 2 Hz, during which significant loss of observable magnetisation occurs through relaxation. This experiment only became practicable after a new nmr instrument with greater sensitivity was available. The most significant of the additional correlations observed were $^{3}J_{C\text{-}H}$ H6-C5 and $^{4}J_{C\text{-}H}$ H3-C5.

Table 1. NMR Data for Variolin B (1)

position	C[a]	mult	J (Hz)	C[b]	mult	J (Hz)	H[c]	J (Hz)	H[d]	J (Hz)	HMBC correlations
2	143.2	dd	180, 2	144.8	dd	183, 2	8.26	5.6	8.38	5.5	C-3, C-4, C-10a, C-4a[e]
3	107.6	dd	162, 8	108.9	d	170	6.90	5.5	7.26	5.6	C-2, C-4, C-4a, C-5[e]
4	159.9	br d	8	159.3	dd	8, 2					
4a	111.2	br d	5	110.3	d	6					
5	99.6	br s	-	103.9	br s	-					
5a	137.1	t	7	137.4	t	7					
6	100.4	dd	171, 8	102.2	dd	180, 5	7.31	6.9	7.85	7.5	C-5a, C-7, C-5[e]
7	144.6	br d	179	135.0	br d	189	7.73	6.6	7.79	7.5	C-5a, C-6, C-9
9	150.3	d	13	148.9	d	9					
10a	145.0	d	14	146.9	d	15					
2'	158.4	br t	4	155.2	d	7					
4'	161.5	d	12	164.6	br d	5					
5'	106.1	dd	169, 7	109	d	170	7.23	5.9	7.65	6.6	C-5, C-4', C-6'
6'	160.1	br d	178	146.6	br d	187	8.37	5.4	8.44	6.6	C-2', C-4', C-5', C-5[e]
4OH							16.1				
9NH_2							8.6	br m	10.0	br m	
9NH_2							9.9	br m	10.7	br m	
2'NH_2							7.07	br m	8.5	br m	

[a]Recorded in $(CD_3)_2SO$ as free base at 75 MHz. [b]Recorded in $(CD_3)_2SO$ as TFA salt at 75 MHz. [c]Recorded in $(CD_3)_2SO$ as free base at 300 MHz. [d]Recorded in $(CD_3)_2SO$ as TFA salt at 300 MHz. [e]J = 2 Hz.

It is interesting to note that there is a difference in chemical shift between the carbons of the two guanidine groupings (δ 150.3 and 158.4 ppm). The unusually shielded value of C9 is possibly due to the incomplete aromaticity of the pyrrolopyrimido ring system. The free base and salt forms of variolin B (**1**) exhibited slight differences in both chemical shifts and coupling constants. Protonation effects for ^{13}C chemical shifts and one-bond ^{13}C-^{1}H coupling constants in nitrogen heterocycles are well-documented.[11,12] The increase in the experimentally observed values of $^{1}J_{C\text{-}H}$ is a consequence of the inductive effect associated with a positively charged nitrogen, compared to substituted pyrimidines,[13] or pyridinium ions in relation to pyridine.[14] The largest effect was observed, as expected, on the carbon adjacent to the protonation site. We have also demonstrated experimentally that the more protonated the compound, the larger the shift of the carbon resonance. This effect was observed again for C6' and C7 adjacent to the protonation site, -13.5 and -9.6 ppm respectively from the base to the salt (after addition by several steps of 50 equivalents of TFA in d_6-DMSO).

With the assignments of the structure and nmr data for variolin B (**1**) now secured, the structure of variolin D (**2**) could be determined. In contrast to variolin B (**1**), variolin D (**2**) is colourless and exhibits a strong fluorescence in the visible spectrum. HREIMS established the molecular formula as $C_{12}H_{10}N_4O_3$. The same pyridopyrrolopyrimidine substructure was deduced from the ^{13}C and HMBC spectra (Table 2) with a signal at δ

167.93 ppm assigned to a carbonyl. The ^{1}H NMR spectrum in DMSO (Table 2) displayed two AB systems, one O-CH_3 singlet at δ 4.09 ppm and signals for three exchangeable protons between δ 12.5 and 8.5 ppm. As observed for variolin B, the broad singlets at δ 8.9 and 9.6 ppm disappeared on heating and were again assigned to a primary amine at C9. Based on the NMR and mass spectral evidence the structure was deduced as **2**. An NOE correlation between H6 and the OCH_3 of the methyl ester suggested that there was hydrogen bonding between the carbonyl and the 4-hydroxyl.

