Synthesis and Structure Determination of Kahalalide F[1,2]

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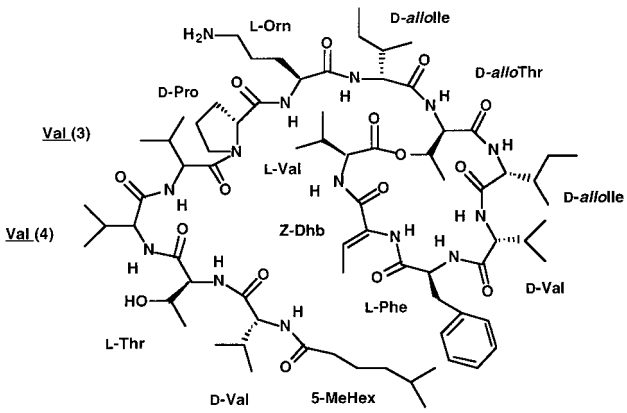
Received July 9, 2001

Abstract: Kahalalide F, the only member of the family of peptides called kahalalides, isolated from the sacoglossan mollusc *Elysia rufescens* and the green alga *Bryopsis sp.*, with important bioactivity, is in clinical trials for treatment of prostate cancer. An efficient solid-phase synthetic approach is reported. Kahalalide F presents several synthetic difficulties: (i) an ester bond between two β-branched and sterically hindered amino acids; (ii) a didehydroamino acid; and (iii) a rather hydrophobic sequence with two fragments containing several β-branched amino acids in a row, one of them terminated with a saturated aliphatic acid. The cornerstones of our strategy were (i) a quasiorthogonal protecting system with allyl, *tert*-butyl, fluorenyl, and trityl-based groups, (ii) azabenzotriazole coupling reagents, (iii) formation of the didehydroamino acid residue on the solid phase, and (iv) cyclization and final purification in solution. HPLC, high-field NMR, and biological activity studies showed that the correct stereochemistry of the natural product is that proposed by Rinehart et al. whereas the stereochemistry proposed by Scheuer et al. is that of a biologically less active diastereoisomer.

Introduction

A family of peptides called kahalalides[3−6] has been isolated from the sacoglossan mollusc *Elysia rufescens* and the green alga *Bryopsis sp.* on which it feeds. Two of them, kahalalide F and its acyclic analogue kahalalide G, incorporate the rare *Z*-didehydroaminobutyric acid (Dhb) moiety. Each peptide also has a saturated aliphatic acid at the *N*-terminus. Among these peptides, the largest, kahalalide F, is a cyclic depsipeptide that contains 13 amino acids and 5-methylhexanoic acid at the *N*-terminus, which recently exhibited very interesting antitumor activity,[1,7] and is in clinical trials for treatment of prostate cancer.[8]

When this work was started, the absolute stereochemistry of kahalalide F was unknown.[1] Further investigations carried out independently by Rinehart et al.[9] (**1a**) and Scheuer et al.[10] (**1b**) allowed them to propose the stereochemistry of kahalalide F. However, these results were not identical, as they differed in the stereochemistry of Val-3 and Val-4.



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

Results and Discussion

Although due to its complexity kahalalide F represents a real synthetic challenge, the development of an efficient synthetic approach should allow the doubt over the stereochemistry to be settled as well as making possible the synthesis of large amounts of the compound and its analogues for further pharmacological investigations. Besides being a cyclic compound, kahalalide F presents several synthetic difficulties:

