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Euryjanicin A: a new cycloheptapeptide from the Caribbean marine sponge *Prosuberites laughlini*

Jan Vicente, Brunilda Vera, Abimael D. Rodríguez *, Idaliz Rodríguez-Escudero, Raphael G. Raptis

Department of Chemistry, University of Puerto Rico, PO Box 23346, UPR Station, San Juan, PR 00931-3346, United States

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ABSTRACT

A new proline-containing cycloheptapeptide, euryjanicin A (**1**), has been isolated from the marine sponge *Prosuberites laughlini* indigenous to Puerto Rico, and its structure established by an X-ray crystal structure determination. The absolute configuration of each amino acid residue was determined by Marfey's method.

Marine sponges, especially those found in tropical ocean areas, continue to represent the single most prolific source of structurally novel natural products of marine origin.[1] Sponge-derived secondary metabolites have also attracted considerable attention because of their relevant and diverse pharmacological actions.[2] During the last 25 years a large body of evidence has been accumulated to indicate that certain marine sponges (especially sponges belonging to the class Demospongiae) also contain amino acid derivatives, usually as trace components, with significant cancer cell growth inhibitory properties.[3] Examples of such biologically active peptides are the axinellins,[4] kapakahines,[5] microsclerodermins,[6] polytheonamides,[7] papuamides,[8] stylisins,[9] hymenamides,[10] wainunuamide,[11] and dominicin.[12] Additional noteworthy examples include the laxaphycins[13] and discodermins[14] (they inhibit tumor promotion), calyculin[15] (tumor promoting), and the antithrombin cyclotheonamides.[16]

As part of our ongoing campaign to find new natural products with potential anticancer activity from marine organisms found near Puerto Rico, a small library of sponge extracts from this location was recently screened for anti-cancer activity. Considerable activity was detected in the crude extract of *Prosuberites laughlini* (Díaz, Alvarez & Van Soest, 1987) (Order Hadromerida, Family Suberitidae).[17] The sponge was collected in April 2006 at a depth of about 15 m by scuba from *El Natural* reef, off the municipality of Aguadilla, in the northwestern coast of Puerto Rico (18°27′46.90″N, 67°10′06.11″W). A freeze-dried sample (623 g) was repeatedly extracted with MeOH (16 L), and the extracts were combined, concentrated in vacuo (83 g), and partitioned between H_2O (1.5 L) and EtOAc (3 × 1 L), followed by *n*-butanol (3 × 1 L). The EtOAc and *n*-butanol extracts were concentrated under vacuum to give 6.1 g and 14.8 g of residue, respectively. Significant anti-cancer activity was detected in the EtOAc fraction; upon treatment with 1.0 mg/mL extract, reduction of cell viability was 15%, 10%, and 19% for DU145 prostate cancer, A2058 melanoma, and MDA-MB-435s breast cancer types, respectively. The EtOAc extract was subjected to normal-phase silica gel (200 g) column chromatography (CC) using a mixture of $CHCl_3$ and MeOH which had been previously saturated with dry NH_3 (80/20). Similar fractions were pooled together on the basis of TLC analysis. Subsequent evaluation of the fraction eluted first (2.8 g) by ^1H and ^{13}C NMR indicated that this pooled fraction contained a mixture of small peptides. Further fractionation was performed by normal-phase silica gel (100 g) CC with a mixture of hexane and EtOAc (95/5). Close inspection by NMR indicated that sub-fractions 10 (183 g) and 11 (156 mg) appeared to contain most of the peptides observed in the initial NMR analysis. Purification of sub-fraction 10 was achieved by C18 silica gel (5 g) reversed-phase CC using a mixture of MeOH and H_2O (85/15) as eluent; this yielded the new compound euryjanicin A (**1**, 44 mg, 0.05%) as well as the known cyclic octapeptide dominicin (**2**, 33 mg, 0.04%).[12] Also isolated was a new cycloheptapeptide, which is currently still under investigation and will be reported separately.

* Corresponding author. Tel.: +1 787 764 0000x4799; fax: +1 787 756 8242.
E-mail address: abrodriguez@uprrp.edu (A.D. Rodríguez).

