Stereochemistry of Kahalalide F

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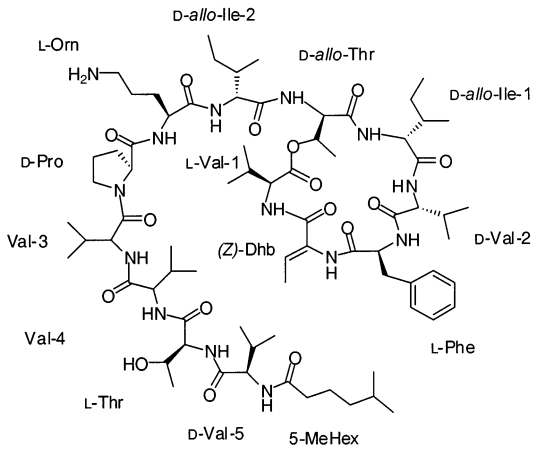
The stereochemistry of the amino acids in the marine-derived cyclic depsipeptide kahalalide F has been defined by a series of degradation reactions (hydrolysis, ozonolysis, Edman degradation, and Marfey derivatization), yielding smaller fragments of the marine natural product. The results from these reactions agree with the structure originally proposed by Hamann and Scheuer and with the same stereochemistry of most of the component amino acids more recently proposed by Goetz, Yoshida, and Scheuer. However, our assignments of D-Val(3) and L-Val(4) are the reverse of previous assignments made as L-Val(3) and D-Val(4). The present (reversed) stereochemistry is crucial for the antitumor activity of kahalalide F.

The kahalalides are depsipeptides isolated by the Scheuer group[1−4] from the sacoglossan mollusk *Elysia rufescens* Pease 1871 (Plakobranchidae) and its green algal diet, a *Bryopsis* sp. (Bryopsidaceae). Seven cyclic depsipeptides, kahalalides A−F and O,[4] and three linear peptides, kahalalides G, H, and J,[3] ranging from a C_{31} tripeptide to a C_{75} tridecapeptide, have been previously described from the mollusk. Kahalalide G, the acyclic analogue of kahalalide F, was found in the diet of the animal.[2] Except for the unusual dehydroaminobutyric acid (Dhb)[5] in kahalalides F and G, all constituent amino acids are of common occurrence. Each peptide also contains an aliphatic acid.

Only kahalalide F exhibits significant bioactivity against human colon and lung cancers and some of pathogenic microorganisms that cause the opportunistic infections of HIV/AIDS. Its mode of action has also been studied,[6] and it is currently in Phase I and II clinical trials in Europe;[7] hence it elicits the most interest chemically. The overall structure was initially assigned by the Scheuer group in 1993,[1] and that structure was subsequently assigned the stereochemistry **1b** by them (see Figure 1).[8]

Since an assured supply of kahalalide F is needed for clinical trials, our group was enlisted to investigate the stereochemistry in view of the conundrum posed by multiple possible stereoisomers in the molecule including three D-Val and two L-Val isomers together with one D-*allo*-Thr isomer and one L-Thr isomer.[8] Only one stereoisomer was found for the isoleucine (two examples), ornithine, proline, and phenylalanine amino acids [D-*allo*-Ile (twice), L-Orn, D-Pro, and L-Phe].[8]

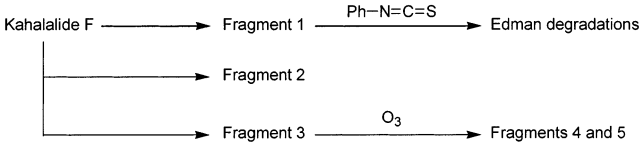
Since the quantities of the natural product available were inadequate for future additional clinical studies, a synthetic route was developed by Albericio, Giralt, et al.,[9] at the University of Barcelona. Their initial synthesis provided stereoisomer **1b**,[9] the Hamann et al. stereoisomer,[8] but it was inactive biologically and differed in its chromatographic and spectroscopic properties. Therefore we elected to reinvestigate the stereochemistry of kahalalide F, and our results have led us to a stereoisomeric structure (**1a**) (see Figure 1), as will be described here. A



1a: Val(3) = D-Val; Val(4) = L-Val
1b: Val(3) = L-Val; Val(4) = D-Val

Figure 1. Possible structures of kahalalide F.

