Rigidin E, a New Pyrrolopyrimidine Alkaloid from a Papua New Guinea Tunicate *Eudistoma* Species

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Abstract: A new pyrrolopyrimidine alkaloid, rigidin E (**1**) and the known metabolites rigidin (**2**) and 1-methylherbipoline (**3**) have been isolated from a Papua New Guinea tunicate *Eudistoma* sp. A combination of spectroscopic data were used to determine the structures of these metabolites.

Keywords: Tunicate; *Eudistoma* sp., rigidin E; cytotoxicity, HCT 116, A431.

Introduction

Tunicates belonging to the genus *Eudistoma* have been the sources of numerous novel and bioactive secondary metabolites. β-Carboline alkaloids have been the predominant structure class isolated from this particular genus to date [1-5], however macrolides [6], indoles [7,8] and aromatic polysulfides [9] have also been reported. In 1990 Kobayashi *et al.* reported the isolation of rigidin (**2**) from an Okinawan marine tunicate *Eudistoma* cf. *rigida*; this novel compound displayed calmodulin antagonistic activity and was the first pyrrolopyrimidine alkaloid to be isolated from a marine source

[10]. More recently three new rigidin congeners, rigidins B-D (**4-6**) have been isolated from the tunicate *Cystodytes* sp. [11]. In our continuing search for unique secondary metabolites from marine tunicates [12,13], we investigated a previously undescribed tunicate *Eudistoma* sp. collected from Papua New Guinea. These chemical studies resulted in the isolation of a new pyrrolopyrimidine derivative, which we have named rigidin E (**1**) along with the known metabolites rigidin (**2**) and 1-methylherbipoline (**3**). Herein we describe the isolation, structure elucidation and cytotoxicity of compounds **1-3**.
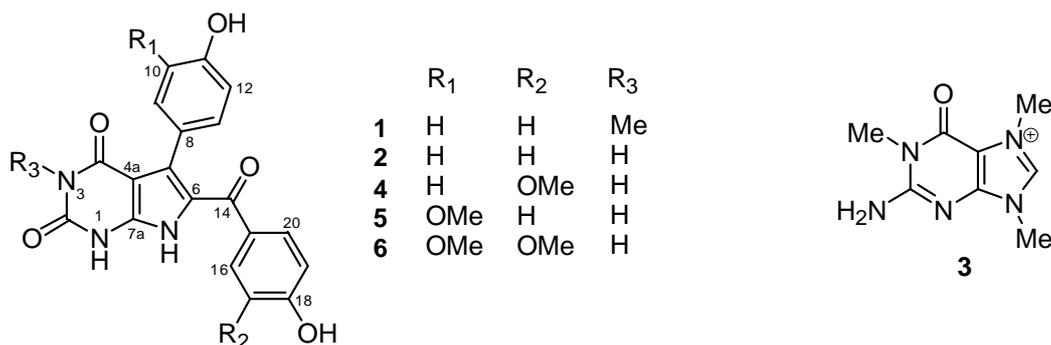
Results and Discussion

A freeze-dried sample of the undescribed *Eudistoma* sp. [14] (family Polycitoridae) was sequentially extracted with DCM, MeOH and H_2O. The MeOH extract was subjected to gel permeation chromatography using Sephadex LH-20 (MeOH) to yield 9 fractions. Fractions 4 and 5 were further purified by reversed-phase C_{18} HPLC using 0.1% aqueous TFA and increasing amounts of MeOH to yield 1-methylherbipoline (**3**, 38.7 mg, 0.198 % dry wt). Fractions 8 and 9 were individually chromatographed by C_{18} HPLC using a 0.1% aqueous TFA/MeOH gradient to afford the new metabolite rigidin E (**1**, 3.6 mg, 0.018 % dry wt) and rigidin (**2**, 2.0 mg, 0.010 % dry wt), respectively. The previously reported metabolites rigidin (**2**) and 1-methylherbipoline (**3**) were readily identified by comparison of their MS and NMR data with literature values [1,10,15].