As variolin D (**2**) could also be obtained in 20% yield from variolin A (**3**) by mild oxidation (MnO_2 in MeOH), this compound is possibly an artefact of the extraction process by aerial oxidation of the major pigments variolins A and B.

Table 2. NMR data for Variolin D (2)

position	C[a]	mult	*J* (Hz)	H[b]		*J* (Hz)	H[c]	mult	*J* (Hz)	HMBC correlations
2	146.9	dd	184, 3	8.36	d	6	8.35	d	5.4	C3, C4, C4a, C10a
3	110.4	dd	166, 8	7.06	d	5.7	7.04	d	5.8	C4, C2, C4a
4	159.4	br s								
4a	111.5	s								
5	101.4	s								
5a	134.9	br s								
6	103.4	d	183	7.44	d	7.5	7.33	d	6.3	C5a, C7
7	128.0	br d	193	7.51	d	8.1	7.89	d	6.4	C9, C5a, C6
9	148.4	br s								
10a	146.5	d	15							
1'	167.9	br s								
OCH_3	53.7	q	149	4.14	s		4.09	s		C1'
$9NH_2$							8.9	br s		
$9NH_2$							9.6	br s		
4OH							12.5	s		

[a]Recorded in $CDCl_3$/TFA at 75 MHz. [b]Recorded in $CDCl_3$/TFA at 300 MHz. [c]Recorded in $(CD_3)_2SO$/TFA at 300 MHz.

The variolins were each tested *in vitro* against P388, for antiviral activity against the DNA virus *Herpes simplex* Type I and the RNA virus *Polio* vaccine Type I, and against a range of bacteria and fungi. Variolin B (**1**) had an IC_{50} of 210 ng/mL against P388, was more effective against *Herpes simplex* than the *Polio* virus by a factor of 4x, but was inactive against the range of microorganisms. Variolin D (**2**) was inactive in all the assays. Clearly, substitution on the pyridopyrrolopyrimidine ring system with the 2-aminopyrimidine was necessary for the biological activity. It is possible that variolin B (**1**) belongs to the same class of DNA-intercalating compounds as ellipticine and its analogs.[15]

This new class of alkaloid is interesting from both the structural and biogenetic points of view as the variolins are the first examples of natural products with a pyridopyrrolopyrimidine moiety. This tricyclo ring system has been described only once before and then as synthetic derivatives only.[16] Variolins B (**1**) and A (**3**) are also the first examples with substitution on the tricycle by an aminopyrimidine substituent.

EXPERIMENTAL

General: Antiviral disk assays were against *Herpes simplex* Type I and *Polio* Type I viruses and antileukemia assays against the P388 murine leukemia cell line. Infrared spectra were recorded on a Perkin-Elmer series 1600 FT-IR spectrophotometer. Ultraviolet spectra on a Perkin-Elmer Lambda 2 UV/VIS spectrophotometer. NMR experiments were recorded on Varian XL300 and UNITY 300 spectrometers. Carbon chemical shifts are reported in ppm relative to $\delta = 77.0$ ppm for $CDCl_3$ or $\delta = 39.6$ ppm for $(CD_3)_2SO$, while proton chemical shifts are reported in ppm relative to $\delta = 7.26$ ppm for $CHCl_3$ or $\delta = 2.62$ ppm for $(CHD_2)_2SO$. Mass spectra were recorded on a Finnegan 4500 (DEI) or VG7070F. Melting points were determined on a Reichert hot stage microscope.