Scheme 1. Summary of Degradation Routes of Kahalalide F



synthetic sample of **1a** prepared by the group of Albericio, Giralt, et al.[9] showed this compound to be fully active biologically as well as identical in physical properties with the natural product.

Results and Discussion

Due to the multiple uncertainties in the stereochemistry of the constituent amino acids of kahalalide F, we elected to assume the overall structure **1b** was correct, or nearly so, and that the discrepancy lay in one or more stereostructures, and we chose to degrade kahalalide F into smaller units, which could be independently examined for their content of D- or L-Val and D-*allo* or L-Thr stereoisomers. The degradation routes are summarized in Scheme 1.

We have compared the products from our degradation scheme with those expected from the structure proposed

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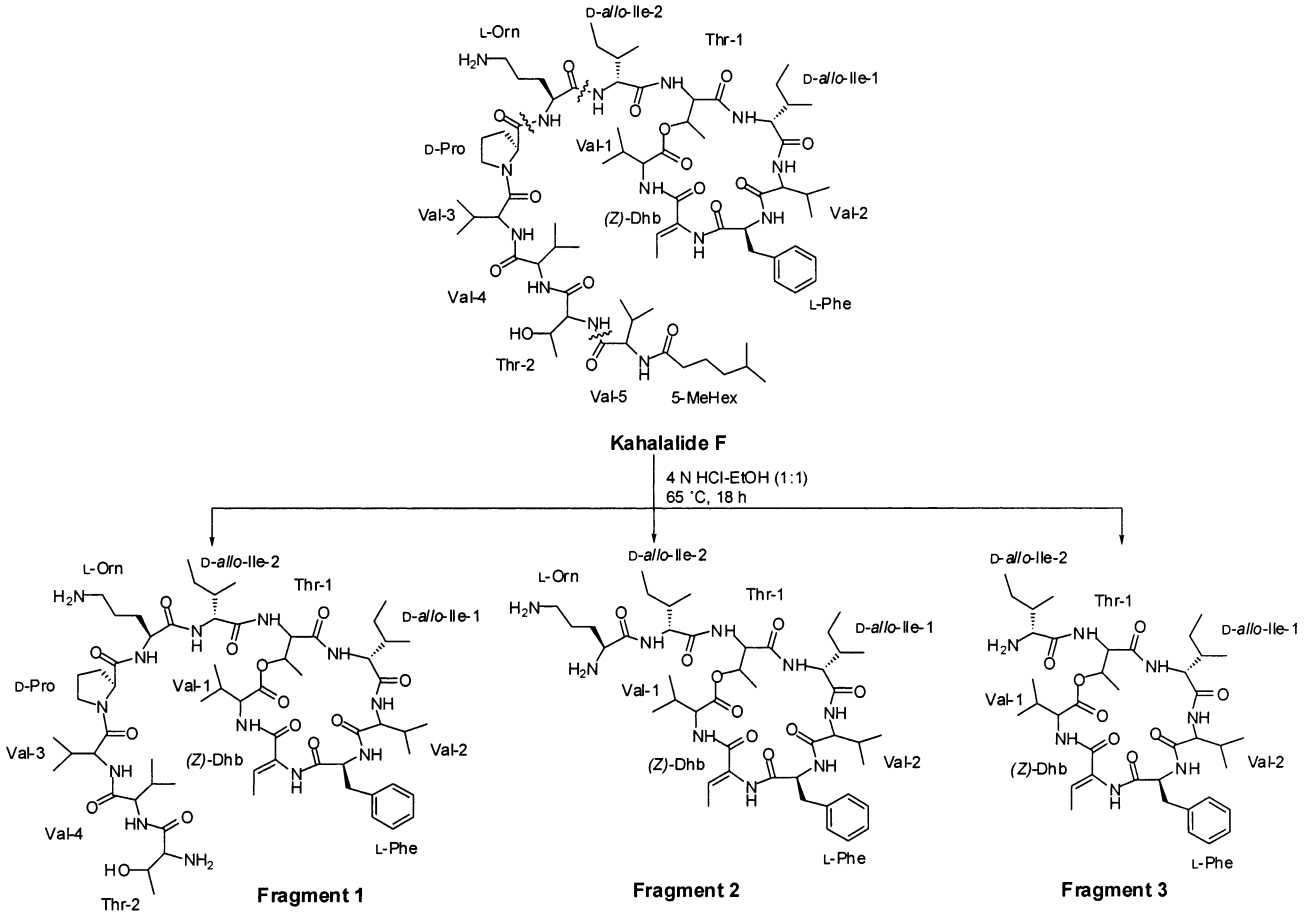
Scheme 2. Mild Acid Hydrolysis of Kahalalide F