(1) Abbreviations used for amino acids and the designations of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in: *J. Biol. Chem.* **1972**, *247*, 977−983. The following additional abbreviations are used: ACH, α-cyano-4-hydroxycinnamic acid; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; *t*-Bu, *tert*-butyl; Cl-TrtCl-resin, 2-chlorotrityl chloride-resin; DHB, 2,5-dihydroxybenzoic acid; *Z*-Dhb, α,β-didehydro-α-aminobutyric acid; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformanide; EDC, 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride; ESMS, electrospray mass spectrometry; FABMS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *N*-[(dimethylamino)-1*H*-1,2,3-triazolo-[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU, *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HOAt, 1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine); HOBt, 1-hydroxybenzotriazole; 5-MeHex-OH, 5-methylhexanoic acid; MeOH, methanol; NMM, *N*-methylmorpholine; PyAOP, 7-azabenzotriazol-1-yl-*N*-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; PyBOP, benzotriazol-1-yl-*N*-oxy-tris-(pyrrolidino)phosphonium hexafluorophosphate; SPS, solid-phase synthesis; TFA, trifluoroacetic acid; amino acid symbols denote the L-configuration unless stated otherwise. All solvent ratios are volume/volume unless stated otherwise.

(2) Taken in part from the Ph.D. Thesis of Àngel López-Macià, University of Barcelona, Spain, December 2000.

(3) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.

(4) Hamann, M. T.; Otto, C. S.; Scheuer, P. J.; Dunbar, D. C. *J. Org. Chem.* **1996**, *61*, 6594−6600. Hamann, M. T.; Otto, C. S.; Scheuer, P. J.; Dunbar, D. C. *J. Org. Chem.* **1998**, *63*, 4856.

(5) Goetz, G.; Nakao, Y.; Scheuer, P. J. *J. Nat. Prod.* **1997**, *60*, 562−567.

(6) Horgen, F. D.; Delossantos, D. B.; Goetz, G.; Sakamoto, B.; Kan, Y.; Nagai, H.; Scheuer, P. J. *J. Nat. Prod.* **2000**, *63*, 152−154.

(7) García-Rocha, M.; Bonay, P.; Avila, J. *Cancer Lett.* **1996**, *99*, 43−50.

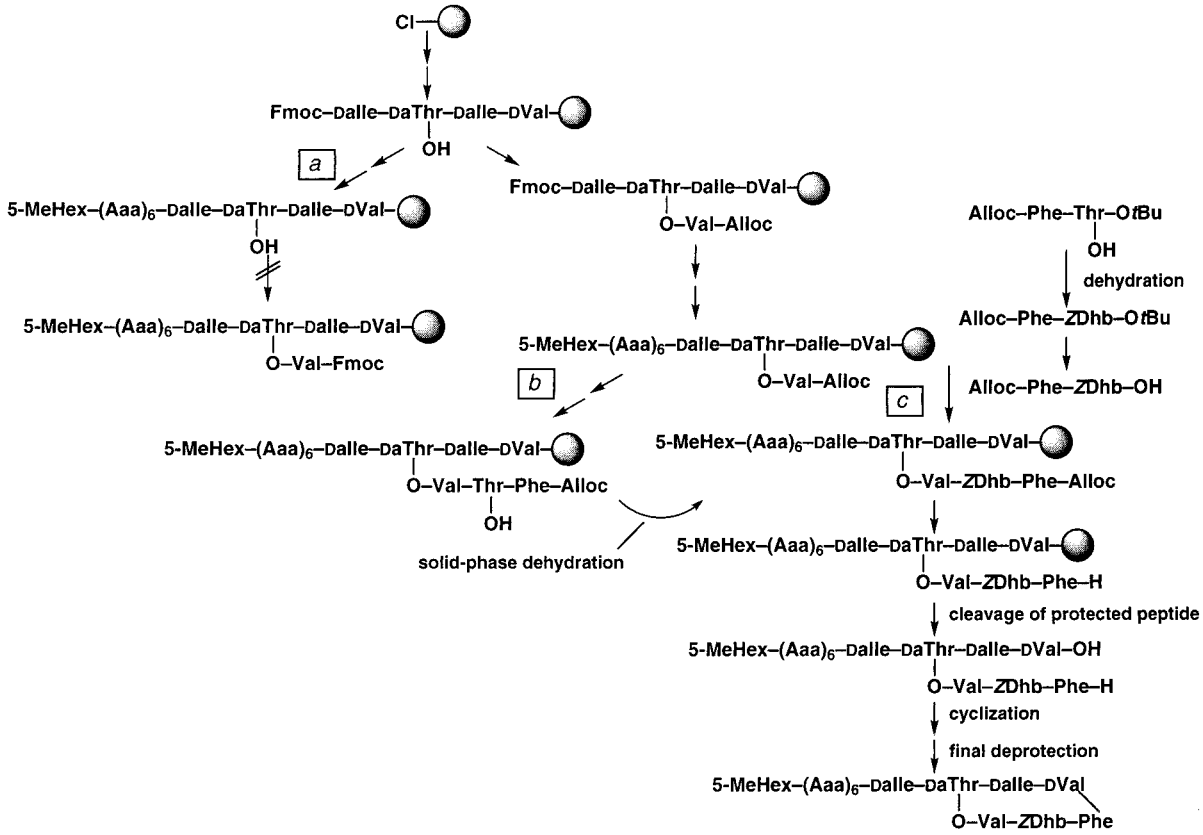
(8) Faircloth, G. Personal communication.

