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Oriamide, A New Cytotoxic Cyclic Peptide Containing a Novel Amino Acid From The Marine Sponge *Theonella* Sp.

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Abstract. A novel cyclic peptide, oriamide (**1**) containing the new 4-propenoyl-2-tyrosylthiazole amino acid (PTT), was isolated from the marine sponge *Theonella* sp. collected in Sodwana Bay. The structure of oriamide, including the absolute configuration of all amino acids, was elucidated by spectroscopic analysis and degradation experiments. © 1997 Elsevier Science Ltd.

In the course of our program to discover potential biomedicinals from marine invertebrates[1], we isolated a new cyclic peptide from the blue marine sponge, *Theonella* sp[2]. Marine Sponges of the genus *Theonella* have been shown to be a rich source of unique secondary metabolites with intriguing structures including cyclic peptides. The *Theonella* peptides reported to date include orbiculamide A[3], keramamides A-H,J,[4-7] theonellamides A-F[8-9], theonellamine B[10], theonellapeptolides Ia-Ie,IId[11-12], cyclotheonamides A-E[13-14], motuporin[15], theonegramide[16], perthamide B[17] and konbamide[18].

The freeze-dried sponge collected in Sodwana Bay, at a depth of -15m, was extracted with chloroform-methanol (1:1) and then with methanol . The extracts were concentrated in vacuo and then partitioned each according to the Kupchan procedure[19]. The chloroform and butanol fractions of the two partitions were combined and were subjected to RP-18 and Sephadex LH-20 columns to afford oriamide (**1**, $6.8 \cdot 10^{-3}$%)[20].

The positive FAB mass spectrum of **1** gave an $(M+H)^+$ ion at *m/z* 1036. Doping the matrix with sodium chloride shifted the FABMS peak to *m/z* 1058 $(M+Na)^+$. The negative FABMS gave an $(MH-Na)^-$ ion at *m/z* 1014 confirming the molecular weight of 1035. Both the ^1H and ^{13}C NMR spectra (Table 1) suggested that **1** was a peptide. The individual amino acids were assigned based upon comparison to literature chemical shifts, HPLC comparisons after hydrolysis, and interpretation of the 2D HMBC, HMQC, COSY and TOCSY experiments as described below. The molecular formula of **1** was established to be $C_{44}H_{54}O_{15}N_9S_2Na$ based upon combination of FABMS and NMR data.

The ^1H NMR spectrum included signals for a 1,2,4 trisubstituted benzene (δ 7.24, 6.82, 6.73) (Table 1) which were identified as a 2,5-dihydroxybenzoyl (DHB)[21] group by the HMQC and HMBC spectra. Gly was proposed based on the ^1H, ^{13}C and the HMBC data[22]. Ala was assigned based upon the COSY correlations observed between the NH and the α-H and between the α-H and the β-CH_3[6]. A diaminopropionic acid (Dpr)

