Tetrahedron Vol. 44, No. 6, pp. 1727 to 1734, 1988
Printed in Great Britain.

0040-4020/88 $3.00+.00
Pergamon Press plc

CYTOTOXIC PIGMENTS FROM NEW ZEALAND SPONGES OF THE GENUS *LATRUNCULIA* : DISCORHABDINS A, B AND C

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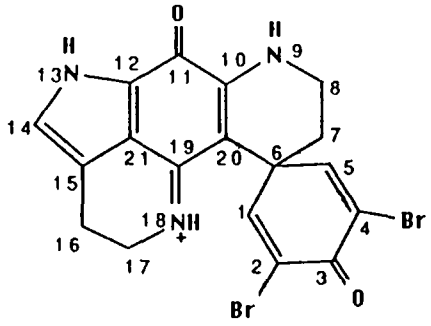
(*Received in UK* 19 *January* 1988)

Abstract—Discorhabdins A, B and C have been isolated as the major cytotoxic pigments of three different *Latrunculia* sponge species from New Zealand. The ^{1}H- and ^{13}C-NMR spectra of the previously reported discorhabdin C 1 have been assigned. The structures of discorhabdins A 2 and B 3 have been established by spectral comparisons (especially NMR) with 1. The discorhabdins are strongly cytotoxic (P388 ED_{50}'s 0.03-0.1 μg/ml) and antimicrobial.

Introduction In a search for antiviral and antitumour compounds from New Zealand's marine invertebrates, some of the most cytotoxic extracts found have been from heavily pigmented green and brown sponges of the genus *Latrunculia* du Bocage (family Latrunculiidae, order Hadromerida). There have already been several reports of metabolites from *Latrunculia* species. *L. brevis* Ridley and Dendy, collected in New Zealand, was included in chemotaxonomic surveys of sponges for their sterol and fatty acid contents.[1,2] A Red Sea sponge, *L. magnifica* Keller, was the source of the latrunculins, toxic macrolides bearing a 2-thiazolidinone function.[3] Latrunculin A has since been reported from a Fijian sponge, *Spongia mycofijiensis* Bakus.[4] *L. magnifica* also yielded the *cis*- and *trans*- N,N-dimethyl-4-hydroxyproline betaines.[3] Most recently, two Australian collections, one an undescribed *Latrunculia* species and the other *L. brevis*, have been reported to contain norditerpene and norsesterterpene peroxides with antimicrobial activity.[5,6]

Initial work on one New Zealand *Latrunculia* extract showed that the cytotoxicity was due to a polar pigment. HPLC analyses of all the extracts of the three distinct *Latrunculia* species in our collection showed that each contained one of three major pigments. These were named discorhabdins A, B and C after the discorhabd microscleres that are characteristic of the genus.[7] Bioassay-directed analyses of bulk extracts led to the isolation of these compounds as the major cytotoxic components of the source sponges.

The structure of discorhabdin C 1, which contains the new pyrrolo[1,7]phenanthroline ring system, was established by a single crystal X-ray diffraction study.[8] We now report the structures of the other major pigments, discorhabdin A 2 and discorhabdin B 3,[9] based on spectral comparisons with discorhabdin C 1, whose ^{1}H- and ^{13}C-NMR spectra are assigned below. Discorhabdin A 2 has also been isolated from a sponge of the genus *Prianos* (family Hymeniacidonidae, order Halichondrida) collected off Okinawa, Japan.[10] Most recently (after this paper was submitted) the structure of prianosin A, from an Okinawan



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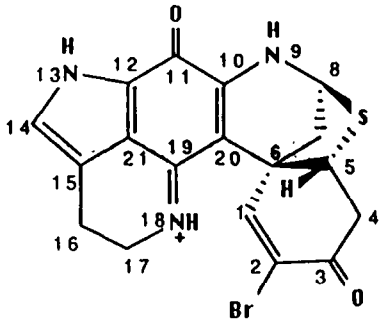

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marine sponge *Prianos melanos*, has been published.[11] This structure is the same as that proposed below for discorhabdin A **2** and comparison of the spectroscopic and biological data confirmed that these compounds were identical.[12]

NMR Assignments for Discorhabdin C 1 The hydrochloride salt of discorhabdin C **1** in CD_3OD gave a ^{1}H-NMR spectrum of stark simplicity (Table 1). A sharp two proton singlet at 7.73 ppm, due to H1 and H5, showed that the two enantiomeric conformations found in the solid state exchange rapidly in solution.[8] The indolic proton H14 was weakly coupled to the two proton triplet of H16, coupled in turn to H17. The protons H7 and H8 gave another pair of coupled triplets. The ^{1}H-NMR spectrum of discorhabdin C **1** in $(CD_3)_2SO$ retained the NH signals with detectable couplings to the vicinal protons, so that these could also be assigned (Table 1).

