JOC*Article*

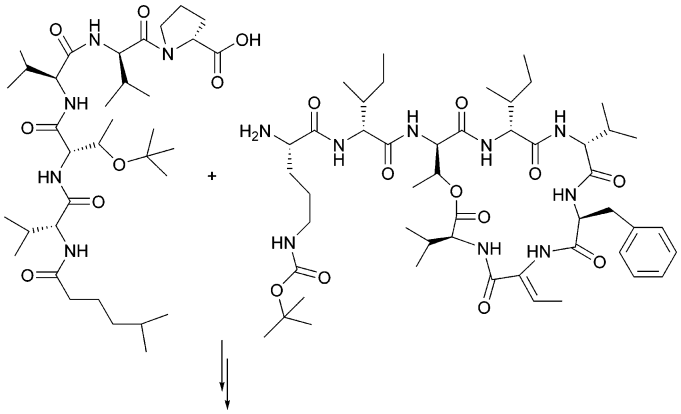
Convergent Approaches for the Synthesis of the Antitumoral Peptide, Kahalalide F. Study of Orthogonal Protecting Groups

Carolina Gracia,[†,||] Albert Isidro-Llobet,[†,||] Luis J. Cruz,[†] Gerardo A. Acosta,[†] Mercedes Álvarez,[†,‡] Carmen Cuevas,[§] Ernest Giralt,[†,⊥] and Fernando Albericio*[,†,⊥]

Institute for Research in Biomedicine, Barcelona Science Park, University of Barcelona, 08028-Barcelona, Spain, Laboratory of Organic Chemistry, Faculty of Pharmacy, University of Barcelona, 08028-Barcelona, Spain, PharmaMar S.A.U., 28770-Colmenar Viejo (Madrid), Spain, and Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1, 08028-Barcelona, Spain

albericio@pcb.ub.es

Received May 10, 2006



Kahalalide compounds are peptides that are isolated from a Hawaiian herbivorous marine species of mollusc, *Elysia rufescens*, and its diet, the green alga *Bryopsis* sp. Kahalalide F and its synthetic analogues are the most promising compounds of the Kahalalide family because they show antitumoral activity. Linear solid-phase syntheses of Kahalalide F have been reported. Here we describe several new improved synthetic routes based on convergent approaches with distinct orthogonal protection schemes for the preparation of Kahaladide analogues. These strategies allow a better control and characterization of the intermediates because more reactions are performed in solution.

Introduction

Kahalalide compounds are peptides that are isolated from a Hawaiian herbivorous marine species of mollusc, *Elysia rufescens*, and its diet, the green alga *Bryopsis* sp. Kahalalide F[1] and analogues are the most promising compounds of the Kahalalide family because of their antitumoral activities.[2] Kahalalide F alters the function of the lysosomal membrane, a characteristic that distinguishes it from all other known antitumor agents. This compound also inhibits TGF-α expression, blocks intracellular signaling pathways downstream of the EGF and ErbB2 receptor family, and induces non-p53-mediated apoptosis.[3] Recent studies demonstrate that Kahalalide F induces cell necrosis in vivo (oncosis) and shows selectivity for tumor cells compared with healthy cells in vitro.[4] Its activity is independent of multidrug resistance (MDR) expression.[5]

Kahalalide F is currently undergoing Phase II clinical trials in various solid tumors: melanoma, non-small-cell lung cancer, and hepatocellular carcinoma. A Phase II trial for the treatment of patients with severe psoriasis is also ongoing.[6]

[†] Institute for Research in Biomedicine, University of Barcelona.
[‡] Faculty of Pharmacy, University of Barcelona.
[§] PharmaMar S.A.U.
[⊥] Department of Organic Chemistry, University of Barcelona.
[||] Contributed equally to this study.

(1) Hamann, M. T.; Scheuer, P. J. *J. Am. Chem. Soc.* **1993**, *115*, 5825−5826.
(2) Hamann, M. T. *Curr. Opin. Mol. Ther.* **2004**, *6*, 657−665.
(3) Janmaat, M. L.; Rodriguez, J. A.; Jimeno, J.; Kruyt, F. A. E. *Mol. Pharmacol.* **2005**, *68*, 502−510.

10.1021/jo060976f CCC: $33.50
Published on Web 08/24/2006

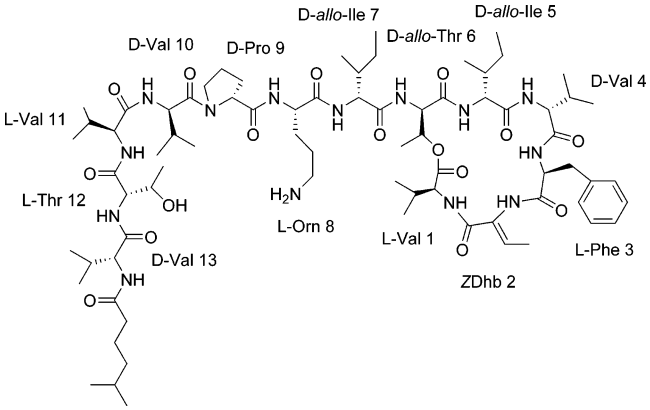


FIGURE 1. Structure of Kahalalide F.

Members of the Kahalalide F family, which are head-to-side chain cyclodepsipeptides, have a complex structure, comprising six amino acids, among these (*Z*)-didehydro-α-aminobutyric acid (*Z*Dhb) (formed from dehydratation of a Thr residue), as a cyclic part between the carboxylic acid of a L-Val 1 and the secondary alcohol of the D-*allo*-Thr 6, and an exocyclic chain of seven amino acids with a terminal aliphatic/fatty acid group (R) (Figure 1).[1,7−9] Compounds in which R is a methylhexanoic acid are of greatest interest.[10]

Linear solid-phase syntheses of Kahalalides involving cyclization and final deprotection performed in solution have been described.[8,11] However, synthetic routes for Kahalalide compounds are still required. Here we report several new convergent strategies for the synthesis of Kahalalide F derivatives. Convergent strategies are defined as those in which peptide fragments are coupled to give the desired target molecule.[12−15] The condensation of peptide fragments should lead to fewer problems in the isolation and purification of intermediates. The difference between the condensation product desired and the segments themselves, in terms of molecular size and chemical nature, should be sufficiently pronounced so as to permit their separation relatively easily. Several orthogonal protecting schemes have been used in these strategies.[16]

SCHEME 1. Convergent Strategy for the Synthesis of Kahalalide F Analogues; AAA, BBB, CCC, DDD, and EEE Are Amino Acid Residues

Although solid-phase strategies are crucial for peptide synthesis,[12b,17−20] the method of choice for a convergent strategy involves a combination of solid-phase and solution chemistries. Thus, protected peptides are prepared in solid phase and then combined in solution.[21,22a] Paradigm of this strategy is the large-scale commercial production of enfuvitide (T-20 or Fuzeon), a 36-amino acid peptide.[23]

Results and Discussion

To render Kahalalide F, a 14-building-block peptide [13 amino acids and the methylhexanoic acid], the most convenient synthetic strategy involves dividing the peptide in two fragments, the *N*-terminal and the fragment containing the cycle Scheme 1). Fragment composition can be modified in order to optimize condensation yields and minimize side reactions, such as epimerization.