Table 1: NMR Data of 1

anmino acid	position	^{1}H (#H, J [Hz])	^{13}C	COSY	TOCSY	HMBC
DHB	1	-	116.8	-	-	6.73
	2	-	151.8	-	-	7.24, 6.82, 6.73
	2-OH	11.2 (1H, s)	-	-	-	-
	3	6.73 (1H, d, 9.0)	118.1	6.82	7.24	-
	4	6.82 (1H, dd, 2.8, 8.8)	121.4	6.73, 7.24	-	8.99, 7.24
	5	-	149.7	-	-	8.99, 7.24, 6.82, 6.73
	5-OH	8.99 (1H, s)	-	-	-	-
	6	7.24 (1H, d, 2.8)	114.4	6.82	6.73	8.99, 6.82
	7	-	168.0	-	-	7.24, 6.73
Gly	NH	8.91 (1H, bt)	-	3.92	-	-
	α	3.92 (2H, dq, 3.6, 6.8)	42.7	8.91	-	-
	CO	-	168.4	-	-	8.13, 3.92
Ala	NH	8.13 (1H, d)	-	4.341	1.17	-
	α	4.341 (1H, m)	48.2	1.17	1.17	1.17
	β	1.17 (3H, d, 7.0)	18.7	4.341	0.72	4.341
	CO	-	171.6	-	-	7.93, 1.17, 4.341
Dpr	NH	7.93 (1H, d, 7.5)	-	4.05	3.75, 2.45	-
	α	4.05 (1H, m)	51.3	3.75, 2.45, 7.93	-	-
	β	3.75 (1H, m) 2.45 (1H, m)	41.0	2.45, 4.05, 7.81 3.75, 4.05, 7.81	- 7.93	-
	β-NH	7.81 (1H, bs)	-	3.75	2.45,4.05	-
	CO	-	171.5	-	-	8.59
nor-Val	NH	8.59 (1H, d, 7.0)	-	4.327	2.25, 1.65, 1.35, 0.92	-
	α	4.327 (1H, m)	54.7	-	8.59, 2.25, 1.55, 0.92	-
	β	2.25 (1H, m) 1.65 (1H, m)	32.4	1.55, 1.35, 4.327 2.25	8.59 0.92, 1.35	0.92
	γ	1.55 (1H, m) 1.35 (1H, m)	20.0	0.92 0.92	1.35, 2.25 2.25, 1.65	0.92
	δ	0.92 (3H, t, 7.3)	14.1	1.35, 1.55	1.65, 4.327, 8.54	-
	CO	-	175.1	-	-	8.29
Cys acid	NH	8.15 (1H,d)	-	4.74	-	-
	α	4.74 (1H, m)	49.7	2.71, 3.16 8.15	2.71	2.71
	β	2.70 (1H, m) 3.16 (1H, d)	50.8	3.16, 4.74 2.71, 4.74	8.15 -	4.74
	CO	-	170.9	-	-	7.81, 4.74, 2.71
PTT	1	-	165.2	-	-	8.15, 7.39, 6.76
	2	6.76 (1H, d, 16.0)	123.7	7.39	-	-
	3	7.39 (1H, d, 15.1)	132.8	6.76	-	-
	4	-	150.0	-	-	7.81, 7.39, 6.76
	5	7.83 (1H, s)	123.4	-	-	-
	7	-	170.7	-	-	5.21
	9	5.21 (1H, m)	54.7	3.10, 3.02, 9.37	-	-
	10-NH	9.37 (1H, d, 8.0)	-	5.21	3.10, 3.02	-
AKMH	11-CO	-	164.0	-	-	9.37
	12-CO	-	197.0	-	-	-
	13	5.04 (1H, dd, 3.6, 9.2)	60.4	8.29	-	0.80
	14-NH	8.29 (1H, d, 9.4)	-	5.04	0.80	-
	15	2.39 (1H, m)	37.2	0.80	-	0.80, 0.72, 5.04
	16	0.80 (3H, d, 6.9)	16.5	2.39	8.29	-

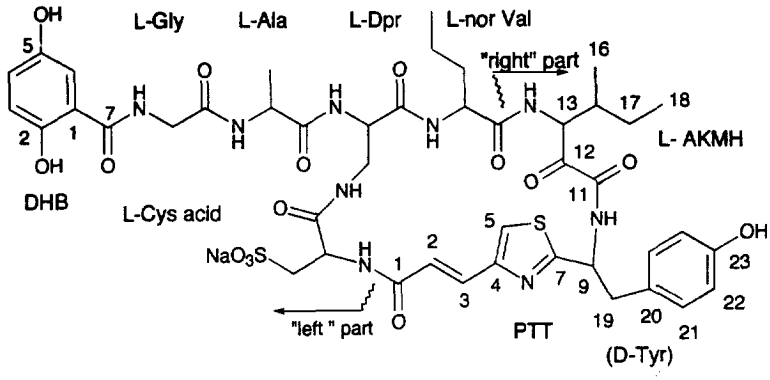
	17	1.11 (1H, m) 0.92 (1H, m)	23.6	0.72 0.80	2.39	0.80, 0.72, 5.05
	18	0.72 (3H, t, 7.4)	12.1	1.11	-	-
PTT	19	3.10 (1H, m) 3.02 (1H, m)	39.5	5.21 5.21	9.37 9.37	7.16, 5.21 7.16, 5.21
	20	-	127.7	-	-	6.69
	21	6.69 (1H, d, 8.3)	130.7	7.16	-	7.16
	22	7.16 (1H, d, 8.3)	115.5	6.69	-	6.69, 9.27
	23	-	156.5	-	-	9.27, 6.69, 7.16
	23-OH	9.27 (1H, s)	-	-	-	-
	24	7.16 (1H, d, 8.3)	115.5	6.69	-	6.69, 9.27
	25	6.69 (1H, d, 8.3)	130.7	7.16	-	7.16