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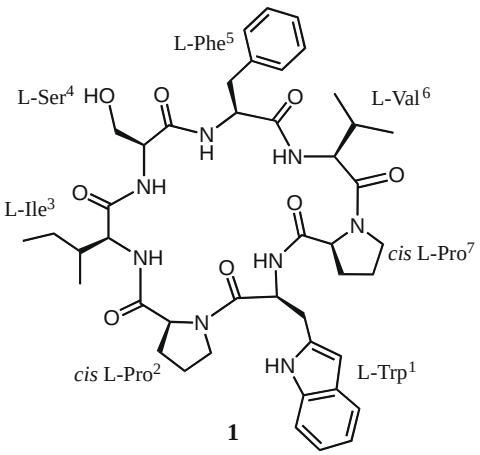
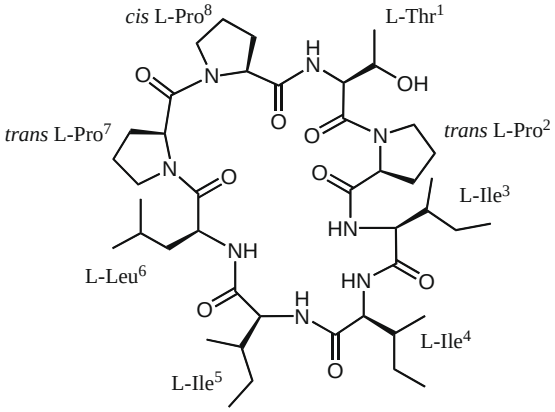


1



2

Euryjanicin A (**1**),[18] was obtained as optically active, $[\alpha]_D^{20}$ −18.0 (*c* 1.0, $CHCl_3$), colorless crystals that showed a pseudomolecular ion peak at *m/z* 827 in the ESIMS spectrum. The molecular formula of **1** was determined to be $C_{44}H_{58}N_8O_8$ by the HRESIMS [*m/z* 827.4439 $(M+H)^+$, $C_{44}H_{59}N_8O_8$, Δ −1.7 mDa], requiring 20 sites of unsaturation. The intense IR absorptions at 3316 and 1644 cm^{-1} were attributed to amino and amide carbonyl groups, respectively, and the UV absorptions at λ_{max} 281 (ε 1800) and 290 (ε 1600) nm indicated the presence of aromatic ring(s). The ^1H NMR spectrum of **1** (Fig. 1A) was indicative of a peptide. Standard amino acid analysis of the acid hydrolysate of **1** revealed the presence of 1 mol each of valine (Val), phenylalanine (Phe), tryptophan (Trp), serine (Ser), and isoleucine (Ile), and 2 mol of proline (Pro) residues, which subsequently, were all confirmed to be of L-configuration by Marfey's derivatization and subsequent HPLC analysis with standards.[19,20] Euryjanicin A (**1**) was negative to a ninhydrin test, which together with its solubility in organic solvents and the 20 degrees of unsaturation, indicated a cyclic structure.

The crystals of **1** gave a sharp, well-resolved peak when analyzed by reversed-phase HPLC using a variety of solvent systems, in addition to a sharp melting point range (mp 174–176 °C) and a single, clean molecular ion in the HRESI. Despite these indications of purity, the initial structural approaches to euryjanicin A (**1**) employing high field 2D NMR techniques were complicated by the existence of a mixture of slowly interconverting conformers in varying proportions depending on the solvent used (e.g., $CDCl_3$, DMSO-d_6, acetone-d_6, MeOH-d_4, pyridine-d_5, MeCN-d_3, and benzene-d_6). Attempts to simplify the ^1H NMR spectra by cooling (to −15 °C) and heating (to 60 °C), or by adjusting the pH by addition of TFA or Et_3N, did not alleviate the problem, and typically resulted in further loss of resolution which precluded structural studies. Likewise, many of the resonance lines in the ^{13}C NMR spectra were doubled, tripled, or broadened when the spectra were recorded in the same solvents (Fig. 1B). Because the 1D and 2D NMR spectra of euryjanicin A in these organic solvents were complex and difficult to interpret, only a handful of NMR spectral assignments could be made with complete confidence.[21] Therefore, efforts to determine the amino acid sequence by 2D NMR experiments were abandoned.

Crystals suitable for X-ray crystallographic analysis were obtained by slow recrystallization from MeOH(NH_3). The results obtained from this analysis (Fig. 2) defined the relative stereo-

