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The Absolute Stereochemistry of Kahalalide F

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Abstract: Kahalalide F (**1**) is a depsipeptide of 14 residues, five of which form a 19-membered ring. It was isolated from a marine mollusk, *Elysia rufescens*, and is currently in preclinical trials against lung and colon cancers. It was known from conventional amino acid analysis that five valine and two threonine residues represented D- and L- enantiomers, but their position in the molecule was not known. After extensive hydrolytic trials, a combination of acid hydrolysis and hydrazinolysis succeeded in definitive stereochemical assignment.

Keywords: Antitumor compounds; Depsipeptides, Marine metabolites; Stereochemistry

The kahalalides are cyclic or acyclic depsipeptides isolated from a marine mollusk *Elysia rufescens*.[1-3] Kahalalide F (**1**) is the largest with one fatty and thirteen amino acid residues. It is the only member of the group with significant bioactivity.[4] It is currently in preclinical evaluation for its selective activity against human lung and colon cancers. Determination of the absolute configuration of all amino acid residues, which is the subject of this report, proved to be challenging. Conventional amino acid analysis combined with spectral data had revealed that the five valine and two threonine residues represented both D- and L- enantiomers. In order to assign correct chirality to each of the seven residues extensive hydrolytic reactions had to be performed.

To delineate the optimal hydrolysis parameters, we subjected kahalalide F(**1**) to a broad spectrum of experimental variants. In the first set-up, 2 mg samples of **1** were treated in sealed vials at 70°C with trifluoroacetic acid at four concentrations, 3-6N, for periods of 0.5 to 18 hours representing seven sets of conditions. All 28 samples were analyzed by HPLC and peaks were scannned by ^{1}HNMR allowing fractions to be recombined as appropriate. These trials yielded, in addition to unreacted **1**, its acyclic analog kahalalide G,[3] a single identifiable fragment **A**, the amide of 5-methylhexanoic acid and valine. NMR data are summarized in Table 1.

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Table 1. ^{13}C, ^{1}H Data and COSY for A

amino acid	#	$^{13}C^{a}$	$^{13}C^{b}$	$^{1}H^{b}$	mult^{b}	J(Hz)^{b}	COSY^{b}
5MeHex	1	non detected	173.9				
	2	36.9	36.9	2.25	m		H: 3
	3	24.9	23.5	1.64	m		H: 2, 4
	4	39.6	38.4	1.21	m		H: 3, 5, 6, 7
	5	29.0	27.8	1.55	m		H: 4, 6, 7
	6	22.9	22.5	0.88	d	6.6	H: 4, 5, 7
	7	22.9	22.5	0.88	d	6.6	H: 4, 5, 6
5MeHex	1	non detected	174.1				
(second	2	34.6	34.4	2.28	m	9.9, 1.8	H: 3_A, 3_B
conformn)	3_A	33.7	32.1	1.46	m		H: 2, 3_B,
	3_B			1.70	m		H: 2, 3_A,5_B
	4_A	30.3	29.1	1.18	m		H: 3, 5, 6, 7
	4_B			1.35	m		H: 4_A, 5
	5	35.4	34.1	1.39	m		H: 4_A, 6, 7
	6	11.6	11.2	0.87	d	6.6	H: 4_A, 5, 7
	7	19.2	18.8	0.88	d	6.6	H: 4_A, 5, 6
Val5	8	non detected	174.6				
	9	59.0	57.0	4.57	ddd	2.0, 5.0, 8.5	H: N_1, 10
	10	31.6	30.7	2.25	m		H: 11, 12
	11	18.4	17.7	0.96	d	6.8	H: 10, 12
	12	19.6	19.0	0.99	dd	6.8, 0.7	H: 10, 11
	N_1			5.94	d	7.9	H: 9