* The numbering in the 'right' part (1-18) is identical to the numbering in Keramamide F[7] .

moiety was suggested based upon the COSY correlations observed between the NH and the α-H, between this α-H and both of the β-H's and between the β-NH and both of the β-protons[7]. The nor-Val unit was assigned based upon interpretation of the 2D and mainly the TOCSY data in which correlations between all α-H through δ-Me protons were observed[23]. The fifth amino acid of the 'left' part of **1**, i.e. a cysteic acid, was determined based upon comparison to literature chemical shifts of this acid[24].

The 'right' part of **1** was also deduced by NMR data (Table 1), and was found to consist of three modified amino acid residues, containing an α,β-unsatutaied carbonyl group conjugated with a thiazole as well as a β-amino acid moiety constituing an isoleucine like moiety[7] *vide infra*. The presence of the thiazole ring in this half was indicated by the ^{1}H and ^{13}C NMR spectral data [δ_H 7.83 (s, 1H); δ_C 170.7s, 123.5d, ($^{1}J_{C-H}$ = 189 Hz), and 150s]. One of the thiazole substituens at C-4 was determined, based on the HMBC correlations, to be an α,β-unsatutared carbonyl moiety, namely, a propenoyl functionality. The presence of AKMH (3-amino-2-keto-4-methyl hexanoic acid), a β-amino α-keto isoleucine like acid,[7] was proposed based upon COSY, HMBC and TOCSY data (Table 1). Most useful in establishing the AKMH-isobutyl side chain were the HMBC correlations observed between : the γ-C and the δ-CH_3 & β-H ; the β-C and the δ-CH_3 & β-H. Two pairs of two protons each in the ^{1}H NMR spectrum [δ_H 7.16 (d, 1H, J=8.3 Hz), 6.69 (d, 1H, J=8.3 Hz)] constitute an aromatic AA'BB' system (p-substituted phenol). Further data from the HMBC and TOCSY spectra allowed the assignement of this aromatic system as part of the amino acid tyrosine. The HMBC correlation between H-9 and C-7 and a NOESY correlation between NH-10 and H-13 concluded the structure determination of the 'right' part of **1**. The latter half incorporates the new 4-propenoyl-2-tyrosylthiazole (PTT) acid.

NOESY data provided further conformative information on the amino acids sequence. The major interresidue NOE correlations observed were : glycine NH to DHB H-6 ; nor-Val NH to Dpr α-H, Dpr β-H and Dpr α-NH ;AKMH NH to nor-Val α-H and nor-Val β-H ; 10-NH to H-13 ; cysteic acid NH to Dpr β-NH cysteic acid α-H to Dpr β-NH.



Further corroborative evidence for the structure of **1** was obtained from the FABMS Fragmentations: 1036 (MH^+), 1014.7(M-Na+H), 908.6 (M-Na-$CH_2C_6H_4OH$+H), 837.5 (M-Cys-acid-H_2O), 750.5 (M-Na-DHB-Gly-Ala), 555.4 (M-Na-$CH_2C_6H_4OH$-DHB-Gly-Ala-Dpr), 423.4(M-Na-DHB-Gly-Ala-Dpr-norVal-AKMH +H), 384.4 (M-Na-CHO-DHB-Gly-Ala-Dpr-Cys-acid-norVal). Noteworthy is the strong resemblance of **1** to keramamide F[7] , both being characterized by a cyclic segment containing amino acids Dpr,AKMH and an α,β-unsaturated carbonyl group conjugated with a thiazole ring.

Chiral TLC analysis [23] of the hydrolysate of **1** and comparison with authentic samples revealed that the Ala, Dpr, nor Val and cysteic acid residues, in **1**, were of the L-form. Ozonolysis of **1**[7] led to degradation of the tyrosylthiazole moiety to yield Tyr , while treatment of **1** with H_2O_2/NaOH[7] transformed the C-11÷N-14 moiety (AKMH) of **1** into isoleucine. The Tyr thus obtained was determined by the chiral TLC method to be D and the isoleucine was determined to be L implying that the tyrosylthiazole in **1** is D and the AKMH is L.