The ^{13}C-NMR spectrum was recorded in $(CD_3)_2SO$ because of the higher concentrations possible in this solvent (Table 2). All the protonated carbon signals except C14 were assigned from a two-dimensional heteronuclear correlation (HETCOR) experiment. A proton-coupled spectrum showed a $^1J_{CH}$ value of 192 Hz for C14, a normal value for such a position,[13] but outside the optimum range for the observation of correlations in the standard HETCOR experiment.[14] A long-range ($^2J_{CH}$ and $^3J_{CH}$) HETCOR experiment assisted with the assignment of the eleven non-protonated carbon signals.[15] The sharp singlet of H1 and H5 was correlated with signals assigned to C3 (lowest field), C2 and C4 (equivalent), C20, and C6 (highest field). The signal at 165.52 ppm weakly correlated with NH9 could be due to either C10 or C11. This signal was assigned to carbonyl C11 because of its chemical shift and because specific decoupling of NH9 gave a sharp singlet. The signal at 153.39 ppm was correlated with a signal at *ca* 3.7 ppm due to either H8 or H17 (the resolution in the proton dimension was too low to distinguish these). However, the X-ray structure of discorhabdin C **1** had shown the N9-C10 and N18-C19 bonds to have similar double bond character[8] so the signals at 153.39 and 151.98 ppm were assigned to C10 and C19 without further discrimination. These assignments for C10, C11 and C19 are supported by ^{13}C-NMR assignments for a similar aminoiminoquinone substructure in the chromophore of actinomycin D.[16] The signal at 123.35 ppm was correlated with H14 and irradiation at NH13 gave a sharp doublet, J = 8 Hz, so it was assigned to C12. The two remaining signals at 123.74 ppm (correlated with H16) and at 120.00 ppm had to be due to either C15 or C21. The ^{13}C-NMR assignments are given in Table 2, along with the $^1J_{CH}$ values.

Further analysis of the COSY and HETCOR results revealed the substructure NH-CH-CH_2-C-CH-CH_2-C. Assuming that the remainder of the molecular framework was also the same as in discorhabdin C **1**, this substructure was assigned as N9 to C3 in structure **2**. The ^{13}C-NMR spectrum of discorhabdin A showed that two double bonds had still to be located, a carbonyl group (186.67 ppm) and a carbon-carbon double bond (quarternary carbon signal at 125.78 ppm and methine carbon signal at 147.96 ppm). These were assigned to C3, C2 and C1 in structure **2**, after comparison with the corresponding shifts (182.17, 119.32 and 145.77 ppm) in another 2-bromo-*spiro* cyclohex-2-enone.[20] This left only the sulphur atom to be located, bridging C5 and C8. This proposed structure of discorhabdin A **2** contained three chiral centres. The configuration at C8 (arbitrarily shown as *S*) governs the configuration of C6 because of the thiolane substructure. Examination of a Dreiding model showed that either configuration at C5 was feasible, but only that shown for **2** put H5 and one H7 in a "W" conformation to give the observed $^4J_{HH}$ coupling of *ca* 1 Hz.[21]

The proposed structural features of discorhabdin A **2** were supported by all the spectroscopic data. The NMR results, which included chemical shifts (δ_C and δ_H), coupling constants (J_{CH} and J_{HH}) and NOE enhancements, reflected a variety of molecular properties and had to be considered separately. The usual method for judging δ_C and δ_H values is by comparisons with model compounds, but the combination of unusual structural features proposed in **2** is unique. However, the δ_C values of C4 to C8 all fell within expectation ranges based on α-substituents.[22] As $^1J_{CH}$ values are largely governed by the hybridization of the CH bond,[13] model compounds for these values did not need to be so close in overall structure. The observed values (Table 2) agreed well with published data.[23] The $^2J_{HH}$ value of 12.5 Hz for the protons at C7 was the same as in methane and cyclohexane.[21] The $^2J_{HH}$ value of 17.0 Hz for the C4 protons showed the influence of the adjacent carbonyl group, with a torsion angle of about 60° between H7α and the π-orbital.[21] $^3J_{HH}$ values were calculated using a modified Karplus equation (which takes into account substituent electronegativities)[24] and torsion angles measured on a Dreiding model. These calculated values were in acceptable agreement with those observed (coupling constants in Hz, observed values in parentheses): H4α-H5, 12.2 (12.0); H4β-H5, 3.7 (6.5); H7α-H8, 1.2 (1.0); H7β-H8, 5.2 (4.0). The most graphic evidence of the three-dimensional structure of discorhabdin A **2** was provided by difference NOE spectra. The observed enhancements, shown in Figure 1, were in complete agreement with the proposed structure **2**.