Collection, Extraction and Chromatography: The red sponge *Kirkpatrickia varialosa* was collected by hand using SCUBA in the shallow water of Cape Armitage in McMurdo Sound, Antarctica in 1988 and 1991 and frozen immediately until extraction. Voucher specimens (88ANT02-04 and 91ANT01-03) are held in the University of Canterbury collection. A typical isolation was as follows: the sponge (wet weight 1040 g) was extracted, by blending and filtering, with MeOH (1 x 1 L), 3:1 MeOH/CH_2Cl_2 (2 x 0.5 L) and 100:1 MeOH/HCl (aq) (2 x 0.5 L). The combined extracts (54.9 g) were subjected to reverse phase C18 flash chromatography.[17] One of the fractions (1.37 g) recovered from the column with 9:1 MeOH/H_2O was loaded on to a silica column. The fractions which displayed a yellow spot on tlc (silica 95:5 CH_2Cl_2/MeOH) were recovered from the column with 95:5 MeOH/CH_2Cl_2. A final purification on Sephadex LH20 (1:1 MeOH/CH_2Cl_2) gave variolin B (**1**) (140 mg, 0.0134% w/w sponge) which was most easily handled as its trifluoroacetate salt, a yellow-brown solid soluble in MeOH and DMSO. Addition of NH_4OH(aq) to a MeOH solution of the salt gave the free base, a yellow solid sparingly soluble in MeOH and DMSO: $R_f = 0.24$ (silica 95:5 CH_2Cl_2/MeOH); mp 45°C (decomposition); UV λ_{max} free base in MeOH, (ε) 422 (8400), 404 (9100), 324 (3900), 280 sh (3400), 244 sh (10200), 221 (14100), + HCl 1M: 394 (6300), 311 (4400), 275 sh (5300), 213 (15500) nm. IR(KBr) υ_{max} 3100, 1684, 1614, 1540, 1497, 1310, 1188, 1135 cm^{-1}. HREIMS (50ev): 293.102 (100%, M^+, $C_{14}H_{11}N_7O$ requires 293.103), 252.089 (55%, $C_{12}H_8N_6O$ requires 252.076). NMR data: see Table 1.

Variolin A (**3**), more polar than variolin B (**1**), was isolated from C18 fractions by chromatography on a Baker Bond® Diol column (1:9 MeOH/CH_2Cl_2), then on Sephadex LH20 (1:1 MeOH/CH_2Cl_2) and finally on a Baker Bond® NH_2 column (1:9 MeOH/CH_2Cl_2) to give 244 mg of the TFA salt after addition of TFA to prevent degradation (0.0234% w/w sponge). Silica tlc (8:2 CH_2Cl_2/MeOH) showed an orange spot which changed to pink on standing, presumably due to aerial oxidation. The physical properties of variolin A (**3**) are given in the following paper.[5]

Variolin D (**2**) was isolated from less polar fractions from the Diol column by means of silica and NH_2 Baker Bond® columns (CH_2Cl_2) to give 14.7 mg of a white solid which displayed a fluorescence in solution: $R_f = 0.42$ (silica 4:6 heptane/EtOAc); mp 248°C; UV λ_{max} free base in MeOH, (ε) 388 (9700), 368 (12000), 354 sh (8800), 246 (14800), 240 (14700), 226 (11700): + HCl 1M, 363 sh (4800), 345 (8500), 330 (8900), 253(15200), 220 (12600) nm. IR(KBr) υ_{max} 3228, 2963, 1711, 1631, 1586, 1499, 1236, 1194, 1110, 803 cm^{-1}. HREIMS (50ev): 258.0741 (100%, M^+, $C_{12}H_{10}N_4O_3$ requires 258.0753), 226.0488 (66%, M^+-

CH_3OH, $C_{11}H_6N_4O_3$ requires 226.0490), 198.0540 (58%, M^+-CH_3OH-CO, $C_{10}H_6N_4O_2$ requires 198.0541). NMR data: see Table 2.

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