by the Scheuer group. In most cases the products agree with those predicted from the Hamann et al. structure (**1b**), as will be seen in the present work, but significant differences occurred at Val(3) and Val(4), with reversal of their earlier proposed stereochemistry and, as noted above, the revised structure **1a** was synthesized by Albericio, Giralt, et al.[9] and proved identical to the natural product.

Our first approach to the structure of kahalalide F involved its partial hydrolysis. Kahalalide F (5 mg) was hydrolyzed with 4 N HCl−EtOH (1:1) at 65 °C for 18 h, and the fragments were separated by HPLC with solvents A (H_2O−0.1% TFA−5% CH_3CN) and B (CH_3CN) and gradients thereof: A 70% to 65% in 10 min, 65% to 40% in 10 min, and linear 40% during 10 min. The separation yielded fragments 1, 2, and 3 (Scheme 2). Fragment 1 was a peptide, kahalalide F − [MeHex-Val(5)] (HRFABMS *m/z* $[M + H]^+$, 1266.7844, calcd for $C_{63}H_{104}N_{13}O_{14}$, found 1266.7826). Fragments 2 and 3 were peptides whose structures are summarized in the formulas shown in Scheme 2 and whose molecular formulas are $C_{44}H_{72}N_9O_9$ ($[M + H]^+$ 870.5) for fragment 2 and $C_{39}H_{62}N_7O_8$ ($[M + H]^+$ 756.4656) for fragment 3, which differs from fragment 2 by the element of ornithine.

Kahalalide F and all of the fragments were analyzed by Edman degradation[10] as well as by Marfey's method[11] on HPLC after total hydrolysis to amino acids. Fragment 1 is kahalalide F less Val(5) and less isoheptanoic acid, as noted in an earlier section. Val(5) must be D-Val since the D-Val peak is relatively more intense than the L-Val peak in the HPLC trace of intact kahalalide F relative to that of fragment 1 (by the approximate ratio of 3:2, see Figures 2a and 2b). Comparisons of retention times showed the amino acids from fragments 2 and 3 to be identical except for Orn, revealing a D-*allo*-Thr, an L-Val, and a D-Val (see Figures 2c and 2d and Table 1). Accordingly, Thr(1) is D-*allo* and Thr(2) must be L, and there are one L-Val and one D-Val in the Val(3)-Val(4) dipeptide cycle.

Ozonolysis of fragment 3, followed by reduction with $NaBH_4$, cleaved fragment 3 at the Dhb residue (HRFABMS *m/z* $[M + H]^+$ of cleaved peptide, 748.4617, calcd for $C_{37}H_{62}N_7O_9$, 748.4609). Then alkaline hydrolysis (1 N NaOH, 1 h at room temperature) gave two fragments: one, fragment 4, containing Val(1) [Val(1)-$COCH_2OH$; FABMS *m/z* $[M + H]^+$, 159.2], in the aqueous layer, and the other, fragment 5, containing Val(2) [NH_2-D-*allo*-Ile(2)-Thr(1)-D-*allo*-Ile(1)-Val(2)-Phe-CO-NH_2; FABMS *m/z* $[M + H]^+$, 591.4] in the organic layer (Scheme 3). Marfey analysis of these fragments, after total hydrolysis of each, showed that Val(1) in fragment 4 is L-Val and Val(2) in fragment 5 is D-Val and Thr(1) is D-*allo*-Thr (see Figures 2e and 2f and Table 2).