(9) Bonnard, I.; Manzanares, I.; Rinehart, K. L. *J. Nat. Prod.* In press.

(10) Goetz, G.; Yoshida, W. Y.; Scheuer, P. J. *Tetrahedron* **1999**, *55*, 7739−7746.

10.1021/ja0116728 CCC: $20.00
Published on Web 10/27/2001

Scheme 1. Solid-Phase Strategies Investigated for the Preparation of Kahalalide F[a]



[a] Cl attached to the shaded circle represents Cl-TrtCl-resin, 2-chlorotrityl chloride-resin.

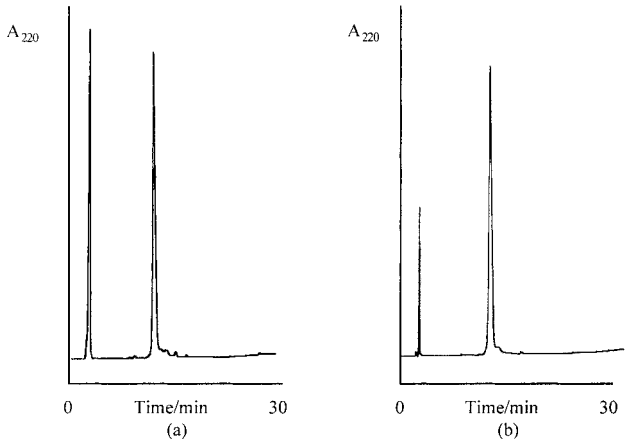


Figure 1. HPLC chromatograms of peptides **1a** (a) and **1b** (b). Reverse-phase C-18 columns were used for the analysis with elution by a linear gradient over 30 min of 0.036% TFA in ACN and 0.045% TFA in H_2O from 45:55 to 90:10, flow rate 1.0 mL min^{-1}.

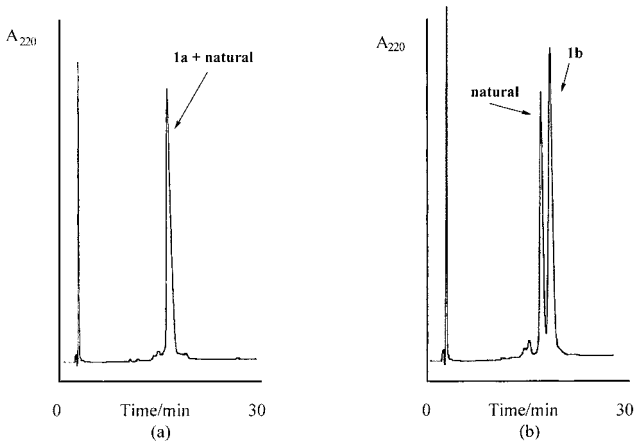


Figure 2. HPLC chromatograms of co-injection of peptide **1a** and natural kahalalide F (a) and co-injection of peptide **1b** and natural kahalalide F (b). Reverse-phase C-18 columns were used for the analysis with elution by a linear gradient over 30 min of 0.036% TFA in ACN and 0.045% TFA in H_2O from 45:55 to 6:4, flow rate 1.0 mL min^{-1}.