^{a} in CD_3OD. ^{b} in $CDCl_3$

In the light of the above meager results, hydrolytic conditions were strengthened in the second experimental series. The samples of **1** (2.2 mg, repeated with 15.4 mg) were treated with 2 and 6N ethanolic HCl at 105°C for periods from 15 to 60 minutes. HPLC separation, ^{1}HNMR scanning, appropriate recombination, and a second hydrolytic round resulted in three identifiable fragments **B**, **C**, and **D**. Again, ^{1}HNMR data (Tables 2,3) and comparison with the corresponding values of intact **1** secured the structures shown in Fig. 1. An alternative structure for **C**, HO_2C-Val 4-Thr 2-Val 5-NH_2 was ruled out by the doublet resonance of the α-proton of Thr 2.

Table 2. ^{13}C, ^{1}H Data and HMBC for C [a]

amino acid	#	^{13}C	^{1}H	mult	*J*(Hz)	HMBC
Val3	1	171.6				H: 2
	2	58.2	4.57	dd	8.9, 5.6	H: 5
	3	32.0	2.17	m		H: 4, 5
	4	17.6	0.91	d	6.8	H: 2, 5
	5	19.6	0.96	d	6.8	H: 3, 4
	N1		8.55	d	8.9	H: 9
Val4	6	173.4				H: N1, 7
	7	58.8	4.35	dd	8.3, 5.3	H: 9
	8	31.5	2.17	m		H: 9, 10
	9	18.2	0.91	d	6.8	H: 7, 10
	10	19.6	0.95	d	6.8	H: 8, 9
	N2		8.18	d	8.6	H: 9
Thr2	11	168.1				H: N2, 12
	12	59.8	4.15	d	6.8	H: 13, 14
	13	67.2	4.09	m		H: 12, 14
	14	20.0	1.29	d	6.5	H: 10, 12
	$N3H_2$		8.21	s		H: 9

[a] in $CDCl_3$

Table 3. ^{13}C, ^{1}H Data for B and D [a]

	Fragment	B				Fragment	D			
amino acid	#	^{13}C	^{1}H	mult	J (Hz)	#	^{13}C	^{1}H	mult	J (Hz)
Val1	1	171.5								
	2	60.5	4.12	t	9.3					
	3	31.0	1.70	m						
	4	18.9	0.96	d	7.0					
	5	17.4	0.81	d	7.0					
	N1		7.18	d	9.2					
Dhb	6	164.6								
	7	131.3	6.44	q	7.0					
	8	130.1								
	9	12.8	1.37	s	7.3					
	N2		9.41	s						
Phe	10	171.8				1	172.4			
	11	56.6	4.67	m		2	54.9	4.57	m	
	12a	36.9	3.01	dd	7.9, 13.4	3a	36.7	2.99	m	
	12b		3.19	dd	7.9, 13.4	3b		3.20	dd	13.8, 4.3
	13	138.2				4	138.7			
	14, 14'	130.0	7.33	m		5, 5'	129.9	7.34	m	
	15, 15'	128.9	7.30	m		6, 6'	128.9	7.29	m	
	16	127.2	7.24	m		7	127.1	7.22	m	
	N3		8.84	d	6.1	N1		8.67	d	8.6
Val2	17	179.1				8	172.3			
	18	58.4	4.44	m		9	58.5	4.54	m	
	19	32.8	2.21	m		10	31.3	2.10	m	
	20	19.8	0.90	d	6.7	11	19.7	0.97	d	6.8
	21	17.2	0.78	d	6.7	12	17.7	0.70	d	6.8
	N4		7.86	d	7.3	N2		7.77	d	9.5
Ile1	22	171.7				13	171.9			
	23	57.4	4.56	dd	10.1, 4.6	14	57.9	4.52	m	
	24	37.8	1.97	m		15	38.0	20.3	m	
	25	14.8	0.75	d	9.5	16	14.0	0.77	m	
	26a	27.0	1.57	m		17a	26.7	1.41	m	
	26b		1.14	m		17b		1.27	m	
	27	11.9	0.85	m		18	11.8	0.92	m	
	N5		8.73	d	10.1	N3		8.38	d	8.3
Thr1	28	170.5				19	170.4			
	29	58.0	4.63	dd	8.1, 14.2	20	59.6	4.34	m	
	30	71.1	5.00	dq	10.2, 6.2	21	68.8	3.97	m	
	31	19.8	1.18	d	6.4	22	20.8	1.21	d	6.2
	N6		8.03	m		N4		8.03	m	
Ile2	32	169.6				23	169.9			
	33	57.3	3.96	m		24	57.3	4.07	d	4.4
	34	39.0	1.95	m		25	37.5	1.97	m	
	35	14.1	0.74	d	9.2	66	14.8	0.84	m	
	36a	25.9	1.57	m		27a	25.9	1.59	m	
	36b		1.14	m		27b		1.27	m	
	37	11.8	0.88	t	9.2	28	12.0	0.93	m	
	$N7H_2$					$N5H_2$				