Rigidin E (**1**) was isolated as a stable dark green film. A molecular formula of $C_{20}H_{14}N_3O_5$ was assigned to **1** by (-)-HRESMS analysis [*m/z* 376.0933 (M-H)⁻], which established that rigidin E was larger than rigidin (**2**) by a CH_2 unit. IR absorption bands for compound **1** at 3600-3000, 1690 and 1590 cm⁻¹ were attributed to OH/NH and carbonyl group(s) respectively. UV absorptions at 276 (ε 7000) and 362 (ε 4000) suggested the presence of conjugated phenol chromophore(s) [14]. The presence of phenol(s) in **1** was further supported by the UV spectrum that underwent a bathochromic shift on addition of base.

The ¹H-NMR spectrum of rigidin E (**1**) displayed three D_2O-exchangeable signals (δ 9.28, 1H, br s; 10.05, 1H, br s; 11.90, 2H, br s), four aromatic resonances corresponding to two A_2B_2 systems (δ 7.28, 2H, d, *J* = 8.5 Hz/δ 6.48, 2H, d, *J* = 8.5 Hz; δ 6.94, 2H, d, *J* = 7.5 Hz/δ 6.45, 2H, d, *J* = 7.5 Hz;)

and one *N*-methyl signal (δ 3.13, 3H, s). In the ^{13}C NMR spectrum all carbon resonances, except for the *N*-methyl carbon (26.8 ppm), were observed between 97 and 186 ppm. Four of the 15 unique low-field carbon resonances were attributed to aromatic methines on the basis of gHSQC analysis and hence the remaining downfield signals were all quaternary carbons. The A_2B_2 aromatic systems were both assigned to *p*-hydroxyphenyl moieties based on strong ROESY correlations between H-10/H-12 (δ 6.45) and HO-11 (δ 9.28), and H-17/H-19 (δ 6.48) and HO-18 (δ 10.05). Two conjugated phenol substructures were further elucidated based on HMBC correlations from H-9/H-13 (δ 6.94) and H-16/H-20 (δ 7.28) to C-5 (128.0 ppm) and C-14 (185.2 ppm), respectively. An *N*-methyl imide moiety was established based on two strong $^3J_{CH}$ correlations between Me-3 (δ 3.13) and the carbonyl carbons C-2 (150.6 ppm) and C-4 (159.1 ppm). Although the three remaining sp^2 carbons (97.6, 124.9 and 139.6 ppm) showed no HMBC correlations to either of the broad exchangeable protons at H-1 and H-7, a pyrrolopyrimidine system was clearly identified since compound **1** had essentially identical carbon chemical shifts compared to the known compound rigidin (**2**). Hence structure **1**, the 3-methyl analog of rigidin, was assigned to rigidin E.

All three compounds were tested for cytotoxicity against the p53 wildtype (p53$^{+/+}$) and p53 deficient (p53$^{-/-}$) HCT 116 human colon carcinoma cell lines, while **1** and **2** were also tested in the A431 human epidermoid carcinoma cell line. Only minimal growth inhibition at 100 μg/mL was identified for all metabolites, hence no further biological evaluations were pursued (see Table 1).

Table 1. Cytotoxicity Data for Compounds **1-3** [a].

Cell line	Rigidin E (**1**)	Rigidin (**2**)	1-Methylherbipoline (**3**)
HCT 116 p53$^{+/+}$	0.75 ± 0.05	1.00 ± 0.02	0.97 ± 0.05
HCT 116 p53$^{-/-}$	1.03 ± 0.09	0.96 ± 0.15	1.02 ± 0.11
A431	1.08 ± 0.04	0.97 ± 0.08	not tested

[a] Data is expressed as fractional survival ($\pm$ standard deviation) for the three human tumor cell lines after exposure to each compound for either 72 h (HCT) or 96 h (A431) at 100 μg/mL.

Interestingly, rigidins B-D (**4-6**) have recently been shown to exhibit moderate cytotoxocity against murine leukemia L1210 cells. Preliminary biological results showed that at 10 μg/mL, compounds **4-6** inhibited leukemia tumor cell growth by 40%, 40% and 20% respectively [11].

