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Renieramide, a Cyclic Tripeptide from the Vanuatu Sponge *Reniera* n. sp.

Linda Ciasullo,[†] Agostino Casapullo,[†] Adele Cutignano,[†] Giuseppe Bifulco,[†] Cécile Debitus,[‡] John Hooper,[§] Luigi Gomez-Paloma,[†] and Raffaele Riccio*[,†]

Dipartimento di Scienze Farmaceutiche, Università di Salerno, 84084 Fisciano (SA), Italy, IRD, Centre de Nouméa, BP A5, 98848 Nouméa, New Caledonia, and Queensland Centre for Biodiversity, Queensland Museum, P.O. Box 3300, South Brisbane, Qld, 4101, Australia

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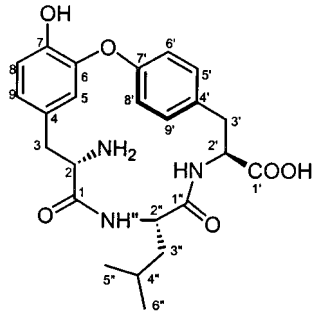
The polar extract of the Vanuatu sponge *Reniera* n. sp., which showed immunomodulating activity in preliminary tests, was found to contain a cyclic tripeptide, which we named renieramide (**1**). This metabolite is identical to a synthetic derivative mentioned in a patent concerning the preparation of cyclic peptides of the OF4949 family of anticancer agents. We describe here the first isolation of this metabolite from natural sources and its complete characterization by spectroscopic and chemical approaches. Renieramide (**1**) possesses a 17-membered cyclic side-chain-linked biphenyl ether skeleton, typical of the class that includes the natural products OF4949 I−IV, K13, and eurypamides. A tridimensional model of **1**, obtained by NMR restrained molecular mechanics and dynamics, is also presented.

Natural and synthetic cyclic peptides containing an isodityrosine moiety are reported in the literature as antimicrobial, antiviral, potential antitumor, and antihypertensive agents.[1−6] A few glycopeptidic antibiotic agents belonging to the vancomycin group, such as teicoplanin, avoparcin, and ristocetin, contain an aryl ether bond between two phenyl moieties of amino acids.[1] The K-13 molecule, produced by *Micromonospora halophytica* subsp. e*xilisia* K-13, is a dityrosine cyclic tripeptide exhibiting a potent noncompetitive inhibition of the angiotensin converting enzyme (ACE).[2] In 1986, Sano et al. isolated from cultures of *Penicillium rugulosum OF4949* four compounds named OF4949 I−IV, a series of tricyclic compounds that inhibited aminopeptidase B in a competitive way, thus showing an antitumor activity due to a strong stimulation of the immune system.[3] Euripamide A from *Microciona eurypa*[4] and bastadin-5 from *Ianthella basta*[5] are further examples of compounds with the isodityrosine feature from marine sponges. Recently, the diphenyl ether scaffold has also been taken in consideration for generating new leads for HIV protease inibition.[6]

Under the auspices of MAST III project "Bioactive Marine Natural Products in the Field of Antitumoral, Antiviral and Immunomodulatory Activity"[7] we investigated the crude extracts of a Vanuatu sponge, Reniera n. sp. (Haplosclerida), that exhibited immunomodulating activity in a preliminary pharmacological screening, and this led to the isolation of renieramide (**1**), a cyclic tripeptide belonging to the class of OF4949 and euripamide A. Renieramide (**1**) has not been reported previously as natural product, although it was mentioned as a synthetic compound in a patent concerning the preparation of a cyclic peptide of the OF 4949 family of anticancer agents.[8]

Besides describing its isolation from a marine source, we also report for the first time its complete spectroscopic characterization.