[a] in DMF-d_4

Marfey's method[5] was used to establish the chirality of the seven ambiguous amino acids, five valines and two threonines. Each of the four hydrolysis fragments **A-D** (100 μg each) was dissolved in 500 μL 5 N HCl, degassed *in vacuo*, and hydrolyzed for 13 h at 105°C. The resulting hydrolysate was dried under nitrogen; to each residue was added 50 μL of 1% Marfey's reagent in acetone and 100 μL of 1 N $NaHCO_3$, followed by heating at 80°C for 3 min. After cooling to room temperature the reaction mixture was neutralized with 50 μL of 2 N HCl and diluted with 100 μL of MeCN/H_2O (1:1) containing 0.05% TFA. The resulting solution was analyzed by reverse phase HPLC (COSMOSIL $5C_{18}$-AR) with two isocratic solvent systems: (I) MeCN/H_2O (42:48) + 0.05% TFA, (II) MeCN/H_2O (2:8) + 50 mM NH_4OAc. The results are shown in Table 4.

Table 4. Results of Marfey's Analysis of Fragments A-D

	Standard	A	B	C	D	C+ $(NH_2)_2$
L-Val[a]	18.5		18.3	18.3		18.2
D-Val[a]	25.5	25.5	25.3	25.3	25.4	
L-Thr[b]	16.8			16.8		
D-*allo*Thr[b]	24.3		24.3		24.3	

[a] solvent I: 42 % MeCN + 0.05 % TFA, [b] solvent II: 20 % MeCN + 50 mM NH_4OAc.

Since fragment **C** contained two valine residues Val 3 and Val 4, one D and one L, hydrazinolysis[3,6] of this fragment became necessary. This procedure separated Val 3 possessing a free carbonyl group from the rest of the fragment. Marfey's analysis proved L configuration for Val 3. Hence Val 4 had D chirality. Similar analysis of fragments **B** and **D** with solvent II detected D-*allo*Thr, while analysis of fragment **C** proved the presence of L-Thr. Figure 1 and Table 5 summarize the final results.