Edman degradation[10] of fragment 1 removed Thr(2) after one step and gave a shortened peptide (HRFABMS *m/z* $[M + H]^+$, 1300.7481, calcd for $C_{66}H_{102}N_{13}O_{12}S$, 1300.7492), where one phenyl isothiocyanate molecule had been added to the Orn residue [FABMS/MS: main peak *m/z* 1103.2 (−197.7, −2Val)]. A second step of Edman degradation removed Val(4) [HRFABMS *m/z* $[M + H]^+$, 1201.6789, calcd for $C_{61}H_{93}N_{12}O_{11}S$, 1201.6808 (including one added phenyl isothiocyanate); FABMS/MS: main peak *m/z* 1103.2 (−98.7, −Val)]. HPLC comparisons between the two peptides after one and two steps of Edman degradation by Marfey's method showed a decrease of the L-Val peak (average for three experiments of 1.8-fold reduction of L-Val vs D-Val) (see Figure 3) and allow us to conclude that Val(4) is an L-Val unit.[12]

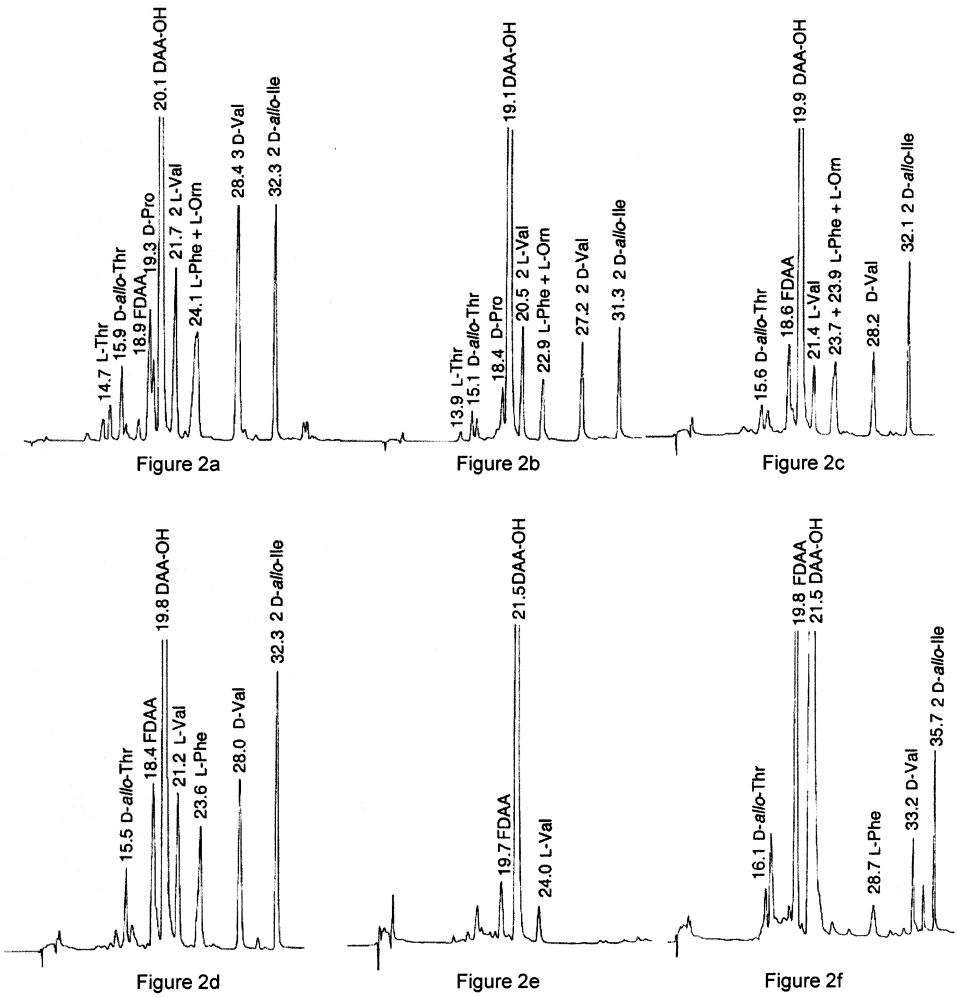


Figure 2. HPLC after total hydrolysis and FDAA derivatization of (a) kahalalide F; (b) fragment 1; (c) fragment 2; (d) fragment 3; (e) fragment 4; and (f) fragment 5 (C_{18} Nova-Pak column, elution with a gradient of 5 mM triethylamine phosphate buffer containing 5% CH_3CN, pH 3.0, and CH_3CN−MeOH, 40:60, UV detection at 340 nm).