The freeze-dried organism (430 g) was preliminarily extracted with MeOH, and after solvent evaporation, the oily residue was partitioned according to a modified Kupchan method,[9] giving *n*-hexane, CCl_4, $CHCl_3$, and *n*-butanol extracts. The butanol-soluble material, which was more interesting by preliminary chromatographic and spectroscopic analysis, was desalted and sequentially fractionated by Sephadex LH20 chromatography and RP-HPLC, affording renieramide (**1**) as a pure compound.



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Complete structural elucidation of renieramide (**1**) was performed by 1D and 2D NMR, ESIMS, and MS/MS data, along with chemical analysis.

The ESIMS spectrum of compound **1** showed an intense pseudomolecular ion at 456 *m*/*z* $[M + H]^+$ corresponding to the molecular formula $C_{24}H_{29}N_3O_6$, as deducted by HRESIMS data (see Experimental Section).

The IR spectrum of **1** contained broad bands centered at 3430 cm^{-1} attributable to OH, NH, and COOH groups and at 1692 cm^{-1} compatible with an amide functionality. These data, together with signals contained in the ^1H NMR spectrum (DMSO-d_6), were suggestive of a peptide.

In fact, the ^1H NMR spectrum of **1** showed two signals typical of amide protons at δ 7.59 (d, $J = 9.8$ Hz) and 7.75 (d, $J = 10.0$ Hz) and three α-methine protons of amino acid residues at δ 4.36 (dt, $J = 9.8$, 3.8 Hz), 4.28 (m), and 3.50 (m).

The 24 signals contained in the ^{13}C NMR spectrum were assigned, on the basis of the HSQC data, to 12 aromatic (of which seven are methines) and nine aliphatic carbons (four methines, three methylenes, and two methyls) and three acylic carbonyls. A complete analysis of homonuclear

* To whom correspondence should be addressed. Tel: +39 089 962818. Fax: +39 089 962828. E-mail: riccio@unisa.it.
[†] Università di Salerno.
[‡] IRD, Centre de Nouméa.
[§] Queensland Museum.

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Table 1. NMR Data for Renieramide (**1**) (DMSO-d_6, 600 MHz)

		^{1}H δ (J = Hz)	^{13}C ppm	HMBC	ROESY
DOPA	1		172.9		
	2	3.50 m	52.8	C-1 C-4	NH″
	3	a 2.68 t (12.8) b 2.66 dd (12.8; 5.7)	38.7	C-4 C-9 C-1	
	4		126.9		
	5	5.85 d (1.9)	116.3	C-3 C-6 C-7 C-9	H-8′ H-6′ H-2″
	6		147.1		
	7		144.9		
	8	6.72 d (8.1)	115.4	C-4 C-6	
	9	6.59 dd (8.1; 1.9)	123.8	C-5 C-7 C-3	
	OH	9.10 s		C-6 C-7 C-8	
Tyr	1′		177.7		
	2′	4.28 m	54.7		H-5′
	3′	a 3.20 dd (12.4; 3.3) b 2.54 bd (12.4)	38.3	C-1′ C-2′ C-4′ C-5′ C-9′	
	4′		135.0		
	5′	7.25 dd (8.1; 1.9)	130.0	C-3′ C-7′ C-9′	H_b−3′
	6′	6.91 dd (8.1; 2.3)	120.9	C-4′ C-7′ C-8′	H-5
	7′		153.1		
	8′	6.66 dd (8.1; 2.3)	120.3	C-3′ C-5′ C-7′	H-5 H-2″
	9′	7.20 dd (8.1; 1.9)	131.7		H_a−3′ H-2″
	NH′	7.75 d (10.0)		C-1″	H-9′ H-2″
Leu	1″		170.4		
	2″	4.36 dt (9.8; 3.8)	49.6		H-5 H-8′ H-9′ NH′
	3″	a 1.41 m b 1.37 m	42.6	C-5″ C-6″	NH′ NH″
	4″	1.54 m	24.0	C-2″	NH″
	5″	0.83 d (6.7)	23.4	C 3″ C-4″ C-6″	
	6″	0.86 d (6.2)	21.6	C-3″ C-4″ C-5″	H-2″
	NH″	7.59 d (9.8)		C-1	H-2 H-4″