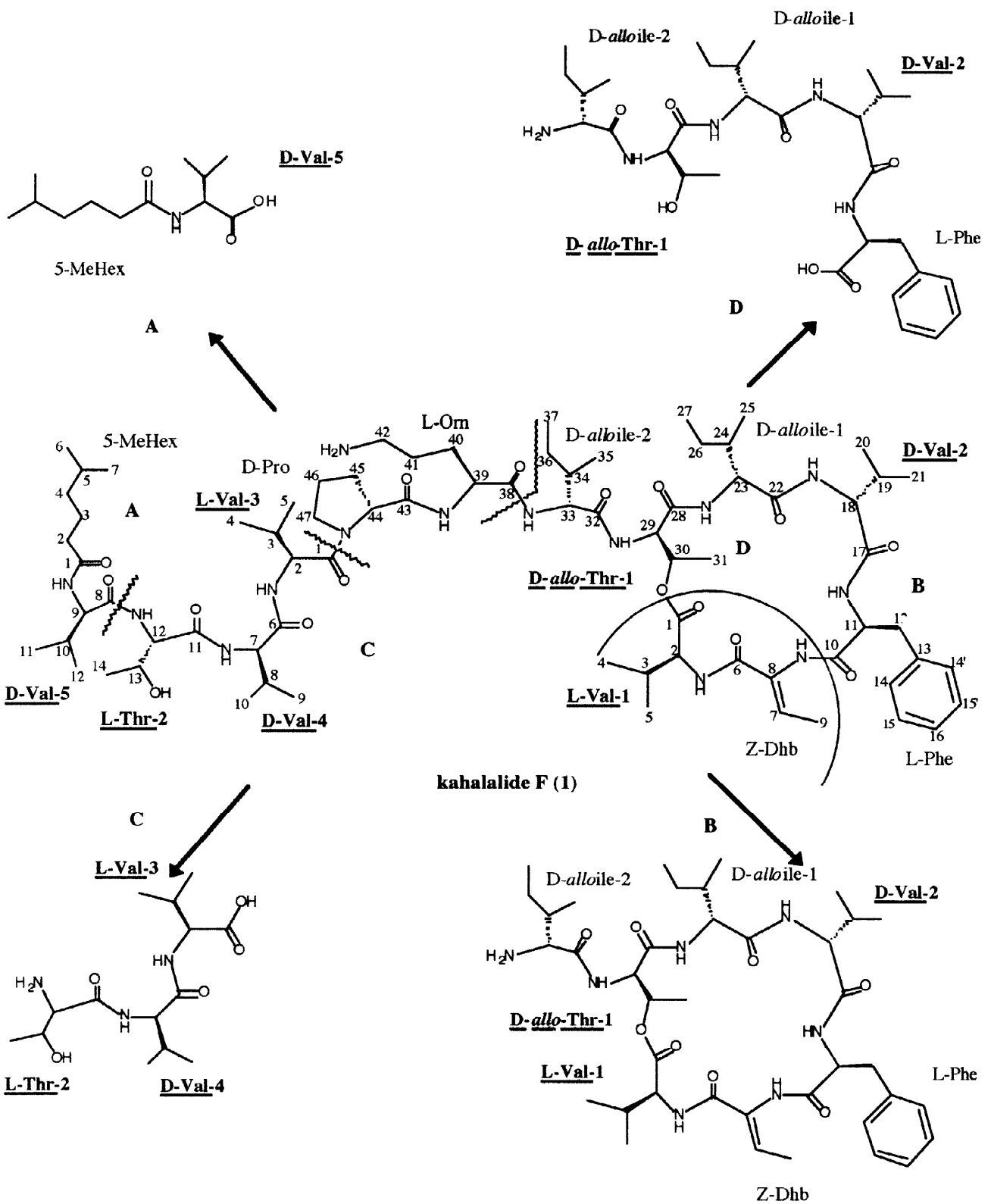

Figure 1. Hydrolysis of 1 (4N HCl/EtOH, 105°C, 25', HPLC).

Table 5. Summary of Results

A	B	C	D
D-Val-5	L-Val-1	L-Val-3	D-Val-2
	D-Val-2	D-Val-4	D-*allo*-Thr-1
	D-*allo*-Thr-1	L-Thr-2	

Experimental Section

General Experimental Procedures: All NMR spectra were recorded on a General Electric GN Omega 500 Mhz NMR spectrometer.

Isolation of A.

Kahalalide F (2 mg each) was suspended in 1 mL of 3N, 4N, 5N or 6N aqueous trifluoroacetic acid in vials (seven replicates of each condition). The vials were sealed and placed in an oil bath regulated at 70°C. Vials were removed and freeze dried after 0.5, 1, 2, 4, 6, 12 and 18 hours.