Table 1. Results of HPLC Analysis Carried out after Total Hydrolysis of Kahalalide F and Fragments 1−3[a,b]

amino acid	standard	kahalalide F	fragment 1	fragment 2	fragment 3
L-Orn δ-DAA	12.8	12.8			
L-Orn α,δ-DAA	14.0	14.0			
L-Thr	14.7	14.7 (1)	13.9 (1)		
D-*allo*-Thr	15.9	15.9 (1)	15.1 (1)	15.6 (1)	15.5 (1)
D-Pro	19.3	19.3 (1)	18.4 (1)		
DAA-OH	20.1	20.1	19.1	19.9	19.8
L-Val	21.7	21.7 (2)	20.5 (2)	21.4 (1)	21.2 (1)
L-Orn α-DAA	23.9	23.9 (1)	22.9 (1)	23.7 (1)	
L-Phe	24.1	24.1 (1)	22.9 (1)	23.9 (1)	23.6 (1)
D-Val	28.4	28.4 (3)	27.2 (2)	28.2 (1)	28.0 (1)
D-*allo*-Ile	32.3	32.3 (2)	31.3 (2)	32.1 (2)	32.3 (2)

[a] The entries are retention times (min) followed by the number of residues of each amino acid in parentheses. [b] The same conditions of elution were used as in Figure 2.

To confirm this result, we tried to use Marfey's reagent (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, FDAA) for analysis of the chiral identity of Val(4) and Val(3) in the course of Edman degradation. After the first and second cycles of degradation, the shortened peptide was subjected to end-group analysis by reaction with FDAA. The peptide was hydrolyzed and submitted to HPLC analysis in order to determine the chirality of the terminal DAA-Val. Unfortunately, the peak intensities of the Marfey derivatives of the shortened peptides were too weak to perform accurate analyses, although the sensitivities of DAA-amino acids on HPLC were in the nanomole range.

Another Edman experiment involving the modified reagent, naphthyl isothiocyanate (NITC) instead of phenyl isothiocyanate, was tested. Naphthyl thiohydantoin enantiomers derived from D and L amino acids can be separated by HPLC on a chiral column, both on normal and reversed phases.[13] The modified Edman degradation was performed under the same conditions employed previously on peptide − [MeHex-Val(5)-Thr(2)] (obtained after a first step of Edman degradation). The second cycle of degradation with NITC gave naphthyl thiohydantoin-Val, NTHD-Val(4) (HRFABMS *m/z* $[M + H]^+$, 285.1062, calcd for $C_{16}H_{17}N_2OS$, 285.1062), and peptide − [Val(4)] (HRFABMS *m/z* $[M + H]^+$ 1201.6801, calcd for $C_{61}H_{93}N_{12}O_{11}S$, 1201.6808), separated by extractions with water/heptane and water/ethyl acetate. The third cycle of modified Edman degradation gave NTHD-Val(3) and peptide − [Val(3)] (HRFABMS