and heteronuclear 2D NMR data (DMSO-d_6) allowed us to identify the amino acid residues of DOPA, Tyr, and Leu, assembled in a cyclic structure as required by the 12 degrees of insaturation deduced from the molecular formula (11 of which could be accounted for by functional groups). The ^{1}H resonances at δ 5.85 (d, J = 1.9 Hz), 6.59 (dd J = 8.1, 1.9 Hz), and 6.72 (d, J = 8.1 Hz) were assigned to the 1,2,4-trisubstistuted aromatic nucleus of DOPA, while the signals at δ 6.91 (dd, J = 8.1, 2.3 Hz), 6.66 (dd, J = 8.1, 2.3 Hz), 7.25 (dd, J = 8.1, 1.9 Hz), and 7.20 (dd, J = 8.1, 1.9 Hz) accounted for the *p*-disubstituted ring of a tyrosine unit showing magnetic inequivalency, as a consenquence of a hindered phenyl ring rotation. Moreover we identified, along with the −CH−CH_2− [δ 3.50 (H-2, m), 2.68 (H-3a, t, J = 12.8 Hz), and 2.66 (H-3b, dd, 12.8, 5.7 Hz)] and −NH−CH−CH_2− [δ 7.75 (NH′, d, J = 10.0 Hz), 4.28 (H-2′, m), 3.20 (H-3a′, dd, J = 12.4, 3.3 Hz), 2.54 (H-3b′, d, J = 12.4 Hz)] spin systems of DOPA and tyrosine, respectively, the NH-isobutyl chain of the leucine residue [δ 7.59 (NH″, d, J = 9.8 Hz), 4.36 (H-2″, dt, J = 9.8; 3.8 Hz), 1.41 (H-3a″, m), 1.37 (H-3b″, m), 1.54 (H-4″, m), 0.83 (H_3-5″, d, J = 6.7 Hz), and 0.86 (H_3-6″, d, J = 6.2 Hz)]. Analysis of HMBC and ROESY key correlations (Tables 1 and 2) allowed us to identify the cyclic side-chain-linked biphenyl ether tripeptide DOPA-Leu-Tyr structure of renieramide (**1**). This structural hypothesis was also confirmed by mass spectrometric analysis with multiple ion fragmentation technique (ESIMS/MS) as shown in Scheme 1.