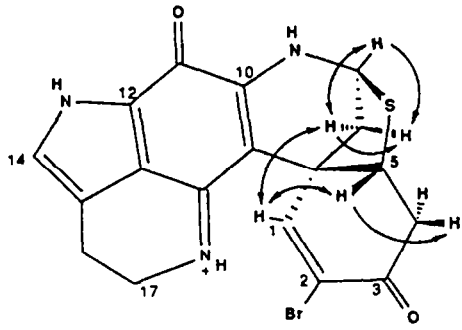


Figure 1. Enhancements observed in difference NOE spectra of discorhabdin A **2**.

Discorhabdin B **3** The third *Latrunculia* species studied was a green-brown sponge, as yet undescribed. Discorhabdin B **3**, the major pigment of the sponge and the major cytotoxin in the extract, was characterised as its hydrochloride, an optically-active green solid. High-resolution mass measurements corresponded to the molecular formula $C_{18}H_{12}BrN_3O_2S$, just two hydrogens less than in discorhabdin A **2**.

Discorhabdin A 2 This cytotoxic compound was the major pigment of a dark-green sponge, *L. brevis* Ridley and Dendy, a *Latrunculia* species different from that which yielded discorhabdin C 1. Discorhabdin A 2 was isolated by bioassay-directed analysis of the sponge extract, using methods similar to those described for 1.[17] Another new compound, 1,3,7-trimethylguanine, was also obtained from this sponge, but it showed no biological activity.[18] The major sterol was identified as 24-methylene-cholesterol by its ^{13}C-NMR spectrum.[19] No sesterterpene peroxides were detected, despite carrying out an extraction by the method used to isolate these compounds from an Australian collection of *L. brevis*.[6]

Discorhabdin A 2 was characterised as its hydrochloride, an optically-active dark-green solid (1 was not optically-active). Various mass spectroscopic methods showed the protonated molecular ion as a 1:1 doublet at 416/418 daltons, so only one bromine atom was present in the molecule. Ions at 383/385 daltons suggested the loss of a sulphur atom from the parent ion. Bromine and sulphur were confirmed by X-ray fluorescence spectroscopy and by high-resolution mass measurements, which gave the molecular formula $C_{18}H_{14}BrN_3O_2S$ (1 was $C_{18}H_{13}Br_2N_3O_2$).

The UV spectrum of discorhabdin A 2 was very similar to that of discorhabdin C 1, so the same major chromophore i.e. NH9 to C21 in 1, was probably present in both molecules. This was confirmed by the ^{1}H- and ^{13}C-NMR spectra of 2. Homonuclear correlation (COSY), HETCOR and specific decoupling experiments showed the substructure C-NH-CH=C-CH_2-CH_2-NH-C with shifts very similar to the protons from NH13 to NH18 in discorhabdin C 1 (Table 1). The δ_C values assigned above to C10 to C21 of 1 were closely matched in the spectrum of 2 (Table 2). Only the signals assigned to C10 and C20 did not have corresponding signals within one ppm.

Table 1
^{1}H-NMR data for 1, 2 and 3[a]

H	1	2	3
1	7.73,s	7.58,s	7.87,s
4	—	2.88,dd	6.24,s
		6.5,17	
		3.07,dd	
		12 ,17	
5	7.73,s	4.53,dd	—
		6.5,12	
7	2.12,t	2.60,dt	2.54,dd *6*
		1 ,1 ,12.5	*3.5,11.5*
		3.00,dd	2.81,d
		4 ,12.5	*11.5*
8	3.73,t	5.38,dd	5.72,d
	6	*1 ,4*	*3.5*
9	10.3,s	10.5,s	10.8,s
13	13.4,s	13.4,s	13.5,s
14	7.22,s	7.19,t	7.21,s
		0.5,0.5	
16	2.90,t	2.97,m	2.92,dd
	7		*6.5,9*
17	3.79,t	3.80,dt	3.83,dt
	7	*10,10,14.5*	*9 ,9 ,14*
		3.94,dt	3.94,dt
		6,6,14.5	*6.5,6.5,14.5*
18	8.3,s	9.8,s	8.7,s