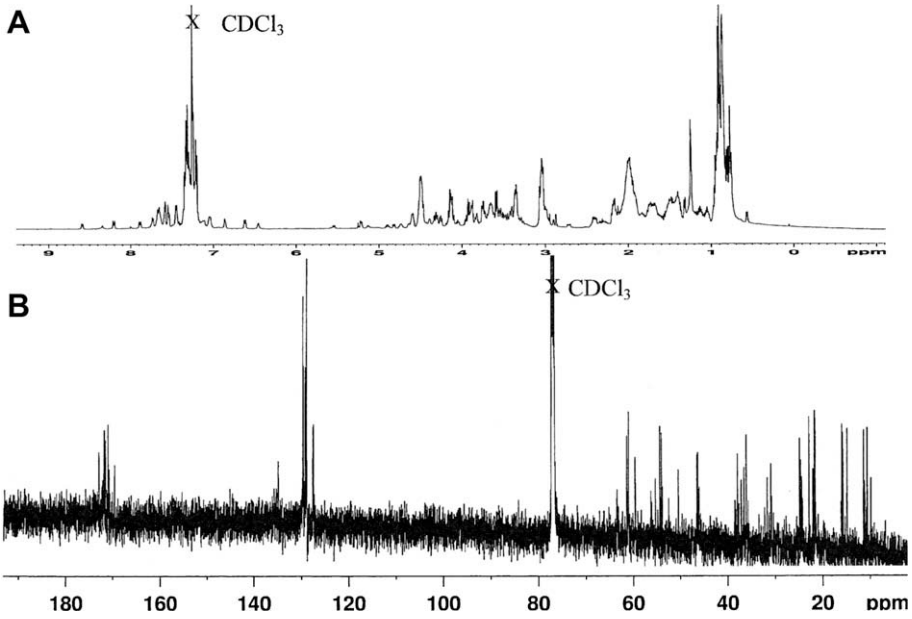


Figure 1. ^1H (500 MHz) (A) and ^{13}C (125 MHz) (B) NMR spectra of euryjanicin A (**1**) in $CDCl_3$ at 25 °C. The doubling and tripling of resonances observed in the NMR spectra is attributed to the existence of slow conformational equilibria.

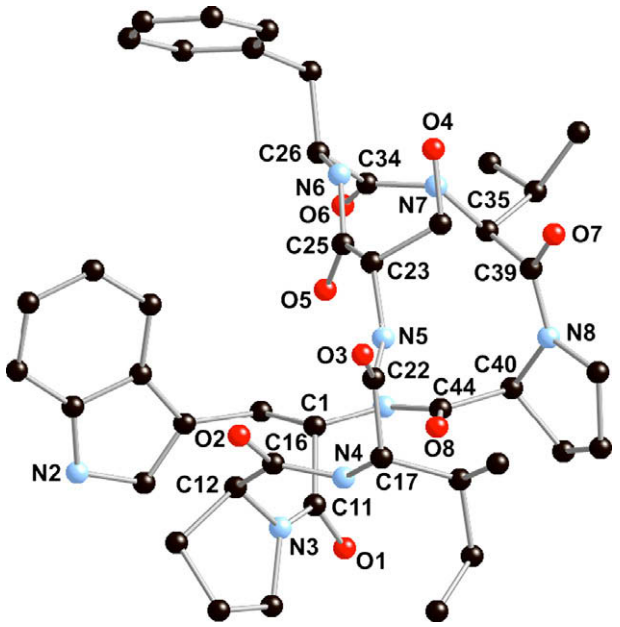
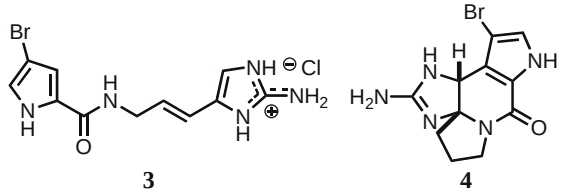


Figure 2. Perspective drawing of the X-ray crystal structure of euryjanicin A (**1**). Hydrogen atoms have been omitted for clarity and selected atoms are labeled. Carbon atoms are indicated in black, nitrogen atoms in blue, and oxygen atoms in red.

chemistry at all centers, and the absolute configuration shown was selected to agree with the known amino acid centers.[22] The geometry of the peptidic linkages about the proline residues was also assigned on the basis of the X-ray diffraction method, which showed that the two proline peptide bonds were cis. The cis peptide bonds and bifurcated transannular hydrogen bonds between the C44 carbonyl oxygen (O8) and N4–H (2.50 Å) and N5–H (2.43 Å) set the sharp turn conformation of the macrocyclic ring. An additional hydrogen bond (2.18 Å) was pinpointed between primary hydroxyl oxygen O4 of the Ser^4 residue and N6–H. This turn results in the side chains of the only two aromatic amino acids, L-Trp^1 and L-Phe^5, being projected in the same direction. The absolute structure **1** of euryjanicin A was thereby completed and thus confirmed as *cyclo*(Trp^1–Pro^2–Ile^3–Ser^4–Phe^5–Val^6–Pro^7). The fact that other investigators have isolated similar cyclic peptides from the extracts of cultured marine bacteria and fungi, suggests a possible microbial or symbiotic origin for metabolites euryjanicin A (**1**) and dominicin (**2**).[23]

Unlike the EtOAc extract, strong anti-tubercular activity was detected in the *n*-butanol partition fraction; thus a small portion of the crude extract (2 g) was subjected to C18 silica gel (50 g) reversed-phase CC using a mixture of MeOH and H_2O (85/15). Similar fractions were combined on the basis of TLC and ^1H NMR analyses. The first (most polar) fraction to elute out (0.90 g) was further chromatographed over normal-phase silica gel (100 g, dry packing), initially with a mixture of $CHCl_3$ and MeOH(NH_3) (90/10), and later using gradients of increasing polarity. This procedure yielded the known compounds hymenidin (**3**, 66 mg, 0.08%) and monobromoisophakellin (**4**, 57 mg, 0.07%).[24,25]