The preparation of the *N*-Component requires cyclization in solution and therefore the *N*-protection should also be kept during the cleavage of the lineal fragment from the resin.

Solid-phase synthesis of protected peptides requires a resin that facilitates the release of the peptide without removing the side-chain protecting groups, which are removed as the last step of the synthesis.[12a,13,14] In this regard, the *t*Bu-based protecting groups are highly convenient because they are removed with high concentrations of trifluoroacetic acid (TFA) in the presence of scavengers and are stable to piperidine, used to remove the α-amino protecting group, and to low concentrations of TFA. The super-acid-labile chlorotrityl chloride polystyrene (CTC, Barlos, Cl-●) resin allows the release of peptides with 1−2% of TFA in CH_2Cl_2 or even with trifluoroethanol or hexafluoroacetone solutions.[24] An additional advantage of the CTC resin is that its hindered structure minimizes the formation of the diketopiperazines (DKP) during removal of the temporal protecting group of the second amino acid.[25,26]

General Strategies. Three sets of fragments were prepared. Schemes 2 and 3 show the *C*-Components and their complementary *N*-Components synthesized.

(4) Suarez, Y.; Gonzalez, L.; Cuadrado, A.; Berciano, M.; Lafarga, M.; Muñoz, A. *Mol. Cancer Ther.* **2003**, *2*, 863−872.

(5) Jimeno, J.; Faircloth, G.; Fernandez Souse-Faro, J. M.; Scheuer, P.; Rinehart, K. *Mar. Drugs* **2004**, *2*, 14−29.

(6) Izquierdo Delso, M. A. PCT Int. Appl. (2004) WO 2004075910, A1 20040910, CAN 141:236712, AN 2004:740175.

(7) Goetz, G.; Yoshida, W. Y.; Scheuer, P. J. *Tetrahedron* **1999**, *55*, 7739−7746 and 11957.

(8) Lopez-Macia, A.; Jimenez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, 11398−11401.

(9) Bonnard, I.; Manzanares, I.; Rinehart, K. L. *J. Nat. Prod.* **2003**, *66*, 1466−1470.

(10) Albericio, F.; Fernandez, A.; Giralt, E.; Gracia, C.; Lopez, P.; Varon, S.; Cuevas, C.; Lopez-Macia, A.; Francesch, A.; Jimenez, J. C.; Royo, M. PCT Int. Appl. (2005) WO 2005023846, A1 20050317, CAN 142:298335, AN 2005:239012.

(11) Lopez, P. E.; Isidro-Llobet, A.; Gracia, C.; Cruz, L. J.; Garcia-Granados, A.; Parra, A.; Alvarez, M.; Albericio, F. *Tetrahedron Lett.* **2005**, *46*, 7737−7741.

(12) (a) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49*, 11065−11133. (b) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997.

(13) Benz, H. *Synthesis* **1994**, *4*, 337−358.

(14) (a) Barlos, K.; Gatos, D. *Biopolymers* **1999**, *51*, 266−278. (b) Barlos, K.; Gatos, D. In *Fmoc Solid-Phase Peptide Synthesis*; Chan, W. C., White, P. D., Eds.; Oxford University Press: Oxford, UK, 2000; pp 215−228.

(15) Sakakibara, S. *Biopolymers* **1999**, *51*, 279−296.

(16) An orthogonal protecting scheme is defined as one based on completely different classes of protecting groups such that each class of groups can be removed in any order and in the presence of all other classes of protecting groups. Barany, G.; Albericio, F. *J. Am. Chem. Soc*. **1985**, *107*, 4936−4942.

(17) Fields, G. B. *Methods in Enzymology, Solid-Phase Peptide Synthesis*; Academic Press: Orlando, Florida, 1997; Vol. 289.

(18) Goodman, M.; Felix, A.; Moroder, L.; Toniolo, C. *Houben-Weyl. Methods of Organic Chemistry. Synthesis of Peptides and Peptidomimetics*; Georg Thieme Verlag: Stuttgart, Germany, 2001; Vol. E 22.

(19) Bruckdorfer, T.; Marder, O.; Albericio, F. *Curr. Pharm. Biotechnol.* **2004**, *5*, 29−43.

(20) Albericio, F. *Curr. Opin. Chem. Biol.* **2004**, *8*, 211−221.

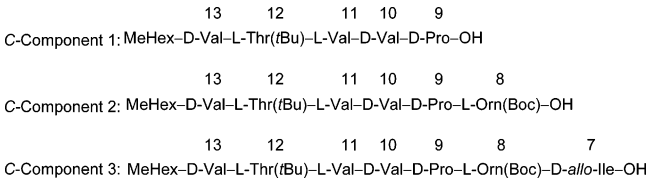
(21) Nisiuchi, Y.; Inui, T.; Nishio, H.; Bodi, J.; Kimura, T.; Tsuji, F. T.; Sakakibara, S. P *Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13549−13554.

(22) Chiva, C.; Barthe, P.; Codina, A.; Gairi, M.; Molina, F.; Granier, C.; Pugniere, M.; Inui, T.; Nishio, H.; Nishiuchi, Y.; Kimura, T.i; Sakakibara, S.; Albericio, F.; Giralt, E. *J. Am. Chem. Soc*. **2003**, *125*, 1508−1517.

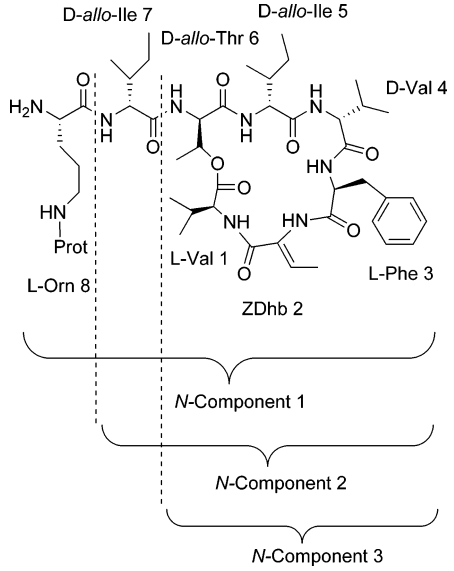
(23) Bray, Brian L. *Natl. Rev. Drug Discovery* **2003**, *2*, 587−593.

(24) (a) Barlos, K.; Gatos, D.; Schäfer, W. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 590−593. (b) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513−520.

SCHEME 2. *C*-Components Synthesized



SCHEME 3. *N*-Components Synthesized



A priori, the main advantage of strategy 1 is that the hardly racemizable Pro is the *C*-terminal amino acid for the *C*-Component. In contrast, the advantage of strategies 2 and 3 is that the *N*-Component does not contain any side-chain protecting group and therefore a level of protecting groups can be avoided for these fragments.

All fragments were synthesized on the CTC resin, starting with a limited incorporation of the first 9-fluorenylmethoxycarbonyl (Fmoc)-amino acid in the presence of *N*,*N*-diisopropylethylamine (DIEA).[27] The remaining resin chloride functions were capped with methanol (MeOH) to prevent the formation of tertiary amines after treatment with the piperidine used to remove the Fmoc group.[28] The Fmoc-protecting group was removed with piperidine−*N*,*N*-dimethylformamide (DMF) (1:4) and the peptide chain was elongated with *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and 1-hydroxybenzotriazole (HOBt) as coupling reagents. Finally, the peptides were cleaved from the resin with TFA−CH_2Cl_2 (1:99), characterized by HPLC and ESMS, and purified by semipreparative HPLC when necessary.