(i) an ester bond between two β-branched and sterically hindered amino acids (Val and D-*allo*Thr); (ii) the didehydroamino acid (*Z*-Dhb); and (iii) a rather hydrophobic sequence with two fragments containing several β-branched amino acids in a row, one of them terminated with the saturated aliphatic acid. Taking these difficulties into account, three different strategies (Scheme 1, a−c) based on the solid-phase approach[11] were studied for the synthesis of **1a** and **1b**, the two proposed structures of kahalalide F, to establish unequivocally the correct stereochemistry of the natural product.

All of these strategies involve the elongation of the synthetic chain on the solid phase, cleavage of the protected peptide from the resin, subsequent cyclization, and final deprotection in solution. Although the macrocyclization can be carried out in any position except that between Phe and *Z*-Dhb because the formation of the double bond is carried out after the amide between these two residues is formed,[12] doing so between Val and Phe looked to be the most favorable option, since the other

(11) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997.

(12) Attempts to perfom the cyclization step before the formation of the *Z*-Dhb failed, presumably because *Z*-Dhb facilitates a favorable conformation for the reaction.

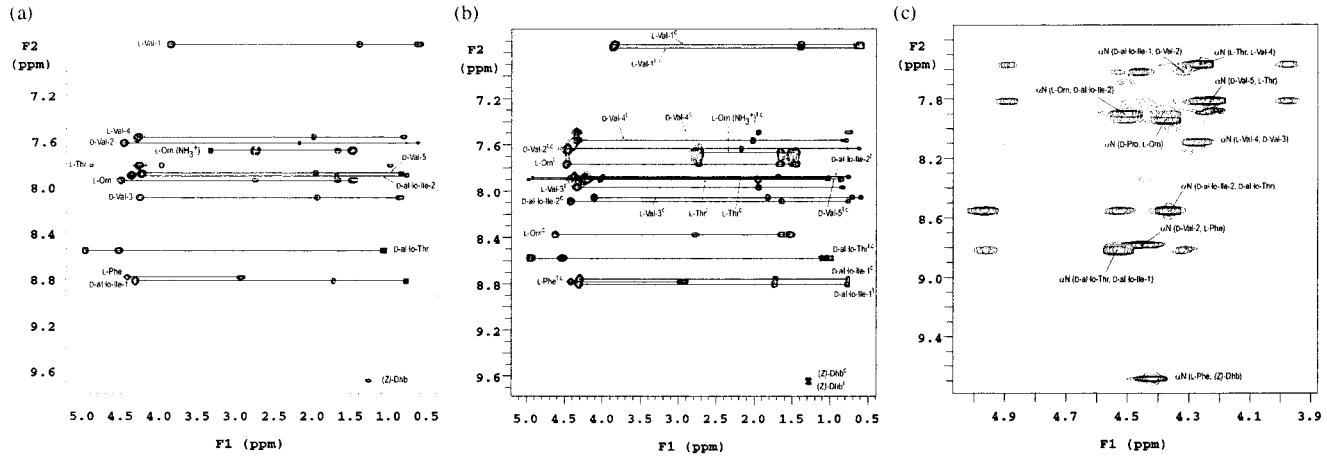


Figure 3. Expansion of the aromatic and amide/aliphatic regio of (a) the TOCSY (500 MHz, 72 ms) spectrum of **1a**, (b) the TOCSY (500 MHz, 72 ms) spectrum of **1b**, and (c) the ROESY (500 MHz, 200 ms) spectrum of **1a**. All the spectra were registered in DMSO-d_6 at 298 K.

positions involve two β-branched amino acids or the poorly reactive carboxyl or amino function of *Z*-Dhb.