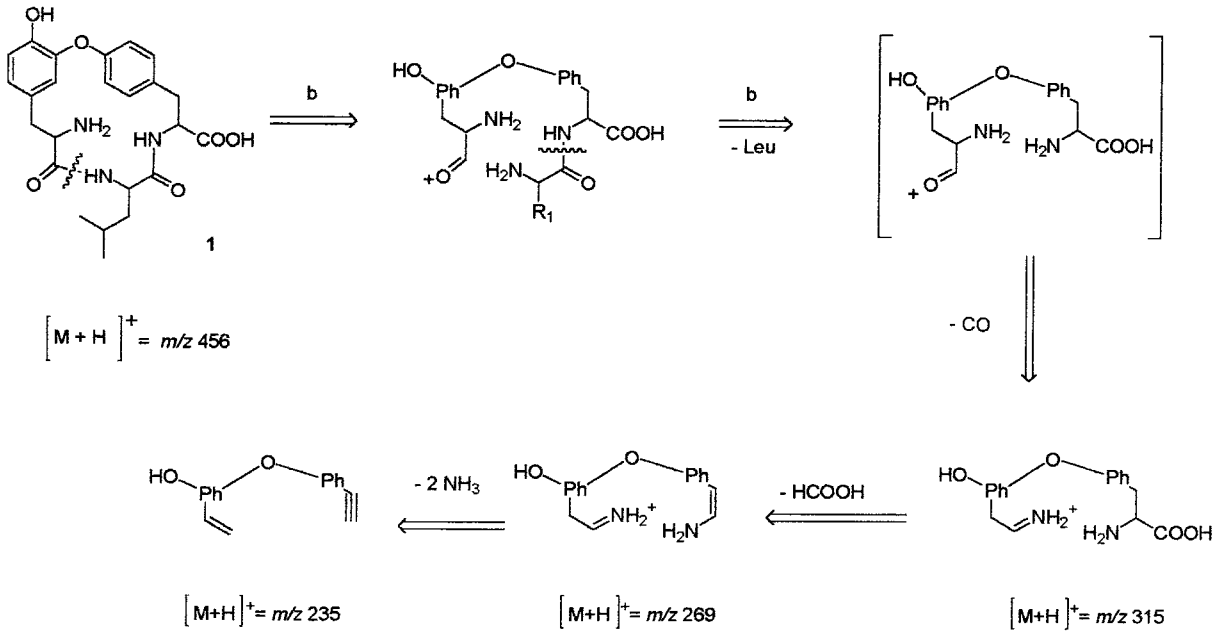
The determination of absolute configuration at C-α of the amino acid residues was achieved through oxidation of the DOPA and Tyr units to Asp by ozonolysis (see Experimental Section), followed by acidic hydrolysis (HCl 6 N, 110 °C, 15 h) and Marfey HPLC analysis of their FDAA derivatives.[10] All amino acid residues were found to belong to the L-series as shown.

Cyclic 17-membered biphenyl ether tripeptides have often been shown to exert interesting biological activities. Sano et al. demonstrated with an extensive in vitro study the immunomodulant activity of synthetic renieramide.[3] Given the identity of synthetic and natural renieramide, it is very likely that this compound is one of the bioactive components of the sponge extracts. Therefore, with the intention of gaining further insight regarding the biological activity of this class of molecules, our contribution was aimed at investigating the structural motifs common to this class of compounds. For this purpose we determined the three-dimensional structure of renieramide (**1**) by means of restrained molecular mechanics and dynamics. This structure has been compared to the crystal structure of H_3N^+-cyclo-[Phe(4-O)-Phe-Phe (3-O)]-OMe·Cl^-[11], a synthetic biphenyl ether cyclic tripeptide investigated for its structural similarity with K13,[2] an ACE inhibitor, and OF4949 I−IV, a family of competitive inhibitors of aminopeptidase.[3] It shows a high degree of superposition in the 17-carbon cyclic framework (Figure 1), and it follows a common rigid motif, already observed for a number of biphenyl ether cyclic tripeptides characterizated by a

Table 2. NMR Data for Renieramide (**1**) (CD_3OD, 600 MHz)

		^{1}H δ (J = Hz)	^{13}C ppm
DOPA	1		170.0
	2	3.93 bd (6.6)	53.8
	3	a 2.90 dd (14.9; 6.6) b 3.16 bd (14.9)	43.6
	4		125.4
	5	6.00 d (1.8)	117.2
	6		149.7
	7		147.0
	8	6.83 d (7.9)	116.7
	9	6.67 dd (7.9; 1.8)	125.0
Tyr	1′		178.3
	2′	4.51 dd (12.5; 3.0)	58.1
	3′	a 2.63 t (12.5) b 3.41 dd (12.5; 3.0)	40.9
	4′		137.1
	5′	7.23 dd (8.3; 1.8)	133.0
	6′	6.89 dd (8.3; 2.6)	122.6
	7′		154.6
	8′	7.03 dd (7.9; 2.6)	123.1
	9′	7.45 dd (7.9; 1.8)	131.6
Leu	1″		172.7
	2″	4.55 dd (11.4; 3.5)	52.1
	3″	a 1.68 m b 1,57 ddd (12.7; 11.4; 2.8)	43.6
	4″	1.65 m	25.9
	5″	0.96 d (6.6)	23.8
	6″	0.98 d (6.6)	21.5

Scheme 1. ESIMS/MS Fragmentation Pathway for Renieramide (**1**); Only Fragments of b Type Are Shown for Clarity, Although an Equivalent Pattern of x Type of Fragments, Carrying the Same Structural Information, Were Identified and Interpreted



β-strand peptide backbone.[6] In the absence of data on the drug−receptor complex, this observation suggests that the different side chains located on this central scaffold could act as molecular discriminants in the recognition process, leading to a modulation of the biological activity.