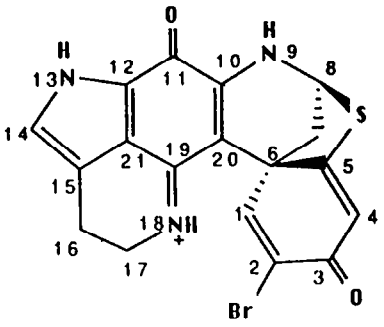
[a]Measured at 300 MHz in CD_3OD, except for NH9, NH13 and NH18 measured in $(CD_3)_2SO$; δ_H values in ppm, followed by multiplicity; J_{HH} *values in Hz.*

Table 2
^{13}C-NMR data for 1, 2 and 3[a]

C	1	2	3
1	151.41	147.96	146.00
	166	*169*	*173*
2	122.78	125.78	128.21
3	171.52	186.67	174.14
4	122.78	44.43	119.32
		127,135	*168*
5	151.41	53.76	169.99
	166	*149*	
6	44.87	49.50	51.44
7	33.83	40.4	42.49
	138	*?*	*?*
8	38.46	58.72	61.30
	144	*168*	*167*
10	151.98[b]	150.16[b]	150.9[b]
11	165.52	165.83	165.16
12	123.35	123.25[c]	123.51[c]
14	127.86	127.28	127.42
	192	*191*	*190*
15	120.00[c]	119.76[c]	120.53[c]
16	18.22	18.04	18.06
	133	*132*	*?*
17	43.87	43.84	45.15
	142	*143*	*?*
19	153.39[b]	153.58[b]	154.22[b]
20	91.89	103.74	97.17
21	123.74[c]	123.13[c]	123.22[c]

[a]Measured at 75 MHz in $(CD_3)_2SO$; δ_C values in ppm; $^1J_{CH}$ *values in Hz.*
[b,c]Interchangeable within columns.

The UV, ^{1}H- (Table 1) and ^{13}C-NMR (Table 2) spectra showed that the same major chromophore was present in discorhabdin B **3** as in both discorhabdins C **1** and A **2**. The NMR data also established the CH_27-CH8 substructure, as in discorhabdin A **2**. However, the remainder of the molecule, C1 to C6, had one more tri-substituted carbon-carbon double bond than in **2**. Thus discorhabdin B **3** had a *spiro* cyclohexadienone system as in discorhabdin C **1** but with only one α-bromine. The signal at 169.99 ppm was ascribed to C5, deshielded relative to C5 in discorhabdin C **1** by the sulphur substituent. The CH4 signals (δ_H 6.24 ppm and δ_C 119.32 ppm) were appropriate for the α-position of an α,β-unsaturated ketone.



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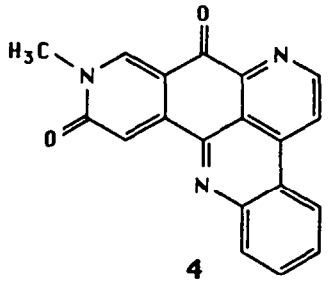
The proposed structure of discorhabdin B **3** was supported by the following NOE enhancements: H7β{H7α} (38%), H8{H7α} (5%), H1{H7α} (9%), H7α{H7β} (31%), H8{H7β} (11%), H1{H7β} (1%) and H4{H7β} (1%). The electron-impact mass-spectrum of discorhabdin B **3** differed considerably from that of discorhabdin A **2**. The first major fragment was a doublet at 228/230 daltons, corresponding to loss of the chromophore by cleavage of the C8-N9 and C6-C20 bonds. The absolute configuration of discorhabdin B **3** has not been determined.

Biological Activities Discorhabdins C **1**, A **2** and B **3** were isolated because of their strong cytotoxicities in *in vitro* antiviral assays.[25] Further testing showed that these compounds were highly active in *in vitro* P388 assays, with respective ED_{50}'s of 0.03, 0.05 and 0.1 μg/ml. However, *in vivo* testing in the P388 leukemia system in mice showed no increase in lifespan with either discorhabdin C **1** or discorhabdin A **2**. These two compounds were toxic to mice at about 2 mg per kg of body weight. Discorhabdin B **3** did show some antitumour effect, with a T/C of 117% at a dose of 0.25 mg/kg, but this did not reach the significance level of 120%.[25]

The discorhabdins also showed antimicrobial activity. In a disk assay, with 30 μg/disk, discorhabdins C **1** and A **2** were active against *Escherichia coli*, *Bacillus subtilis* and *Candida albicans*, but not against *Pseudomonas aeruginosa*. Discorhabdin B **3** was active against *E. coli* and *B. subtilis*, but not against *P. aeruginosa* or *C. albicans*.