Synthesis of the *N*-Components. The *Z*Dhb moiety was incorporated through the dipeptide allyloxycarbonyl (Alloc)-Phe-*Z*Dhb-OH, wich is much faster than incorporating sequentially Thr and Phe and performing the dehydratation of Thr to ZDhb on solid phase.[8,11]

Four synthetic strategies were used to prepare the three distinct *N*-Components (Scheme 4). All strategies are based on the solid-phase synthesis of a branched peptide using a tri- or tetra-orthogonal protecting scheme and subsequent cyclization and deprotection of the *N*-terminal function in solution. Strategy 1, which produces the *N*-Component 1, follows the procedure previously described[8,11] and starts with the incorporation of Fmoc-D-Val-OH on the CTC resin, followed by elongation until the D-*allo*-Ile,[7] esterification of the β-hydroxyl of the D-*allo*-Thr with Alloc-Val-OH. The incorporation of protected Orn-(Boc) residue on the main chain is then followed by the incorporation of the Alloc-Phe-*Z*Dhb-OH on the branched one. Strategy 3, which leads to the *N*-Component 2, is similar to 1, but elongation is stopped after incorporation of D-*allo*-Ile.[7] In Strategies 2 and 4, which yield *N*-Component 1 and *N*-Component 3, respectively, synthesis starts with the incorporation of the Alloc-Phe-*Z*Dhb-OH onto the CTC resin (see below for the discussion of the strategy).

This study addressed (i) the most convenient strategy in terms of coupling yield and racemization of the *C*-terminal amino acid of the *C*-Component (Strategies 1 or 2 vs 3 and 4); (ii) for Strategies 1 and 2 (*N*-Component 1), the best semi-permanent protecting group [Fmoc, *p*-nitrobenzyloxycarbonyl (*p*NZ)[11,29,30] in Strategy 1 and Alloc in Strategy 2][31] for the N^{α}-amino of Orn, taking into account that the *tert*-butoxycarbonyl (Boc) group is reserved as permanent protecting group for the N^{ϵ}-amino of the same Orn; and (iii) whether the dipeptide Alloc-Phe-*Z*Dhb-OH is a good starting point for the elongation of the peptidic chain (Strategies 2 and 4) when compared with the traditional Fmoc-D-Val-OH.[4]

As protecting group for the N^{α}-amino of Orn, Fmoc, Alloc, and the recently described *p*NZ[11,29,30] were examined (Scheme 4, Strategies 1 and 2) as well as Boc (Scheme 4, Strategies 3 and 4). Alloc was removed by Pd(0) in rather neutral conditions in the presence of a scavenger of the allyl carbocations.[32] *p*NZ is orthogonal to the most common solid-phase peptide synthesis (SPPS) protecting groups such as *tert*-butyl (*t*Bu)/Boc, Fmoc, and Alloc. It was removed under simple and practical neutral conditions by $SnCl_2$ in the presence of catalytic amounts of acid.[29] When the Fmoc group (Strategy 1) was removed under the classical conditions piperidine−DMF (1:4), the major product was the linear peptide, which formed by aminolysis of the ester bond.[33] However, the use of diethylamine (DEA) (30 equiv of DEA for 90 min at 25 °C) minimized the undesired side reaction. In this case, although no opening of the cycle was detected, some starting product (around 5%) remained in

(25) Rovero, P.; Vigano, S.; Pegoraro, S.; Quartara, L. *Lett. Pept. Sci.* **1996**, *2*, 319−323.

(26) Chiva, C.; Vilaseca, M.; Giralt, E,; Albericio, F. *J. Pept. Sci.* **1999**, *5*, 131−140.

(27) If the loading is greater than 1 mmol/g, as most of the commercial available resins of this kind are, the analysis by HPLC-ESMS of the crude product shows additional peaks, corresponding to terminated sequences (see ref 26).

(28) Garcia-Martin, F.; Bayo, N.; Cruz, L. J.; Tulla, J.; Bohling, J.; Albericio, F. Manuscript in preparation.

(29) Isidro-Llobet, A.; Guasch-Camell, J.; Alvarez, M.; Albericio, F. *Eur. J. Org. Chem.* **2005**, 3031−3039.

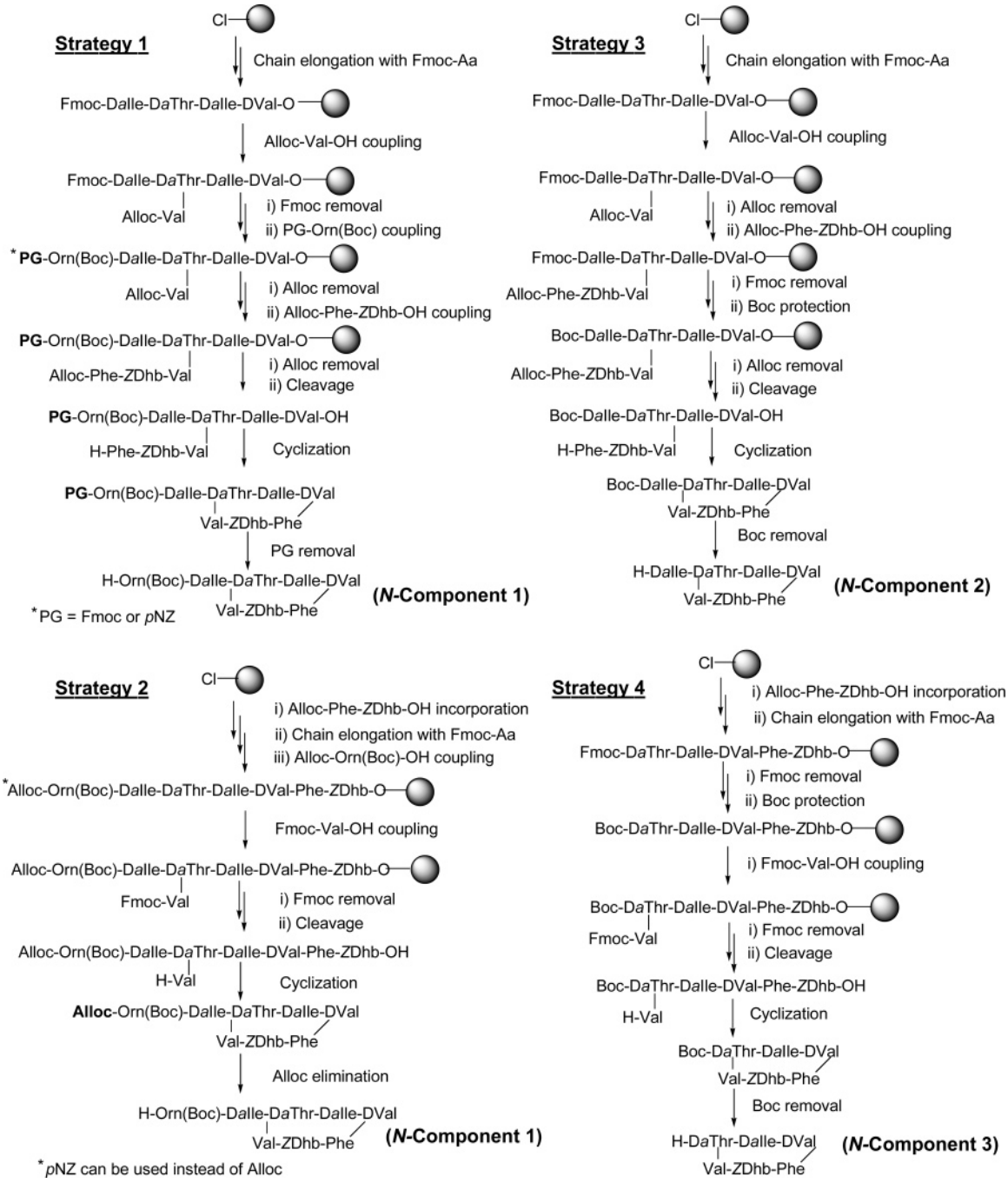
(30) Isidro-Llobet, A.; Alvarez, M.; Albericio, F. *Tetrahedron Lett.* **2005**, *46*, 7733−7736.