3 **4**

Pure euryjanicin A (**1**) and dominicin (**2**) were evaluated for their inhibitory activity against cell viability in melanoma (A2058), breast cancer (MDA-MB-435s), and prostate cancer (DU145). Surprisingly, none of them displayed significant inhibitory activity.[9] On the other hand, in the National Cancer Institute's one-dose (10^{-5} M) 60-cell-line assay, euryjanicin A (**1**) inhibited a non-small cell lung and renal cancer cell line: NCI-H522 displayed growth at 72% of the mean and UO-31 at 73% of the mean. Dominicin (**2**), in turn, inhibited a leukemia (SR) and a renal (UO-31) cancer cell line, displaying growths at 80% and 32% of the mean, respectively. Preliminary tests for anti-tuberculosis activity of alkaloids **3** and **4** demonstrated that these compounds significantly inhibited the growth of *Mycobacterium tuberculosis* $H_{37}Rv$ (MIC values = 6.1 and 64.0 μg/mL, respectively). On the other hand, alkaloids **3** and **4** lacked significant anti-plasmodial activity as their IC_{50}s against the malaria parasite *Plasmodium falciparum* (chloroquine-resistant W2 clone) were $\geqslant$10 μg/mL.

Acknowledgments

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17. This sponge was originally described as *Eurypon laughlini*.
18. *Euryjanicin A* (**1**): colorless crystals, mp 174–176 °C; $[\alpha]_D^{20}$ −18.0 (*c* 1.0, $CHCl_3$); ν_{max} (thin film) 3316, 2961, 2928, 1644, 1531, 1454, 1348, 750, 702 cm^{-1}; UV (MeOH) λ_{max} 211 (ε 12,500), λ_{max} 274 (ε 1700), λ_{max} 281 (ε 1800), λ_{max} 290 (ε 1600) nm; ^1H ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) spectra, see Figure 1; HRESIMS *m/z* 827.4439 $[M+H]^+$ (calcd for $C_{44}H_{59}N_8O_8$, 827.4456).
19. Pure euryjanicin A (0.5 mg) was hydrolyzed in 6 N HCl (100 mL) at 110 °C for 12 h. The cooled reaction mixture was evaporated to dryness, and traces of HCl were removed from the residual hydrolysate by repeated evaporation from H_2O (3 × 0.5 mL) using N_2 gas.
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21. The NMR spectra of dominicin (**2**) recorded in a variety of solvents also resulted in poor resolution. Acceptable NMR spectra, however, could be obtained using pyridine-d_5 as the solvent (see Ref. 12). Slowly interconverting conformations have been observed in other cyclic peptides such as schizotrin A and pahayokolides; see (a) Pergament, I.; Carmeli, S. *Tetrahedron Lett.* **1994**, *35*, 8473–8476; (b) An, T.; Krishnaswamy, T.; Kumar, S.; Wang, M.; Liu, L.; Lay, J. O.; Liyanage, R.; Berry, J.; Gantar, M.; Marks, V.; Gawley, R. E.; Rein, K. S. *J. Nat. Prod.* **2007**, *70*, 730–735.
22. Single-crystal X-ray diffraction measurements were made on a Bruker SMART 1 K CCD diffractometer with graphite-monochromated Mo Ka radiation (λ = 0.71073 Å). Crystals were mounted on a glass fiber. Crystal data for **1** at 293(2) K: $C_{44}H_{58}N_8O_8$, M_r = 826.98, orthorhombic, space group $P2_12_12_1$, *a* = 13.7230(13), *b* = 17.275(2), *c* = 19.026(3) Å, *b* = 90°, *V* = 4510.3(9) $Å^3$, *Z* = 4, ρ_{calc} = 1.218 Mg/m^{-3}, F_{000} = 1768, μ = 0.085 mm^{-1}. Data collection and reduction: crystal size, 0.39 × 0.38 × 0.16 mm, θ range = 1.59–28.28°, 32,630 reflections collected, 10,396 independent reflections (R_{int} = 0.0304), final *R* indices [$I > 2s(I)$]: R_1 = 0.0580, wR_2 = 0.1418 for 541 variable parameters, GOF = 1.034. The crystallographic data for euryjanicin A (**1**) have been deposited at CCDC under the registry number 721666. They can be obtained free of charge at www.ccdc.cam.ac.uk/conts/retrieving.html [or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, CB2 1EZ, UK; fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk].
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