Conclusions

We have isolated and spectroscopically characterized a new pyrrolopyrimidine alkaloid, which we have named rigidin E (**1**). The known metabolites rigidin (**2**) and 1-methylherbipoline (**3**) were also isolated during our chemical investigations of a Papua New Guinea tunicate *Eudistoma* sp.

Experimental

General

NMR spectra were recorded on a Varian Mercury 400 MHz spectrometer (^{1}H: 399.880 MHz, ^{13}C: 100.559 MHz) at 25 °C. ^{1}H and ^{13}C chemical shifts are reported in parts per million relative to the reference solvent peaks at δ 2.49 and 39.51 ppm for DMSO-d_6. FT-IR and UV spectra were recorded on a Jasco 420 spectrophotometer and a Hewlett Packard 8452A diode array spectrophotometer respectively, and LRESMS experiments were performed on a Micromass Quattro II Triple Quadrupole mass spectrometer. HRESMS were recorded on a Bruker BioAPEX 47e mass spectrometer equipped with a Bradford CT 06405 electrospray ion source. Gel permeation chromatography was performed on Lipophilic Sephadex LH-20 (45 × 730 mm). An Agilent 1100 quaternary solvent module equipped with a 7725i Rheodyne injector and an Agilent PDA detector was used for HPLC separations. A semi-preparative Phenomenex Luna C_{18}(2) 5 μm 100 Å (10 × 250 mm) column was used for HPLC. All solvents for HPLC, UV, and MS were Fisher HPLC grade and water used was Barnstead E-pure 0.2 μm filtered.

Animal Material

A specimen of the undescribed *Eudistoma* sp. [14] was collected during May of 2001 by SCUBA diving (-10 m) at Milne Bay (S 10° 14.278', E 150° 54.782'), Papua New Guinea, and kept frozen prior to freeze-drying and extraction. Taxonomic voucher specimen MZUSP15712 has been deposited at the Museu de Zoologia, Universidade Federal de São Paulo, São Paulo, Brazil.

Extraction and Isolation

The freeze-dried *Eudistoma* sp. (19.6 g) was sequentially extracted with DCM (2 × 400 mL), MeOH (2 × 400 mL) and H_2O (2 × 400 mL). The MeOH extract (4.0 g) was subjected to gel

permeation chromatography using a Sephadex LH-20 column with 100% MeOH as the eluant at a flowrate of 3.5 mL/min to yield 9 fractions. Fractions 4 and 5 (209.7 mg) were purified by semi-preparative HPLC using a C_{18} column with a gradient from 100% aqueous TFA (0.1%) to 100% MeOH in 20 min at flowrate of 4.0 mL/min, which afforded 1-methylherbipoline (**3**, 38.7mg, 0.198 % dry wt). Fraction 8 (11.0 mg) was purified by C_{18} HPLC using a gradient from 100% aqueous TFA (0.1%) to 100% MeOH in 18 min at flowrate of 4.0 mL/min to yield rigidin E (**1**, 3.6 mg, 0.018 % dry wt). Fraction 9 (8.8 mg) was also purified by C_{18} HPLC using 100% aqueous TFA (0.1%) to 100% MeOH in 20 min at flowrate of 4.0 mL/min which afforded pure rigidin (**2**, 2.0 mg, 0.010 % dry wt).