Experimental Section.

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-600 Avance spectrometer, and the solvent was used as internal standard (CD_3OD: ^{1}H δ 3.34, ^{13}C δ 49.0. DMSO-d_6: ^{1}H δ 2.50, ^{13}C δ 39.5). The IR spectrum was recorded on a Shimadzu FTIR-8101M spectrophotometer. The UV spectrum was obtained on a Beckman DU-70 spectrophotometer. A low-resolution mass spectrum was recorded on a LCQ-DECA Finnigan spectrometer equipped with electrospray source. High-resolution mass spectra was recorded on an API QSTAR Applied Biosystem mass spectrometer.

Animal Material. The sponge *Reniera* n. sp. was collected in Vanuatu (Island of Santo) and taxonomically identified by Dr. John Hooper (Museum of Queensland, Brisbane, Australia; accession number QM G306887). *Reniera* n. sp. (species number 2115) (Porifera, Demospongiae, Haplosclerida, Chalinidae). Growth form: arborescent, cylindrical, slightly flattened, digitate branches; branching pattern simple, with few bifurcations; tips of branches with rounded flattened margins. Oscules: several oscules, moderate in size, up to about 4 mm in diameter, conspicuous, discrete, found only on one side of slightly flattened branches, each with a very slightly raised membranous lip. Texture: soft, spongy, compressible, feels like velvet. Color: brownish-maroon with white tips when alive; white in ethanol. Surface ornamentation: translucent, membranous, optically smooth. Surface is even, unornamented, although subdermal drainage canals can be seen beneath the surface membrane in the preserved specimen. Ectosomal skeleton: tangential unispicular isodictyal reticulation of oxeas. Choanosomal skeleton: isotropic reticulation of ascending paucispicular tracts of oxeas, up to about 4−5 spicules side-by-side in each tract, interconnected by unispicular or paucispicular transverse tracts of oxeas, giving the skeleton a rectangular mesh appearance. Many oxeas are loose, scattered between the tracts. Mesohyl collagen is moderately dense and homogeneous. Choanocyte chambers are not observed (poorly preserved material). Megascleres: two size classes of oxeas. The larger is substantial, relatively thick (115−154 × 3−5μm). The smaller is hair-like, extremely thin (88−106 μm × 0.3−0.5 μm). All megascleres are gently curved, with abruptly pointed ends. Microscleres: absent.

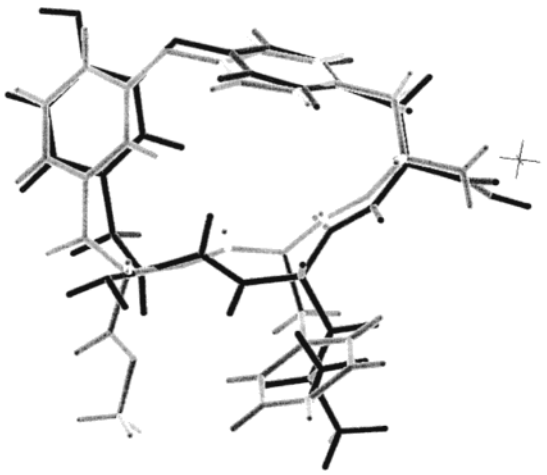


Figure 1. Superposition of the solution structure of renieramide (**1**) with crystal structure of H_3N^+-cyclo-[Phe (4-O)-Phe-Phe (3-O)]-OMe.Cl^-.