(31) The synthesis of peptides by convergent approaches and/or the cyclic peptides require the use of semi-permanent protecting groups, which must be stable to the conditions used to remove the temporal protecting group, which are those that are removed after each synthetic cycle and should be removed without affecting any permanent protecting groups, which removed in the final step of the synthetic process: Albericio, F. *Biopolymers* **2000**, *55*, 123−139.

(32) Guibe, F. *Tetrahedron* **1997**, *53* (40), 13509−13556; **1998**, *54*, 2967−3042.

(33) It is interesting to point out that the ester bond between D-*allo*-Thr[6] and L-Val[1] is perfectly stable to the repetitive piperidine treatments during the stepwise elongation of the peptidic chain where the cycle is still not formed, the same ester bond is unstable to just one piperidine treatment once the cycle is already formed. This is a illustrative example of that the rigidity conferred by a cycle can change the physchem properties of a molecule.

SCHEME 4. Strategies for the Syntheses of the Cyclic Fragment



the reaction crude. (The starting peptide contains a Fmoc group, which makes a difference in the ϵ of both compounds and therefore makes the yield calculation difficult.) In the same strategy, the use of *p*NZ-Orn(Boc)-OH instead of Fmoc-Orn-(Boc)-OH was examined. The ester bond was stable to the $SnCl_2$ treatment used to remove *p*NZ.

Furthermore, the same stability was achieved when the Alloc group (Scheme 4, Strategy 2) was removed with Pd(0) in the presence of triethylsilane (TES). To use Alloc as a semi-permanent protecting group, the classical strategy 1 used for the synthesis of Kahalalide F could not be followed because it would have been necessary to remove Alloc as N^{α}-protecting group of the Phe in the presence of the semi-permanent protecting group of the Orn (Scheme 4, Strategy 1). Thus, a new strategy to circumvent this problem was assayed. This strategy began with the incorporation of Alloc-Phe-ZDhb-OH as the first building block to the CTC resin using the same conditions required for the introduction of protected amino acids (Scheme 4, Strategy 2). The peptide chain was elongated using Fmoc for the α-amino protection of D-Val, D-*allo*-Ile, D-*allo*-Thr, and D-*allo*-Ile, and Alloc for the Orn(Boc). (In this strategy the Orn(Boc) could be also introduced as *p*NZ-Orn(Boc)-OH.) This new strategy worked well in terms of cyclization yield and in addition showed two key advantages. Due to the defect of the first protected building block required for the incorporation onto the CTC resin, a smaller amount of the precious Alloc-Phe-ZDhb-OH was required compared with Strategy 1, in which the dipeptide was introduced in excess. Second and more importantly, ZDhb does not show chirality and therefore no

TABLE 1. Screening of *C*- and *N*-Components Coupling Methods

conditions	% of final product in the HPLC
DIPCDI/HOAt (1.5 equiv, 1.5 equiv)	47% (t = 48 h)
EDC/HOBt/DMAP (4 equiv, 4 equiv, 0.4 equiv)	16% (t = 6 h); 23% (t = 20 h)
PyAOP/DIEA (1 equiv, 3 equiv) + 1 equiv PyAOP at t = h	35% (t = 30 min); 54% (t = overnight)
HATU/DIEA (1 equiv, 3 equiv) + 1 equiv PyAOP at t = 3 h	43% (t = 30 min); 49% (t =overnight)
EDC/HOBt (3 equiv, 3 equiv)	5.8% (t = 30 min); 53% (t = overnight)

epimerization occurred during the cyclization step, which is the most prone for inducing racemization.

In Strategies 3 and 4, and as the *N*-Components 2 and 3 did not contain any side-chain protecting group, the Boc group was used as N^{α}-amino protecting group of the *N*-terminal residue (D-*allo*-Ile[7] in Strategy 3 and D-*allo*-Thr in Strategy 4). In addition to the full stability of the lactone bond to the conditions (TFA) used to remove that protecting group, the main advantages associated with the use of the Boc group were that the reagent to remove this group as well as the side products formed were volatile and therefore could be removed by simple evaporation.

Condensation of Linear and Cyclic Fragments. First of all, and using *C*- and *N*-Components as models, we screened the coupling methods [DIPCDI/7-aza-1-hydroxybenzotriazole (HOAt) (1.5 equiv each); 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide hydrochloride (EDC)/HOBt (3 equiv each); EDC/HOBt/*N*,*N*-(dimethylamino)pyridine (DMAP) (4 equiv, 4 equiv, 0.4 equiv); (7-azabenzotriazol-1-yloxy)-tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)/DIEA (1 equiv, 3 equiv) + PyAOP (1 equiv after 1 h), and 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU)/DIEA (1 equiv, 3 equiv)]. On the basis of this screening, we concluded that the method based on PyAOP gave the best results in terms of yields and the absence of side products in the HPLC chromatograms (Table 1). Phosphonium derivatives such as PyAOP are more convenient for slow couplings compared with aminium/uronium reagents such as HATU, because the latter can terminate the peptide chain through a guanidination reaction.[34] Furthermore, PyAOP contains HOAt, which is the most reactive benzotriazole.[35]

Each linear *C*-Component was then condensed with its corresponding *N*-Component in solution phase using PyAOP and DIEA. The three protected peptides obtained were treated with TFA−H_2O (9:1) to remove the Boc and *t*Bu groups and yielded Kahalalide F.

A range of results were obtained depending on which *C*- and *N*-terminal amino acids of the *C*- and *N*-Components, respectively, were involved. From the point of view of the purity of the final product, the strategies that involved the concourse of *C*- and *N*-Components 1 and 2 were superior to that corresponding to fragments 3. This can be explained because the latter involves coupling between two hindered β-branched amino acids (D-*allo*-Ile and D-*allo*-Thr). Furthermore, D-*allo*-Thr is part of the cycle, which may also impair the coupling of the protected peptide. The *C*- and *N*-Component 1 strategy also gave slightly superior results.