Spectral Data

Rigidin E (**1**): stable dark green film; UV (MeOH) λ_{max} 276 (ε 7000), 362 (ε 4000); UV (MeOH/NaOH) λ_{max} 244 (sh, ε 9000), 284 (ε 7000), 340 (ε 5000), 404 (ε 5000); IR ν_{max} (NaCl) 3600-3000, 1690, 1590, 1440, 1380, 1260, 1200, 1160, 1030, 800, 730 cm^{-1}; ^{1}H-NMR (400 MHz, DMSO-d_6) δ 3.13 (3H, s, Me-3), 6.45 (2H, d, J = 7.5 Hz, H-10/H-12), 6.48 (2H, d, J = 8.5 Hz, H-17/H-19), 6.94 (2H, d, J = 7.5 Hz, H-9/H-13), 7.28 (2H, d, J = 8.5 Hz, H-16/H-20), 9.28 (1H, br s, HO-11), 10.05 (1H, br s, HO-18), 11.90 (2H, br s, H-1/H-7); ^{13}C NMR (100 MHz, DMSO-d_6) δ 26.8 (Me-3), 97.6 (C-4a), 113.8 (2C, C-10/C-12), 114.3 (2C, C-17/C-19), 122.7 (C-8), 124.9 (C-6), 128.0 (C-5), 128.6 (C-15), 131.5 (2C, C-16/C-20), 132.3 (2C, C-9/C-13), 139.6 (C-7a), 150.6 (C-2), 156.4 (C-11), 159.1 (C-4), 160.7 (C-18), 185.2 (C-14); (+)-LRESMS (rel. int.) *m/z* [MeCN] 194 (50), 378 (25), 400 (100); (+)-LRESMS (rel. int.) *m/z* [1:1 MeCN/D_2O] 383 (100); (-)-LRESMS (rel. int.) *m/z* [MeCN] 249 (10), 362 (20), 376 (100); (-)-LRESMS (rel. int.) *m/z* [1:1 MeCN/D_2O] 249 (15), 379 (100); (-)-HRESMS *m/z* 379.0933 ($C_{20}H_{14}N_3O_5$ requires (M-H)$^-$ 379.0939).

Rigidin (**2**): identified by comparison of spectroscopic data with literature values [10].

1-Methylherbipoline (**3**): identified by comparison of spectroscopic data with literature values [1,10,15].

Cells and Culture Conditions

All cell cultures were grown at 37 °C, 5% CO_2 and maximal humidity (by equilibration). All media reagents were purchased from Invitrogen Corporation (Carlsbad, CA). The HCT 116 strains were donated by Dr. Bert Vogelstein (Johns Hopkins University) and grown in McCoy's 5A media supplemented with 10% v/v heat inactivated fetal bovine serum (FBS), 1% v/v MEM (Mimimal Essential Media) sodium pyruvate, 1% v/v penicillin-streptomycin and 1% v/v L-glutamine. The A431 cell line was purchased from American Type Culture Collection (Manassas, VA) and grown in MEM supplemented with 10% v/v heat inactivated FBS, 1% v/v MEM sodium pyruvate, 1% v/v

penicillin-streptomycin and 1% v/v L-glutamine. Cells were counted using a Beckman Coulter cell counter (Fullerton, CA) [16].

Cell Proliferation Assay

The ability of drug to inhibit cellular growth was determined using the MTT assay [16]. For the HCT 116 cell lines, 8000 cells per well were plated in 96-well plates in 200 µL of the appropriate media (see above) and the cells were allowed to grow overnight. Serial dilutions of drug were then added to wells in quadruplicate. Control wells were treated with vehicle (DMSO). After 72 h of incubation at 37 °C and 5% CO_2, the media was removed and 100 µL fresh media was added along with 10 µL of 2.5 mg/mL aqueous 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT). The cells were then incubated for 4 h under the same conditions as above. The media was then removed and 100 µL DMSO added to each well. The amount of formazan resulting from MTT metabolism was measured by observing absorbance at 540 nm using a Labsystems Multiskan Plus plate reader. Using the absorbance in the vehicle treated lanes as 100% survival (1.00 fractional survival) the fractional survival of each treatment was observed. Because no growth inhibition was observed after most of the diluted treatments only the survival at 100 µg/mL is presented. The protocol using the A431 cells was essentially identical to the HCT 116 assays except that 16000 cell per well were initially plated and the cells were exposed to drug for 96 h. Both the HCT 116 and A431 cell lines responded typically when treated with laboratory standards. For example, the IC_{50} of makaluvamine C towards HCT 116 cells was 0.37 µg/mL

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Sample Availability: Samples are available from the authors.