Discussion The discorhabdins, with their pentacyclic carbon-nitrogen framework, are a new type of nitrogenous pigment.[26] Other fused pentacyclic aromatic alkaloids have been reported from three phyla of marine animals: a sponge,[27] an ascidian[28] and a sea anemone.[29] Strangely, all three of these compounds have $C_{18}N_3$ frameworks, as do the discorhabdins. The structure of amphimedine **4**, from a sponge of the genus *Amphimedon* (family Haliclonidae, order Haplosclerida), was based on extensive long-range HETCOR and carbon-carbon correlation experiments.[27] Amphimedine **4** was active against P388 cells *in vitro* (ED_{50} 2.8 μg/ml) but was inactive *in vivo*.[25] 2-Bromoleptoclinidone **5** (*in vitro* P388 ED_{50} 0.4 μg/ml) was isolated from an ascidian and its structure determined by long-range HETCOR experiments.[28] Two alternative structures have been proposed for calliactine (**6** or **7**), a pigment from a sea anemone.[29] It may be that the discorhabdins and these other compounds are produced by related microorganisms, which are either part of the diets of these filter feeders or are present as symbionts.[30]



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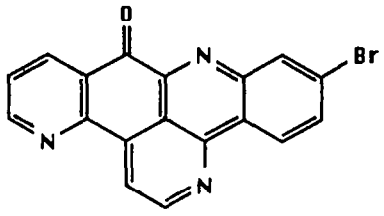

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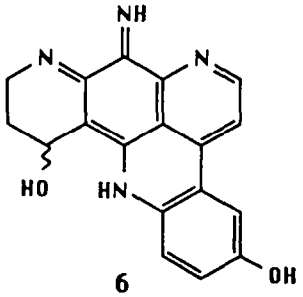

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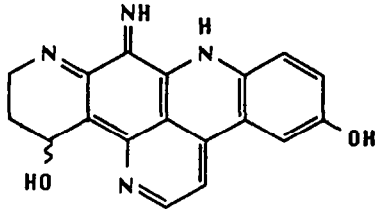

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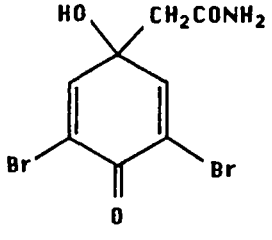
The 2,6-dibromocyclohexadienone portion of discorhabdin C **1** is echoed in compound **8**, a product of tyrosine metabolism in the sponge *Aplysina fistularis* (family Aplysinidae, order Verongida).[31] This compound **8** is cytotoxic and antimicrobial.[32]



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Consideration of the biological properties of the discorhabdins and their relatively high levels in the *Latrunculia* sponges (>1% of dry weight) suggests that they may have defensive roles. Sponges containing antimicrobial substances are rarely overgrown[33] and underwater observations of these *Latrunculia* species showed no evidence of predation or of epibionts.

EXPERIMENTAL

Instrumental Methods ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL300 spectrometer. Chemical shifts are given in ppm on the δ scale, referenced to the solvent peaks: CHD_2OD at 3.30 ppm in CD_3OD; $(CHD_2)_2SO$ at 2.60 ppm and $(CD_3)_2SO$ at 39.60 ppm in $(CD_3)_2SO$. Difference NOE experiments were performed using a low power cycling technique.[34] Ultraviolet spectra were recorded on a Varian DMS 100 UV/Visible spectrometer. $[\alpha]_D$ measurements were made on a Perkin Elmer 241 Polarimeter. Mass spectra were recorded on Finnegan 4500 (DCI/DEI) or VG7070F (high resolution) mass spectrometers. Infrared spectra were recorded on a Pye Unicam SP3-300 spectrometer as KBr discs. X-ray fluorescence measurements were done on a Philips PW 1400 X-ray spectrometer.