Furthermore, when the *C*-terminal amino acids of the *C*-Component were Orn [*C*-Component 2 and *N*-Component 2 (Strategy 3, Scheme 4)] or D-*allo*-Ile [*C*-Component 3 and

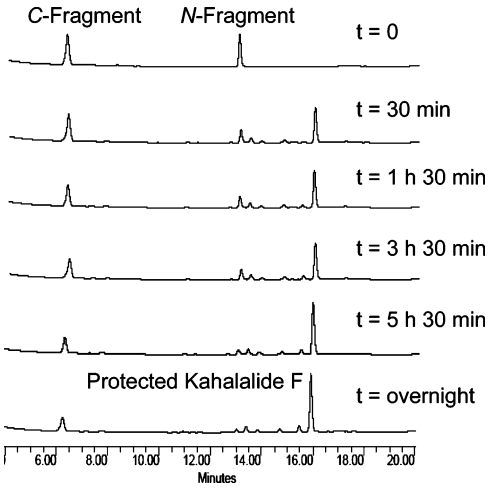


FIGURE 2. HPLC analysis of the solution coupling between *C*- and *N*-Components 1 over time. Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

N-Component 3 (Strategy 4, Scheme 4)], epimerization of these amino acids was observed [4% for the case of Orn (Figure 3) and more than 10% for D-*allo*-Ile (data no shown)].[36] However, when the *C*-terminal amino acid of the linear fragment was D-Pro [*C*- and *N*-Components 1 (Strategy 1 or 2, Scheme 4)], epimerisation was not observed (Figure 4). These results were obtained by comparing the HPLC crudes of the Kahalalide F compounds synthesized by the strategies and the three epimers, which were also synthesized by a stepwise strategy (substitution of D-Pro for L-Pro, of L-Orn for D-Orn, and of D-*allo*-Ile for Ile). Our observations confirm that, as a result of its cyclic secondary amino function, Pro is less prone to racemize during fragment coupling.

Conclusions

Here we developed several convergent strategies for the synthesis of the analogues of the antitumor peptide Kahalalide F. The best approaches were those in which the *C*-terminal amino acid of the *C*-Component is D-Pro because it avoids epimerization during the coupling of the fragment in solution. The choice of the semi-permanent protecting group for the N^{α}-amino of Orn-protecting groups requires additional discussion. Thus, the use of the less common groups, *p*NZ, and Alloc, which ensure total stability of the lactone, implies the concourse of metallic cations or metals, which may demand a purification step before the final fragment coupling. Furthermore, the Fmoc group requires fine-tuning of the experimental process, which can be detrimental for the development of a large-scale process in which conditions may be more difficult to control. The use of the *N*-Component prepared by Strategy 2 (Scheme 4) shows

(34) Albericio, F.; Bofill, J. M.; El-Faham, A.; Kates, S. A. *J. Org. Chem.* **1998**, *63*, 9678−9683.

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

(36) These epimers did not show any remarkable biological activity in the typical cell lines used for evaluation the antitumorals activity of Kahalalide F and its analogues.

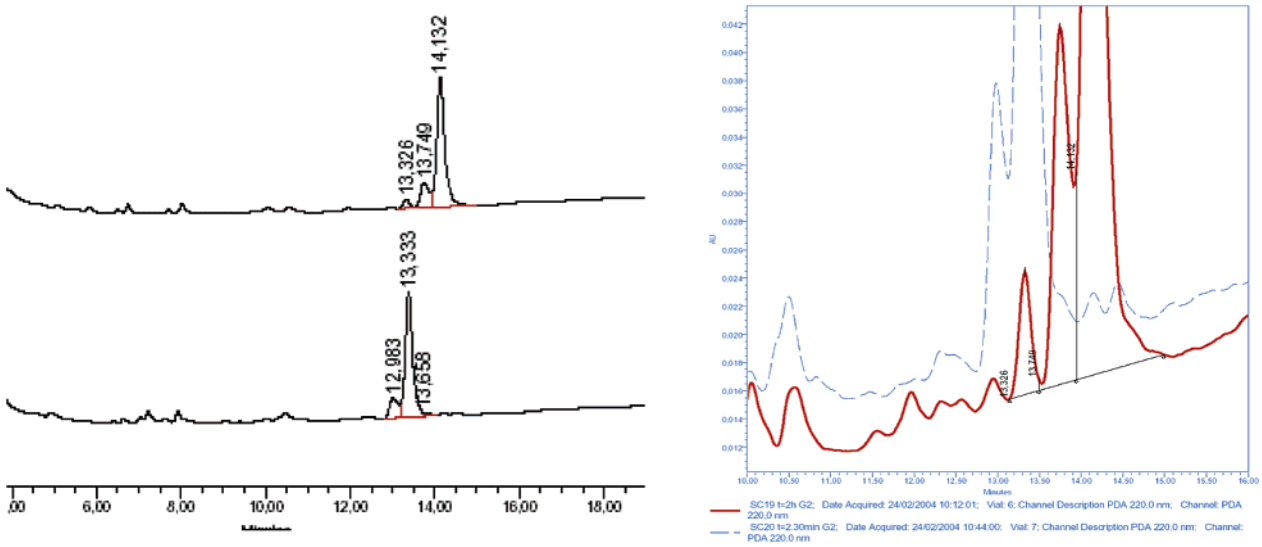


FIGURE 3. HPLC analysis of the Boc-protected Kahalalide F prepared from *C*- and *N*-Components 2 (up and red) compared with the (DOrn[8])-Kahalalide F (down and blue). Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

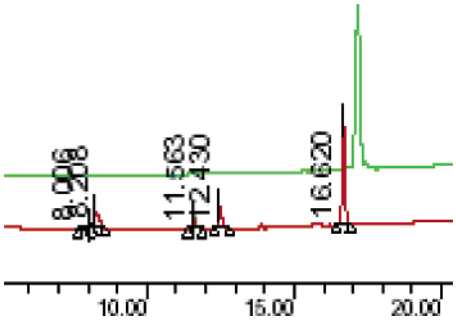


FIGURE 4. HPLC analysis of the Boc-protected Kahalalide F prepared from *C*- and *N*-Components 1 (green) compared with the (LPro[9])-Kahalalide F (red). Linear gradient of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

several advantages because it allows the preparation of Kahalalide F without risk of epimerization during cyclization and uses lower amounts of the precious Alloc-Phe-ZDhb-OH.

Experimental Section

General Procedures. Cl-TrtCl-resin, protected Fmoc-amino acid derivatives, HOBt, and HOAt were purchased from different sources as well as DIPEA, DIPCDI, EDC·HCl, piperidine, TFA, DMF, MeCN (HPLC grade), and CH_2Cl_2. All commercial reagents and solvents were used as received with the exception of DCM, which was passed through an alumina column to remove acidic contaminants.