The linear sequence was synthesized on a 2-chlorotrityl chloride-resin (ClTrt-Cl-resin),[13] which allowed the cleavage of the peptide under very mild acid conditions and in the presence of other acid-labile protecting groups. The limited incorporation of Fmoc-D-Val-OH (0.2 equiv) was performed in the presence of DIEA (2 equiv).[14,15] The elongation of the peptide chain was carried out by using the Fmoc/*t*Bu strategy. The D-*allo*Thr and the Thr precursor of the *Z*-Dhb were both introduced without protection of the hydroxyl function. HATU-DIEA[16] was used for all the amide formations (in all cases single coupling for 90 min with 5 equiv of Fmoc-amino acid and HATU and 10 equiv of base in DMF gave a ninhydrin negative test).

In strategy ***a***, the incorporation of Fmoc-Val-OH did not take place in good yield (30% with DIPCDI-DMAP), presumably due to the hydrophobicity of the peptide chain, which favors interchain aggregation. Thus, protected Val should be incorporated in a previous step (strategies ***b*** and ***c***), requiring the use of a protecting group for the α-amino function, such as the Alloc group, which is orthogonal to the Fmoc and the acid-labile protecting groups.[17] Removal of the Alloc group was carried out with $Pd(PPh_3)_4$ (0.1 equiv) in the presence of $PhSiH_3$ (10 equiv) under an atmosphere of Ar.[18] The double bond of the didehydroamino acid was formed on the solid phase through a β-elimination reaction (strategy ***b***) with use of a method developed recently in our laboratory,[19] with the modification described by Fukase et al.[20] that uses EDC (100 equiv) as the activating reagent of the hydroxyl function in the presence of CuCl (60 equiv) in DMF−CH_2Cl_2 (5:1) for 6 days. This reaction led, as demonstrated in a model peptide,[17] exclusively to the *Z* isomer that is thermodynamically more stable. Alternatively in strategy ***c***, the dipeptide Alloc-Phe-(Z)-Dhb-OH (5 equiv), which was prepared in solution from Alloc-Phe-OH and H-Thr-O*t*Bu with EDC and subsequent dehydration with EDC (6.5 equiv) in the presence of CuCl (2.7 equiv) and treatment with TFA, was coupled with HATU-DIEA (5:10) for 16 h with a further recoupling for 3 h.[21]

Before the cleavage of the protected peptide from the resin [TFA−CH_2Cl_2 (1:99), 5 × 0.5 min, 65−87% yield)], the Alloc group was removed as described above.[22] The cyclization step was performed with PyBOP−DIEA (3:6 equiv) in DMF for 1 h.[23] Final deprotection was carried out with TFA−H_2O (95:5) for 1 h.

Although strategies ***b*** and ***c*** both led to the correct product in the case of both stereoisomers of kahalalide F, the HPLC quality of the crude products obtained by strategy ***b*** was in both cases clearly superior. Crude **1a** and **1b** were purified by medium-pressure chromatography[24] to give the title products [10−14% overall yield (synthesis and purification)], which both showed high purity by HPLC (Figure 1) and a correct MALDI-TOF-MS.

Product **1a** coeluted by HPLC with an authentic sample of kahalalide F (Figure 2a), while **1b** showed a longer retention time than the natural compound (Figure 2b).

Furthermore, the ^{1}H NMR spectra [(500 MHz, DMSO-d_6) 1H, TOCSY (72 ms), ROESY (200 ms)] of **1a** were identical with those of the natural peptide, while the ^{1}H NMR spectra for **1b** showed a chemical shift pattern different from that of natural kahalalide F as well as the presence of two conformations due to a cis−trans equilibrium around the L-Val-D-Pro peptide bond, an equilibrium that was not observed in the natural product

(13) Barlos, K.; Gatos, D.; Schäfer, W. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 590−593.

(14) The use of ClTrt-Cl-resins of high loading (>0.5 mmol/g) for the preparation of hydrophobic peptides such as kahalalide F usually leads to impure peptide. Chiva, C.; Vilaseca, M.; Giralt, E.; Albericio, F. *J. Pept. Sci.* **1999**, *5*, 131−140.