Scheme 3. Ozonolysis of Fragment 3

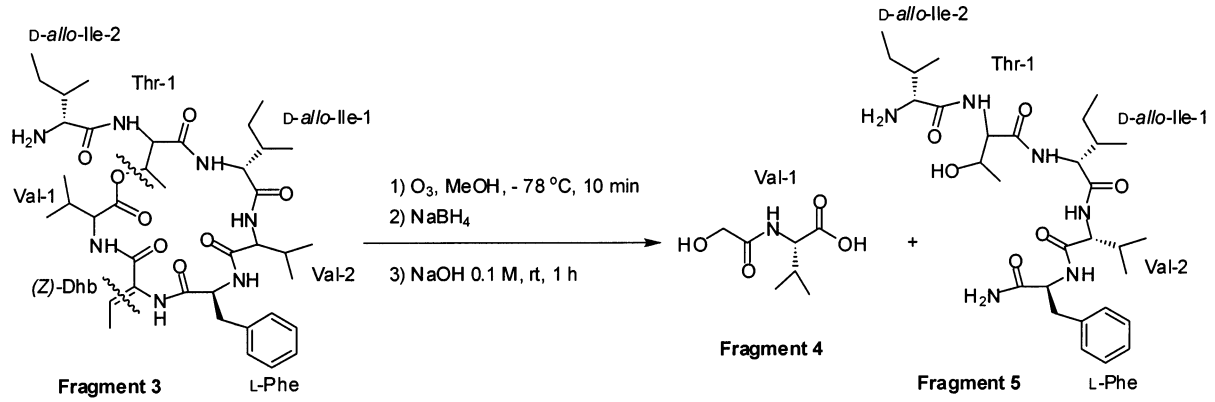


Table 2. Results of HPLC Analysis Carried Out after Total Hydrolysis of Fragments 4 and 5[a,b]

amino acid	standard	fragment 4	fragment 5
L-Orn δ-DAA	12.8		
L-Orn α,δ-DAA	14.0		
L-Thr	14.7		
D-*allo*-Thr	15.9		16.1 (1)
D-Pro	19.3		
DAA-OH	20.1	21.5	21.5
L-Val	21.7	24.0 (1)	
L-Orn α-DAA	23.9		
L-Phe	24.1		28.7 (1)
D-Val	28.4		33.2 (1)
D-*allo*-Ile	32.3		35.7 (2)

[a] The entries are retention times (min) followed by the number of residues of each amino acid in parentheses. [b] The same conditions of elution were used as in Figure 2.

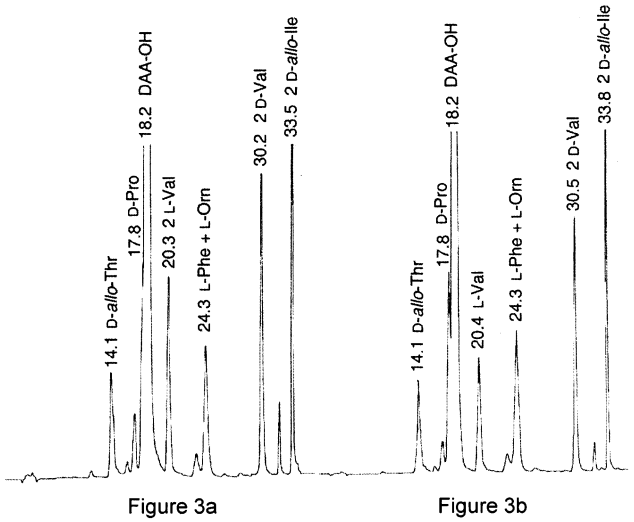


Figure 3. (a) HPLC following the first step of Edman degradation of fragment 1 and after total hydrolysis and FDAA derivatization and (b) HPLC following the second step of Edman degradation of fragment 1 and after total hydrolysis and FDAA derivatization (C_{18} Nova-Pak column, elution with a gradient of 5 mM triethylamine phosphate buffer containing 5% CH_3CN, pH 3.0, and CH_3CN−MeOH, 40:60, UV detection at 340 nm).

m/z $[M + H]^+$, 1102.6112, calcd for $C_{56}H_{34}N_{11}O_{10}S$, 1102.6123).

Analysis and separation on a Co-poly Whelk-O chiral column under reversed-phase conditions (MeOH−*i*-PrOH−H_2O, 10:20:70) showed for the NTHD-Val(4) fraction a peak corresponding to NTHD-L-Val at 7.13 min (standard retention times: 5.15 min for NTHD-D-Val and 7.10 min for NTHD-L-Val) (Figure 4).

Therefore, based on these results, kahalalide F possesses structure **1a**, that is, *cyclo*[L-Val(1)-*Z*-Dhb-L-Phe-D-Val(2)-D-*allo*-Ile(1)-D-*allo*-Thr(1)]-D-*allo*-Ile(2)-L-Orn-D-Pro-D-Val(3)-L-Val(4)-L-Thr(2)-D-Val(5)-5-MeHex.