Extraction and Isolation. The lyophilized material (430 g) was extracted with MeOH (3 × 1.5 L) at room temperature. The combined methanolic extracts, filtered through paper and concentrated under reduced pressure, gave a dark brown oil. The oily residue was partitioned using a modified Kupchan partition method: the extract was dissolved in 0.5 L of a mixture of 9:1 MeOH/H_2O and partitioned against 0.5 L of *n*-hexane. The water content (% v/v) of the methanolic fraction was adjusted to 20% and 40% and partitioned against 0.7 L of CCl_4 and 1 L of $CHCl_3$, respectively. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-butanol (0.5 L). The *n*-BuOH-soluble material (6.9 g) was then subjected to LH-20 chromatography, using MeOH as eluent. The collected fractions 75−79 purified by RP-HPLC on Vydac column (H_2O (0.1% TFA)/90% CH_3CN−10% H_2O (0.1% TFA) gradient from 100% to 60% of H_2O) afforded renieramide (**1**) as a pure compound (66.7 mg).

Renieramide (1): colorless white powder. $[\alpha]_D$ −31° (1 mg/mL MeOH); IR (neat) 3432; 1692; 1513; 1030 cm^{-1}; UV (MeOH) λ_{max} (ϵ) 203 (4000), 230 (ϵ = 8900) e 278 (2000) nm;

ESIMS$^+$ *m/z* 456 [M + H]$^+$; HR-ESIMS$^+$ *m/z* 456.2148 ($C_{24}H_{30}N_3O_6$ requires 456.2134); NMR data, see Tables 1 and 2.

Ozonolysis of Renieramide (1). Compound **1** (3.8 mg) was dissolved in 2 mL of MeOH and maintained at −78 °C (CO_2/acetone) under a stream of ozone (0.7 bar) for 2 h. After this time, 50 drops of H_2O_2 (30%) were added to the reaction mixture, which was allowed to stand with strirring at room temperature for 1 h. The solvent was then evaporated under reduced pressure, and the product was hydrolyzed and derivatized according to the Marfey procedure.[10]

Molecular Mechanics/Dynamics Simulations. Molecular mechanics/dynamics (MM and MD) calculations were performed on a Silicon Graphics Indigo2 using the InsightII/Discover package[12] and the CVFF force field.[13] MD calculations (500 K, 50 ps) were first performed without distance restraints in order to allow a full exploration of the conformational space. The lowest minimum energy structures, achieved by energy minimization of different snapshots of the MD trajectory, resulted in fair agreement with the experimental ROESY and homonuclear *J* values. Subsequently, to refine these structures, molecular mechanics and dynamics were performed using a set of distance restraints obtained by the ROESY data. A distance calibration (r^{-6}, two-spin approximation) was used to convert the most intense and significative ROESY cross-peak volumes in the following distance restraints list:

nucleus 1	nucleus 2	lower bound	upper bound
NH′	H-9′	2.30	3.30
H-3′a	H-9′	2.00	3.00
H-3′b	H-5′	2.70	3.70
H-2′	H-5′	2.10	3.10
NH′	H-2″	1.80	2.40
NH′	H-3″a	2.50	3.40
NH″	H-3″b	2.50	3.20
NH′	H-3′b	2.50	3.50
H-2	NH″	1.80	2.40

A penalty of 32 kcal/Å^2 was applied for the distance violations. A temperature of 500 K was used during the dynamics simulations, and the Verlet algorithm was used to integrate the equations of motions. A distant-dependent dielectric term was used in all the calculations for reducing the artifacts derived from the absence of the solvent. All the structures were minimized using the steepest descendent and Newton−Raphson algorithms. In the lowest energy structures, together with the accordance of the internuclear distances with the ROESY data, all the most significant dihedral angles obtained showed a good agreement with the expected dihedrals obtained by the experimental NMR *J* values.

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