*p*NZ-Orn(Boc)-OH was prepared as described previously[29] and Alloc-amino acids as essentially described by Dangles et al. and Cruz et al.[37,38] Alloc-ZDhb-Phe-OH was prepared as before.[39]

Solid-phase syntheses were performed in polypropylene syringes (10−50 mL), each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. The Fmoc group was removed with piperidine−DMF (2:8, v/v) (1 × 2 min, 2 × 10 min). Washings between deprotection, coupling, and again, deprotection steps were performed with DMF (5 × 0.5 min) and CH_2Cl_2 (5 × 0.5 min) using 10 mL solvent/g resin each time. Peptide synthesis transformations and washings were done at 25 °C. Syntheses carried out on solid phase were controlled by HPLC of the intermediates obtained after cleaving an aliquot (approximately 2 mg) of the peptidyl-resin with TFA−H_2O (1:99) for 1 min. HPLC reversed-phase columns Symmetry C_{18} 4.6 mm × 150 mm, 5 μm (column A) and Symmetry300 C_{18} 4.6 mm × 50 mm, 5 μm (column B) were used. Analytical HPLC was performed on an instrument comprising two solvent delivery pumps, automatic injector dual wavelength detector, and system controller (Breeze V3.20) and on an instrument comprising two solvent delivery pumps, automatic injector, and a variable wavelength detector (photodiode array). UV detection was at 215 or 220 nm, using linear gradients of MeCN (+0.036% TFA) into H_2O (+0.045% TFA), from 30% to 100% in 15 min.

MALDI-TOF and ES-MS analysis of peptide samples were performed using ACH matrix. Peptide-resin samples for amino acid analysis were hydrolyzed in 12 N aqueous HCl−propionic acid (1:1), at 155 °C for 1−3 h, and peptide-free samples were hydrolyzed in 6 N aqueous HCl at 155 °C for 1 h.

The "&" symbol is used in the nomenclature for cyclic peptides and precursors.[40] The appearance of "&" in a given position of the one-line formula indicates the location of one end of a chemical bond and the second "&" the point to which this bond is attached. Thus, "&" represents the start or the end of a chemical bond, which is "cut" with the aim to facilitate the view of a complex formula. In this way, two "&" symbols indicate one chemical bond.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from Fmoc-Orn(Boc)-OH.**

***H*-D-Val-*O*-TrtCl-resin (1).** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), and a solution of Fmoc-D-Val-OH (238 mg, 0.7 mmol, 0.43 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added. The mixture was then stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was stopped by adding MeOH (800 μL) and stirring for 10 min. The Fmoc-D-Val-O-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), piperidine as indicated in General

(37) Dangles, O.; Guibe, F.; Balavoine, G.; Lavielle, S.; Marquet, A. *J. Org. Chem.* **1987**, *52*, 4984−4993.

(38) Cruz, L. J.; Beteta, N. G.; Ewenson, A.; Albericio, F. *Org. Process Res. Dev.* **2004**, *8*, 920−924.

(39) Albericio, F.; Giralt, E.; Jimenez, J. C.; Lopez, A.; Manzanares, I.; Rodrigues, I.; Royo, M. PCT Int. Appl. (2001) WO 2001058934, A2 20010816, CAN 135:167039, AN 2001:598019.

(40) Spengler, J.; Jimenez, J. C.; Burger, K.; Giralt, E.; Albericio, F. *J. Pept. Res.* **2005**, *65*, 550−555.

Procedures, and DMF (5 × 0.5 min). The loading was 0.50 mmol/g, as calculated by Fmoc determination.

[Fmoc-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (2). Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Thr-OH (free hydroxy group) (683 mg, 2 *mmol*, 4 equiv), and Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv) were added sequentially to the above obtained *H*-D-Val-O-TrtCl-resin (**1**) using DIPCDI (310 μL, 2 mmol, 4 equiv) and HOBt (307 mg, 2 mmol, 4 equiv) in DMF (2.5 mL). In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures. Alloc-Val-OH (502 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 mg, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated twice in the same conditions. An aliquot of the peptidyl-resin was treated with TFA and the HPLC (R_t 14.2 min, column A) of the crude obtained after evaporation showed a purity of >98%. ESMS calcd for $C_{45}H_{63}N_5O_{11}$ 849.45; found *m*/*z* 850.1 $[M + H]^+$, 871.9 $[M + Na]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (3). The Fmoc group of the peptide resin (**2**) was removed, and Fmoc-Orn(Boc)-OH (912 mg, 2 mmol, 4 equiv) was added using DIPCDI (310 μL, for 2.0 mmol and 4 equiv; and 388 μL, for 2.5 mmol and 5 equiv) and HOBt (307 mg, for 2.0 mmol and 4 equiv; and 395 mg, for 2.5 mmol and 5 equiv) for 90 min. Ninhydrin test after the incorporation was negative. An aliquot of the peptidyl-resin was treated with TFA and the HPLC (R_t 12.8 min, column A) of the crude obtained after evaporation showed a purity of 90%. ESMS calcd for $C_{56}H_{81}N_7O_{14}$ 1,063.58; found *m*/*z* 1,086.77 $[M + Na^+]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Phe-ZDhb-Val&] (4). The Alloc group of the peptide resin (**3**) was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), followed by washings with diethyldithiocarbamate 0.02 M (3 × 15 min). Alloc-Phe-ZDhb-OH (666 mg, 2 mmol, 4 equiv) and HOAt (273 mg, 2 mmol, 4 equiv) were dissolved in DMF (1.25 mL) and added to peptidyl-resin. DIPCDI (310 μL, 2 mmol, 4 equiv) was then added, and the mixture was stirred for 5 h. The ninhydrin test was negative. After washings with DMF and CH_2Cl_2, an aliquot of the peptidyl-resin was treated with TFA−H_2O (1:99) for 1 min, and the product was characterized by MALDI-TOF-MS: calcd for $C_{68}H_{95}N_9O_{16}$ 1,293.69; found *m*/*z* 1,294.35 $[M + H]^+$, 1,316.39 $[M + Na]^+$, 1,333.34 $[M + K]^+$.

[Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-OH][*H*-Phe-ZDhb-Val&] (5). The Alloc group of the peptide resin (**4**) was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), the resin was washed with sodium diethyldithiocarbamate in DMF 0.02 M (3 × 15 min), and the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). The filtrate was collected on H_2O (4 mL) and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve solid that formed during the removal of H_2O, and the solution was lyophilized to give 700 mg of title compound (578 μmol, 99% yield) of the title compound with a purity of > 91% as checked by HPLC (Column A, R_t 8.59 min)], which was used without further purification. MALDI-TOF-MS calcd for $C_{64}H_{91}N_9O_{14}$ 1,209.67; found *m*/*z* 1,210.45 $[M + H]^+$, 1,232.51 $[M + Na]^+$, 1,248.45 $[M + K]^+$.