Experimental Section

General Experimental Procedures. FABMS were run on either a ZAB-SE or a 70-SE4F mass spectrometer. NMR spectra were obtained on a Varian 500NB spectrometer with DMSO-d_6 as solvent. HPLC was carried out on a Phenomenex C_{18} column (3.2 × 250 mm) at 1 mL/min, with UV detection at 225 nm for analytical separation, and on a Nova-Pack C_{18} column (3.9 × 300 mm) at 0.5 mL/min, with UV detection at 340 nm for analysis of FDAA-derivatized amino acids (Marfey analysis).[11] Naphthyl thiohydantoins of amino acids were analyzed on a Co-poly Whelk-O chiral column (2.5 × 250 mm), at 2 mL/min with UV detection at 254 nm. Kahalalide F was provided by PharmaMar, S.A.

Amino Acid Analysis and Molecular Formula. Amino acid hydrolysis of a sample of kahalalide F[11] isolated from extraction of *Elysia rufescens* and purification gave the following amino acid composition: Pro, Orn, Phe, Val (5 mol), Thr (2 mol), *allo*-Ile (2 mol), plus 1 mol each of 5-methylhexanoic acid and dehydrobutyrine (Dhb), identified from comparison of the ^1H NMR spectrum of kahalalide F with those of the microcystins.[4] The stereochemistries of the amino acids were defined as 2 mol of L-Val, 3 mol of D-Val, 1 mol of L-Thr, 1 mol of D-*allo*-Thr, and 2 mol of D-*allo*-Ile by comparison with Marfey derivatives of authentic samples. The stereochemistry of Dhb was assigned as *Z* from its coupling constants. Addition

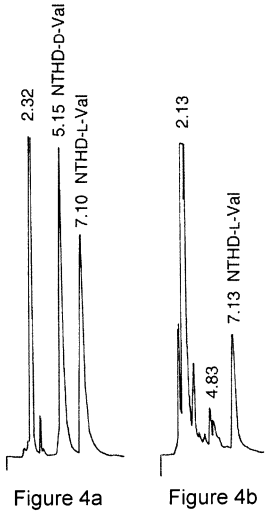


Figure 4. HPLC chromatograms of (a) NTHD-Val authentic racemic and (b) NTHD-Val(4) fraction (chiral column Whelk-O, eluent MeOH−*i*-PrOH−H_2O, 10:20:70, UV detection at 254 nm).

of the unit formula weights of the components translates to *m/z* 1478 $[M + H]^+$, 1500 $[M + Na]^+$, which corresponds to a molecular formula of $C_{75}H_{124}N_{14}O_{16}$ ($[M + H]^+$ 1478).

Hydrolysis of Peptides and Derivatization of Amino Acids. All peptides (parent and fragments, 100 μg each) were hydrolyzed with 6 N HCl at 125 °C for 5 h. The reaction products were treated with a 1% acetone solution (100 μL) of FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, Marfey's reagent)[11] and 1 N $NaHCO_3$ (20 μL) at 40 °C for 1 h. After being cooled to room temperature, the reaction mixtures were neutralized with 2 N HCl (20 μL), diluted with DMSO or methanol (1 mL), and subjected to HPLC analysis. The FDAA derivatives of standard amino acids were prepared by the same procedure. HPLC analysis was carried out with a gradient of solvent A (5% CH_3CN containing 5 mM triethylamine phosphate buffer, pH 3.0) and solvent B (CH_3CN−MeOH, 40:60), eluted as follows: A, 70% to 50% in 6 min, 50% for 14 min, 50% to 5% in 12 min, and 5% for 10 min. The retention times (min) of the 1-FDAA derivatives in the hydrolyzates were as follows: L-Orn δ-DAA (12.8), L-Orn α,δ-DAA (14.0), L-Thr (14.7), D-*allo*-Thr (15.9), D-Pro (19.3), DAA-OH (20.1), L-Val (21.7), L-Orn α-DAA (23.9), L-Phe (24.1), D-Val (28.4), D-*allo*-Ile (32.3) (see also Table 1).