(15) Unreacted reactive sites were capped with MeOH-DIEA.

(16) Carpino, L. A.; El-Faham, A.; Minor, C. A.; Albericio, F. *J. Chem. Soc., Chem. Commun.* **1994**, 201−203.

(17) The incorporation of Alloc-Val-OH (7 equiv) was carried with an equimolar amount of DIPCDI and 0.7 equiv of DMAP for 2 h. This protocol was repeated twice and the yield by amino acid analysis was quantitative. Other methods based in the use of Fmoc-Val-Cl in the presence of base gave less satisfactory results.

(18) Thieriet, N.; Alsina, J.; Giralt, E.; Guibé, F.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 7275−7278.

(19) Royo, M.; Jiménez, J. C.; López-Macià, A.; Giralt, E.; Albericio, F. *Eur. J. Org. Chem.* **2001**, 45−48.

(20) Fukase, K.; Kitazawa, M.; Sano, A.; Shimbo, K.; Horimoto, S.; Fujita, H.; Kubo, A.; Wakamiya, T.; Shibe, A. *Bull. Chem. Soc. Jpn.* **1992**, *65*, 2227−2240.

(21) Other coupling reagents based on HOBt, such as HBTU or DIPCDI-HOBt, led to incomplete incorporations of the dipeptide.

(22) The α-amino function of the Phe also can be protected with Fmoc, because this protecting group is totally stable to the dehydration reaction conditions.

(23) Kates, S. A.; Solé, N. A.; Johnson, C. R.; Hudson, D.; Barany, G.; Albericio, F. *Tetrahedron Lett.* **1993**, *34*, 1549−1552.

(24) Vydac C_{18} (15−20 μm, 300 Å, 240 × 24 mm), linear gradient from 20 to 60% of acetonitrile (+0.05% TFA) in water (+0.05% TFA) in 5 h (300 mL each solvent), 120 mL/h, detection at 220 nm.

(Tables 1−3, Supporting Information).[25] Finally, while the biological activity of **1a** was in the same range as that of natural kahalalide F, **1b** was more than 10 times less active.[26] In conclusion, this first synthesis of kahalalide F and a diastereomer unequivocally established the stereochemistry of the natural compound to be that proposed by Rinehart et al.[6] whereas the stereochemistry proposed by Goetz et al.[7] corresponds to a biologically less active diastereomer. The synthetic method reported herein may find general application for the preparation of other peptides containing didehydroamino acids. All reactions except cyclization and the final deprotection take place on the solid phase.

(25) The ^{1}H NMR chemical shifts in DMF-d_7 containing 1 equiv of TFA of natural kahalalide F were slightly different from those shown in Table 1 of the Supporting Information. However, when natural kahalalide F was treated in a manner similar to the synthetic kahalalides (solution in TFA, evaporation under reduced pressure, and lyophilization), all chemical shifts were identical with those for synthetic kahalalide F (**1a**).

(26) The biological test was performed in Pharma Mar s.a.

Acknowledgment. We thank Dr. I. Manzanares (Pharma Mar, s.a) for his helpful discussions. This work was partially supported by Pharma Mar, s.a., CICYT (BIO1099-0484 and BQU2000-0235), and Generalitat de Catalunya [Grup Consolidat (1999SGR 00042) and Centre de Referència en Biotecnologia].

Supporting Information Available: Experimental data corresponding to the synthesis of kahalalide F (**1a**) and **1b** via both dehydration on solid-phase and incorporation of the dehydrated dipeptide; Tables 1−3 giving ^{1}H NMR (500 MHz, DMSO-d_6) data for kahalalide F (**1a**) and **1b** (both isomers); Figures 4−12 showing ^{1}H NMR spectra (500 MHz) (5 mM), TOCSY (72 ms), and ROESY (200 ms) for kahalalide F (**1a**), **1b**, and natural kahalalide in DMSO-d_6 at 298 K (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA0116728