Fmoc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (6). The protected peptide (**5**) was dissolved in CH_2Cl_2 (580 mL, 1 mM), and HOBt (137 mg, 2.3 mmol) dissolved in the minimum volume of DMF to dissolve HOBt, DIPEA (302 μL, 1.73 mmol, 3 equiv), and DIPCDI (356 μL, 2.3 mmol, 4 equiv) were added. The mixture was stirred for 1 h, and the course of the cyclization step was then checked by HPLC (column A, R_t 12.4 min). The solvent was removed by evaporation under reduced pressure and the product was used without further purification. MALDI-TOF-MS calcd for $C_{64}H_{89}N_9O_{13}$ 1,191.66; found *m*/*z* 1,092.17 $[M + H]^+$, 1,214.14 $[M + Na]^+$, 1,230.10 $[M + K]^+$.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** The protected peptide (**6**) (50 mg, 42 μmol) was dissolved in DMF (5 mL), then DEA (130 μL, 30 equiv) was added and the mixture was stirred for 90 min. The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 × 100 mm), linear gradient of MeCN (30% to 75% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm. The product was characterized by HPLC (R_t 8.7 min, Condition A) and MALDI-TOF-MS calcd for $C_{49}H_{79}N_9O_{11}$ 969.59; found *m*/*z* 970.87 $[M + H]^+$, 870.78 $[M - Boc]^+$.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from pNZ-Orn(Boc)-OH.**

pNZ-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (7). The synthesis was performed in the same way as for compound **6**, but Fmoc-Orn(Boc)-OH was replaced by *p*NZ-Orn(Boc)-OH.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** The protected peptide (14.7 mg, 12.8 μmol) was dissolved in 1.6 mM HCl in DMF (10 mL), $SnCl_2$ (3.8 g, 20 mmol) was then added, and the mixture was stirred until HPLC (Column A) showed the completion of the reaction (1 h). The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), gradient of MeCN (30% to 75% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (4.8 mg, 4.9 μmol, 40% yield). The product was characterized by HPLC (R_t 8.2 min, Column A) and by MALDI-TOF-MS: calcd for $C_{49}H_{79}N_9O_{11}$ 969.59; found *m*/*z* 992.35 $[M + Na]^+$, 870.34 $[M - Boc]^+$, 892.34 $[M + Na - Boc]^+$.

***N*-Component 1: *H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from Alloc-Orn(Boc)-OH**

***H*-Phe-ZDhb-*O*-TrtCl-resin (8).** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), and a solution of Alloc-Phe-ZDhb-OH (232 mg, 0.7 mmol, 0.42 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added. This mixture was then stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was arrested by adding MeOH (800 μL), after stirring for 10 min. The Alloc-Phe-ZDhb-*O*-TrtCl-resin was subjected to washings with CH_2Cl_2 (3 × 0.5 min) and DMF (3 × 0.5 min), and the Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv) in CH_2Cl_2. The resin was washed as described in General Procedures. The loading was 0.68 mmol/g, as calculated by Fmoc determination.

[Alloc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-OH][*H*-Val&] (9). Fmoc-D-Val-OH (678 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Thr-OH (free hydroxy group) (683 mg, 2 mmol, 4 equiv), Fmoc-D-*allo*-Ile-OH (707 mg, 2 mmol, 4 equiv), and Alloc-Orn-(Boc)-OH (630 mg, 2 mmol, 4 equiv) were added sequentially to the above obtained *H*-Phe-ZDhb-*O*-TrtCl-resin (**8**) using DIPCDI (310 μL, 2 mmol, 4 equiv) and HOBt (307 mg, 2 mmol, 4 equiv) in DMF (2.5 mL). In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures. Fmoc-Val-OH (848.2 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 mg, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated twice in the same conditions. After removal of the Fmoc group as described in General Procedures, the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL), and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that formed during H_2O removal,

and the solution was lyophilized to give 650 mg of target compound (606 μmol, 90% yield) of the title compound with a purity of >75% as checked by HPLC (Column A, R_t 9.93 min). ESMS calcd for $C_{53}H_{85}N_9O_{14}$ 1072.29; found *m/z* 1074.4 $[M + H]^+$.

Alloc-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (10). Peptide **9** (250 mg, 233 μmol) was dissolved in CH_2Cl_2 (240 mL, 1 mM), and HOAt (126 mg, 9.325 mmol, 4 equiv) in the minimum volume of DMF, and DIPCDI (143 μL, 9,325 mmol, 4 equiv) were added. The mixture was stirred for 24 h, and the course of the cyclization step was then checked by HPLC (column A, R_t 12.82 min). The solvent was removed by evaporation under reduced pressure, and the product was used without further purification. MALDI-TOF-MS calcd for $C_{53}H_{83}N_9O_{13}$ 1,054.28; found *m/z* 1,056.4 $[M + H]^+$.

***H*-Orn(Boc)-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& (*N*-Component 1).** Peptide **10** was dissolved in 10 mL of CH_2Cl_2, then $Pd(PPh_3)_4$ (8 mg, 6,94 μmol, 0.03 equiv) in the presence of $PhSiH_3$ (94 μL, 763,6 μmol, 3.3 equiv) was added, and the mixture was stirred for 90 min. The solvent was removed by evaporation under reduced pressure. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), linear gradient of MeCN (20% to 80% in 15 min) MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm. The product was characterized by HPLC (R_t 9.19 min, Condition A) and by MALDI-TOF-MS: calcd for $C_{49}H_{79}N_9O_{11}$ 970.21; found *m/z* 972.1 $[M + H]^+$.

***C*-Component 1: MeHex-D-Val-Thr(tBu)-Val-D-Val-D-Pro-OH.** Cl-TrtCl-resin (1 g, 1.64 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was then washed with CH_2Cl_2 (5 × 0.5 min), a solution of Fmoc-D-Pro-OH (237 mg, 0.7 mmol, 0.43 equiv) and DIPEA (0.41 mL) in CH_2Cl_2 (2.5 mL) was added, and the mixture was stirred for 15 min. Extra DIPEA (0.81 mL, total 7 mmol) was added, and the mixture was stirred for an additional 45 min. The reaction was arrested by stirring the resin in MeOH (800 μL) for 10 min. The Fmoc-D-Pro-*O*-TrtCl-resin was subjected to the following washings/treatments with CH_2Cl_2 (3 × 0.5 min), DMF (3 × 0.5 min), piperidine as indicated in General Procedures, and DMF (5 × 0.5 min). The loading was 0.27 mmol/g, as calculated by Fmoc determination.

Fmoc-D-Val-OH (458 mg, 1.32 mmol, 5 equiv), Fmoc-Val-OH (360 mg, 1.06 mmol, 4 equiv), Fmoc-Thr(*t*Bu)-OH (527 mg, 1.32 mmol, 5 equiv), Fmoc-D-Val-OH (360 mg, 1.06 mmol, 4 equiv), and MeHex-OH (138 mg, 1.06 mmol, 4 equiv) were added sequentially to the above peptide resin using DIPCDI (165 μL, for 1.06 mmol and 4 equiv; and 205 μL, for 1.32 mmol and 5 equiv) and HOBt (162 mg, for 1.06 mmol and 4 equiv; and 203 mg, for 1.32 mmol and 5 equiv) for 90 min. In all cases, after 90 min of coupling, the ninhydrin test was negative. Removal of Fmoc group and washings were performed as described in General Procedures.