Ozonolysis of Fragment 3. A solution of fragment 3 (0.3 mg) in MeOH (1 mL) was treated with O_3/O_2 at −78 °C for 10 min. The reaction mixture was reduced with $NaBH_4$ (20 μL of a 0.5 M solution in MeOH) and stirred at 0 °C for 10 min and then at room temperature for 20 min. During the reduction, MeOH was removed by N_2 and H_2O (0.5 mL) was added. The reaction mixture was acidified with 1 N HCl and then another aliquot of 0.5 M $NaBH_4$ was added. After being stirred at room temperature for another 20 min, the solution was acidified with 1 N HCl a second time and extracted with ethyl acetate. The organic layer (EtOAc) yielded an ozone-cleaved fragment, HRFABMS *m/z* $[M + H]^+$, 748.4617 (calcd for $C_{37}H_{62}N_7O_5$, 748.4609).

Alkaline hydrolysis of the ozone-cleaved fragment with 1 N NaOH for 1 h at room temperature followed by extraction with ethyl acetate gave two fragments: one with Val(1) [Val(1)-$COCH_2OH$; FABMS *m/z* $[M + H]^+$, 159.2] in the aqueous layer and the other containing Val(2) [NH_2-Ile(2)-Thr(1)-Ile(1)-Val-(2)-Phe-CO-NH_2; FABMS *m/z* $[M + H]^+$, 591.4] in the organic layer.

Edman Degradation[10] of Fragment 1. A mixture (100 μL) of PITC (phenyl isothiocyanate)−H_2O−EtOH−Et_3N (molar ratio 1:1:7:1) was added to fragment 1 (0.8 mg), and the reaction was allowed to stand at 50 °C for 30 min under a nitrogen atmosphere. The solution was evaporated to dryness and treated with TFA (50 μL) at 50 °C for 15 min (under N_2). After evaporation, the residue was taken up in H_2O (100 μL) and extracted with 15:1 heptane−EtOAc and then with EtOAc. The ethyl acetate layer was evaporated to dryness to yield the dethreonyl peptide [HRFABMS *m/z* $[M + H]^+$, 1300.7481, calcd for $C_{66}H_{102}N_{13}O_{12}S$, 1300.7491 (one phenyl isothiocyanate molecule had been added onto the Orn residue); FABMS/MS: main peak *m/z* 1103.2 (−197.7, −2Val)].

A second Edman degradation cycle was performed to remove the Val(4) residue [HRFABMS *m/z* $[M + H]^+$, 1201.6789, calcd for $C_{61}H_{93}N_{12}O_{11}S$, 1201.6807; FABMS/MS: main peak *m/z* 1103.2 (−98.7, −Val)].

Edman Degradation with NITC. Edman degradation with naphthyl isothiocyanate (NITC) was performed on the peptide − [MeHex-Val(5)-Thr(2)] (obtained after a first step of Edman degradation). The peptide (0.2 mg) was dissolved in 40 μL of 1:7:1 H_2O−EtOH−Et_3N, and 5 equiv of NITC in 10 μL of 1:7:1 H_2O−EtOH−Et_3N was added. The reaction was allowed to stand at 50 °C for 1.5 h under nitrogen. The solution was evaporated to dryness and treated with TFA (50 μL) at 50 °C for 15 min (under N_2). After evaporation, the residue was taken up in H_2O (100 μL), and the products were isolated by extractions with heptane, heptane−EtOAc, 15:1, and EtOAc. The ethyl acetate layer yielded the peptide without Val(4) (HRFABMS *m/z* $[M + H]^+$, 1201.6801, calcd for $C_{61}H_{93}N_{12}O_{11}S$, 1201.6808), while the heptane layer afforded the naphthylthiohydantoin-Val, NTHD-Val(4) (HRFABMS *m/z* $[M + H]^+$, 285.1062, calcd for $C_{16}H_{17}N_2OS$, 285.1062), and excess reagent.

A third cycle of Edman degradation with naphthyl isothiocyanate was performed to give NTHD-Val(3) and the peptide without Val(3) (HRFABMS *m/z* $[M + H]^+$, 1102.6112, calcd for $C_{56}H_{54}N_{11}O_{10}S$, 1102.6123).

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