Each of these 28 samples was analyzed by reverse phase HPLC (COSMOSIL 5C18-AR MeOH/H_2O, 8:2, 0.05% TFA). Peaks were recombined based on NMR spectral features and reanalyzed by sequential reverse phase HPLC (COSMOSIL 5C18-AR, 1^{st} MeCN/H_2O, 5:5, 0.05% TFA – 2^{nd} MeCN/H_2O, 7:3, 0.05% TFA – 3^{rd} MeCN/H_2O, 4.5:3.5, 0.05% TFA) yielding a single identifiable fragment, the amide of 5-methylhexanoic acid with valine **A.**

Isolation of B.

Kahalalide F (2.2 mg and subsequently 15.4 mg) was dissolved in 100 μL of EtOH, to which 50 μL each of 2N and 6N HCl were added (3 replicates). Hydrolysis was performed at 105°C for 15, 25, 30 and 60 minutes respectively. The hydrolates were dried under N_2, then separated by reversed-phase HPLC (COSMOSIL 5C18-AR, MeCN/H_2O 45:55, 0.05% TFA). Fractions which had undergone hydrolysis were recombined and again subjected to HPLC (same column, gradient from 0.45% MeCN + 0.05% TFA to 45% MeCN + 0.05% TFA) leading to unidentified white powders. These were combined and hydrolyzed in EtOH/4N HCl (500μL) at 100°C for 1 hour. The hydrolysate was dried under N_2, then subjected to reversed-phase HPLC (same column) with a gradient of MeCN: H_2O:TFA from 1:99:0.05 to 50:50:0.05, yielded cyclo Val 1-Dhb-Phe-Val 2-*allo*ile 1-Thr 1-*allo*ile 2-NH_2 **B.**

Isolation of C and D.

All unidentified fractions were recombined and hydrolyzed once more in EtOH/4N HCl (500μL) at 100 °C for 1 hour. After being dried under N_2, the hydrolysate was separated by reversed-phase HPLC (COSMOSIL 5C18-AR) using a gradient of MeCN: H_2O:TFA from 1:99:0.05 to 50:50:0.05, leading to

additional peptide fragments, HOOC-Val 3-Val 4-Thr 2-NH_2 (**C**) and HOOC-Phe-Val 2-*allo*ile 1-Thr 1-*allo*ile 2-NH_2 **D**.

Marfey Analysis.

Each of the four hydrolysis fragments **A-D** (100 μg each) was dissolved in 5N HCl (500 μL), degassed under vacuum, and hydrolyzed for 13 h at 105°C. The resulting hydrolysate was dried under N_2; to each residue was added 50 μL of 1% FDAA solution in acetone and 100 μL of 1 N $NaHCO_3$, followed by heating for 3 minutes at 80°C. After cooling to room temperature the reaction mixture was neutralized with 50 μL of 2 N HCl and diluted with 100 μL of MeCN:H_2O (1:1) containing 0.05% TFA.

The resulting solution was analyzed by reverse phase HPLC (COSMOSIL 5C 18-AR) with two isocratic solvent systems: (I) MeCN/H_2O (42:48) + 0.05% TFA, (II) MeCN/H_2O (2:8) + 50 mM NH_4OAc.

Hydrazinolysis of C.

To 9 mg of dry Amberlite GC50, was added under argon, fragment **C** (100 μg) and 600 μL of freshly distilled hydrazine. The reaction mixture was heated for 60 h at 80°C. After cooling to room temperature the reaction mixture was frozen and lyophilized, suspended in water (2 mL), filtered and again frozen and freeze-dried. To the residue was added 50 μL of 1% FDAA solution in acetone and 100 μL of 1 N $NaHCO_3$, followed by heating at 80°C for 3 minutes. After cooling to room temperature the reaction mixture was neutralized with 50 μL of 2 N HCl and diluted with 100 μL of MeCN:H_2O (1:1) containing 0.05% TFA. The resulting solution was analyzed by reverse phase HPLC (COSMOSIL 5C 18-AR, MeCN/H_2O (42:48) + 0.05% TFA).

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