Oriamide contains the unprecedented amino acid, 4-propenoyl-2-tyrosylthiazole (PTT). A related thiazole amino acid was earlier reported as a constituent of the cyclic peptide keramamide F[7].

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Experimental Section

General experimental procedures - The ^1H and 2D NMR spectra were recorded on a Bruker ARX-500 spectrometer. The ^{13}C spectrum was recorded on a Bruker 360 Spectrometer. All chemical shifts were reported in parts per million referenced against residual undeuterated DMSO (δ_H 2.49 ppm, δc 39.5 ppm). ^1H,

^{13}C, DEPT, COSY, HMQC, TOCSY, and HMBC spectra were recorded using standard Bruker pulse sequences. A NOESY experiment was acquired with mixing time of 300ms. FABMS were recorded on a Fisons Autospec Q mass spectrometer. TLC was conducted on MERCK precoated Kieselgel 60 F_{254} plates and the spots were detected by iodine. Chiral TLC analysis was conducted on Macherey-Nagel 811056 chiralplates eluted with MeOH-H_2O-ACN (1:1:4) and the spots were detected by spraying with ninhydrin [23].

Collection and extraction.- The marine sponge *Theonella* sp. was collected in Sodwana Bay, -15m. The Freeze-dried sponge (103g) was extracted two times with EtOAc, once with MeOH-$CHCl_3$ (1:1) and once with MeOH.

The MeOH-$CHCl_3$ and MeOH extracts (1.13g and 2.87g, respectively) were concentrated in vacuo before being each partitioned according to the Kupchan procedure[19]. The chloroform and butanol fractions of the two partitions were combined and were applied to a reversed phase RP-18 column, successively collecting about 11x50ml fractions of a gradient of water - MeOH. Fractions 5-11 (116mg) were combined and were subjected to a sephadex LH-20 column to affort oriamide (10mg).

Oriamide (1): amorphous powder; negative FABMS (70ev) m/z (rel int) 1014.7 ($MH-Na^+$,18), 908.6 (9), 837.5 (10), 750.5 (9), 555.4 (15), 423.4 (14), 384.4 (18), 339.3 (87), 265.2 (95), 215.1 (100). For ^1H and ^{13}C NMR data see Table 1.

Determination of the Stereochemistry of the amino acids Ala, Dpr, nor Val, Cys acid.

A 0.5 mg portion of 1 was dissolved in 6N HCl (ca. 0.5 ml) and was heated at 110°C for 20h, the HCl was then removed under vacuo[7]. HPLC of the hydrolysed amino acids by Marfey's method[25] established 4 amino acids i.e. L-Ala, L-Cys-acid, L-nor-Val and L-Dpr. The residue and authentic samples of chiral amino acids were also subjected to chiral TLC analysis on Macherey-Nagel Chiralplates eluted with MeOH-H_2O-ACN (1:1:4) and the spots were detected by spraying with ninhydrin [23]. The Ala, Dpr, nor-Val and Cys-acid residues were established to be of the L form.

Determination of the stereochemistry of the tyrosyl-thiazole.

A fine stream of 0_3 was bubbled into a solution of compound **1** [0.5mg of compound **1** in MeOH (0.5 ml) - CH_2Cl_2 (5ml)] for 20 sec. Dimethyl sulfide (2 drops) was then added and the reaction mixture was left over night at rt. After removal of the CH_2Cl_2 and DMSO/DMS, the residue was dissolved in 6N HCl (0.5ml) and was subjected to acid hydrolysis as described above. Chiral TLC analysis, as described above [23], established the D form of the Tyr.

Determination of the Stereochemistry of the 3-amino-2-keto-4-methyl hexanoic acid (AKMH) moiety in PTT: To a stirred solution of compound **1** (0.5mg) in 5% NaOH (1ml) was added, dropwise, 30% H_2O_2 (200µl)[7]. After atirring the solution at 65°C for 20 min the reaction mixture was subjected to hydrolysis and chiral TLC analysis as described above [25]. The Ile thus obtained, was determined to be in the L form.

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