Discorhabdin A 2 Specimens of *L. brevis* Ridley and Dendy were dredged from depths of 110 to 145 m, off the Otago Peninsula, in October 1983. A voucher specimen, J047-1, has been kept. The sponges (168 g) were blended and extracted with CH_3OH and CH_2Cl_2 to give, after removal of solvents, a green extract (11.3 g). This was partitioned on a reverse phase column[17] to give a number of cytotoxic fractions containing largely discorhabdin A 2 (0.35 g, 0.2% of wet weight). RPLC (Alltech C8 column, 250 x 10 mm; 5 ml/min 65% CH_3OH, 35% {H_2O + 0.05% CF_3COOH}; 210 nm detection) gave pure discorhabdin A (2). This was characterised as its hydrochloride salt, a green solid, mp > 360°, $[\alpha]_D$ +400° (*c* 0.05, CH_3OH). HREIMS: M^+ 414.99751, calculated for $C_{18}H_{14}{}^{79}BrN_3O_2S$ 414.99908. DCI/NH_3: 416/418 (MH^+, 43%/35%), 384/386 (MH^+-S, 15%/14%), 288 (74%), 270 (56%), 234 (43%), 216 (100%), 204 (85%). UV (CH_3OH): 249 (ε 29500), 351 (ε 10500), 567 nm (ε 900). UV (CH_3OH/KOH): 335 (ε 14000), 473 nm (ε 1000). IR: 3700-2400, 1680, 1620, 1585, 1530, 1410, 1385 cm^{-1}. ^{1}H-NMR in Table 1, ^{13}C-NMR in Table 2.

Discorhabdin B 3 Specimens of an undescribed *Latrunculia* species were collected by SCUBA diving at a depth of 25 m, off the Kaikoura Peninsula in January 1985. A voucher specimen, 5K1-1, has been kept. The sponges (300 g) were blended and extracted with CH_3OH and toluene to give, after removal of solvents, a green extract (21.4 g). This was partitioned on a reverse phase column [17] to give a number of fractions containing largely discorhabdin B 3 (2.5g, 0.8% of wet weight). Two further stages of preparative RPLC (Merck LOBAR RP-8 column, 250 x 25 mm; 4 ml/min 40% CH_3OH, 60% {H_2O + 0.05% CF_3COOH}) followed by semipreparative RPLC (Alltech C8 column, 250 x 10 mm; 5 ml/min 75% CH_3OH, 25% {H_2O + 0.05% CF_3COOH}; 210 nm detection) gave discorhabdin B 3. Discorhabdin C 1 was also isolated (about 3:1 B:C) and identified by its ^{1}H-NMR spectrum.[8] Discorhabdin B 3 was characterised as its hydrochloride salt, a green solid, mp > 360°, $[\alpha]_D$ +400° (*c* 0.17, CH_3OH). HREIMS: M^+ 412.98529, calculated for $C_{18}H_{12}{}^{79}BrN_3O_2S$ 412.98343. DEI: 228/230 (90%/93%), 184 (35%), 150 (100%), 121 (42%), 95 (38%). DCI/NH_3: 414/416 (MH^+, 21%/26%), 386 (21%), 279 (41%), 270 (100%), 256 (45%), 245 (38%), 234 (48%), 218 (33%), 204 (48%). UV (CH_3OH): 248 (ε 30600), 309 (ε 10800), 357 (ε 10600), 567 nm (ε 1100). UV (CH_3OH/NaOH): 231 (ε 25500), 306 nm (ε 14700). IR: 3700-2400, 1740, 1650, 1620, 1520, 1410 cm^{-1}. ^{1}H-NMR in Table 1, ^{13}C-NMR in Table 2.

ACKNOWLEDGEMENTS

We thank the crew of the R. V. James Cook, Mr. J. D. McCombs, Mr. J. van Berkel (Edward Percival Field Station, Kaikoura), and Dr. G. D. Fenwick for assistance with collections; Dr. G. D. Fenwick, Dr. C. N. Battershill and Professor P. R. Bergquist (University of Auckland) for sponge identifications; Mrs. V. L. Calder, Mrs. G. Barns, Dr. M. Lui and Dr. S. Cross (H.B.O.I./S.P. Project) and Dr. B. C. Baguley (Cancer Research Laboratory, University of Auckland) for bioassays; Ms. M. M. Brennan for technical assistance; Dr. S. Bloor and Dr. D. Crump (D.S.I.R., Wellington) for the rotation measurements; Mr. A. Alloway for XRF work; Dr. P. Holland (Soil Research Institute, Ruakura) and Dr. L. K. Pannell (National Institute of Diabetes, Digestive and Kidney Diseases, N.I.H., Bethesda) for the mass spectrometric measurements; Dr. R. J. Capon (Australian National University) for reference samples of trunculins A and B; and the New Zealand University Grants Committee and the Canterbury Medical Research Foundation for equipment.

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