The partially protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL) and H_2O was partially removed in a rotavapor. MeCN was then added to dissolve the solid that formed during H_2O removal, and the solution was lyophilized to give 154.4 mg (226 μmol, 86% yield) of the title compound with a purity of >94% as checked by HPLC (Column A, R_t 12.13 min). The crude obtained after evaporation showed a purity of >94%. The product was characterized by ES-MS: calcd for $C_{35}H_{63}N_5O_8$, 681.9; found *m/z* 682.15.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val& from *C*- and *N*-Components 1. *N*-Component 1 (8.25 mg, 8.5 μmol) and *C*-Component 1 (7 mg, 10.2 μmol, 1.2 equiv) were dissolved in DMF (10 mL), and PyAOP (5.32 mg, 10.2 μmol, 1.2 equiv) and DIPEA (5.3 μL, 30.6 μmol, 3.6 equiv) were added at room temperature. The mixture was stirred for 1 h, when extra PyAOP (5.32 mg, 10.2 μmol, 1.2 equiv) was added. The mixture was allowed to react for 2 h at room temperature until HPLC (Column A) showed completion of the reaction. HPLC showed that the crude obtained after evaporation had a purity of >75%.

The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), linear gradient of MeCN (+0.05% TFA) in water (+0.05% TFA) (30% to 100% in 15 min), 20 mL/h, detection at 220 nm, to give the protected Kahalalide F (6.9 mg, 4.2 μmol, 49% yield). MALDI-TOF-MS calcd for $C_{84}H_{140}N_{14}O_{18}$ 1,633.05; found *m/z* 1,534.33 $[M - Boc]^+$,1,556.26 $[M - Boc + Na]^+$ 1,656.33 $[M + Na]^+$.

Protected Kahalalide F was dissolved in TFA−H_2O (19:1, 700 μL), and the mixture was stirred for 1 h. The solvent was removed by evaporation under reduced pressure, and dioxane was added (245 μL). Again, the solvent was removed by evaporation under reduced pressure (the process was repeated three times), H_2O (1 mL) was then added, and the solution was lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), isocratic 44% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (5 mg, 3.4 μmol, 80% yield, 93.3%).

The HPLC of the crude product did not show the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-*Pro*-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& (Epimer 1). This observation indicates racemization during the coupling step between the two protected peptides. MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$ 1,476.93; found *m/z* 1,478.17 $[M + H]^+$ 1,500.14 $[M + Na]^+$, 1,516.12 $[M + K]^+$.

Epimer 1: MeHex-D-Val-Thr-Val-D-Val-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-ZDhb-Val&. Experimental procedures were as described for the stepwise synthesis of Kahalalide F, except that Fmoc-D-Pro-OH was replaced by Fmoc-Pro-OH.[8] The product was characterized by HPLC (R_t 11.23 min, Column A) and by MALDI-TOF-MS: calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m/z* 1,500.23 $[M + Na]^+$, 1,515.97 $[M + K]^+$.

***N*-Component 2: *H*-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&.** Starting with [Fmoc-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-*O*-TrtCl-resin][Alloc-Val&] (**2**), the Alloc group was removed with $Pd(PPh_3)_4$ (58 mg, 0.05 mmol, 0.1 equiv) in the presence of $PhSiH_3$ (617 μL, 5 mmol, 10 equiv), and the resin was washed with sodium diethyldithiocarbamate in DMF 0.02 M (3 × 15 min). Alloc-Phe-*Z*Dhb-OH (666 mg, 2 mmol, 4 equiv) and HOAt (273 mg, 2 mmol, 4 equiv) were dissolved in DMF (1.25 mL) and added to peptidyl-resin. DIPCDI (310 μL, 2 mmol, 4 equiv) was then added, and the mixture was stirred for 5 h; at which point the HPLC showed completion of reaction (R_t 7.09 min, Column A).

The Fmoc group was removed and after extensive DMF washings, Boc_2O (546 mg, 5 equiv) and DIPEA (0.87 mL, 10 equiv) in DMF were added and left to react for 2 h. The ninhydrin test was negative at this time point. (Alternatively, Boc-D-*allo*-Ile-OH can be introduced instead of the Fmoc derivative.) After DMF washings, the Alloc group was removed, and the protected peptide was cleaved from the resin with TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL) and H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that formed during H_2O removal, and the solution was then lyophilized.

Cyclization was performed as for compound **6**, and Boc was then removed with TFA−H_2O (19:1) (1 h). The solvent was removed under reduced pressure, and dioxane was added (245 μL). Again, the solvent was removed by evaporation under reduced pressure (the process was repeated three times), and then H_2O (1 mL) was added and lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 × 100 mm), isocratic 44% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (207 mg, 273 μmol, 55% yield, 93.3%). The product was characterized by HPLC (R_t 7.27 min, Column A) and by MALDI-TOF-MS: calcd $C_{39}H_{61}N_7O_8$, 755.46; found *m/z* 756.56 $[M + H]^+$, 778.55 $[M + Na]^+$, 794.53 $[M + K]^+$.

***C*-Component 2: MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-OH.** Experimental procedures as for *C*-Component 1, except that the peptide synthesis was initiated by incorporation of Fmoc-Orn(Boc)-OH to the Cl-TrtCl-resin. The product was characterized by HPLC (R_t 13.27 min, Column A) and by Electrospray: calcd $C_{45}H_{81}N_7O_{11}$, 895,60; found *m*/*z* 895,10.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from *C*- and *N*-Components 2. The synthesis was performed as described above except that the condensed fragments were *N*-Component 2 and *C*-Component 2. The HPLC of the crude final product showed the presence of the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-D-Pro-D-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& (4.1%) (Epimer 2), which indicates racemization during the coupling step between both protected peptides. The product was characterized by HPLC (R_t 10.5 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,477.99 $[M + H]^+$ 1,499.97 $[M + Na]^+$, 1,515.93 $[M + K]^+$.

Epimer 2: MeHex-D-Val-Thr-Val-D-Val-D-Pro-D-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&. The synthesis was carried out as described for the stepwise synthesis of Kahalide except that Fmoc-Orn(Boc)-OH is replaced by Fmoc-D-Orn(Boc)-OH.[8] The product was characterized by HPLC (R_t 9.89 min, Column A). MALDI-TOF-MS calcd $C_{75}H_{124}N_{14}O_{16}$ 1,476.93; found *m*/*z* 1,478.06 $[M + H]^+$ 1,500.15 $[M + Na]^+$, 1,516.04 $[M + K]^+$.

***N*-Component 3: *H*-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&.** Starting with Fmoc-D-*allo*-Thr-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-*O*-TrtCl-resin, the Fmoc group was removed, and after extensive DMF washings, Boc_2O (546 mg, 5 equiv) and DIPEA (0.87 mL, 10 equiv) in DMF were added and left to react for 2 h, after which the ninhydrin test was negative. After DMF washings, Fmoc-Val-OH (848.2 mg, 2.5 mmol, 5 equiv) was coupled with DIPCDI (387 μL, 2.5 mmol, 5 equiv) in the presence of DMAP (30.6 mg, 0.25 mmol, 0.5 equiv) and DIPEA (88 μL, 0.5 mmol, 1 equiv) for 45 min. This coupling was repeated in the same conditions twice. After removal of the Fmoc group as described in General Procedures, the protected peptide was cleaved from the resin by TFA−CH_2Cl_2 (1:99) (5 × 30 s). Filtrate was collected on H_2O (4 mL), and the H_2O was partially removed under reduced pressure. MeCN was then added to dissolve the solid that appeared during the H_2O removal, and the solution was lyophilized, to give 57 mg (75 μmol, 90% yield) of the title compound with a purity of >95%, as checked by HPLC (Column A, R_t 7.95 min). ESMS calcd for $C_{38}H_{60}N_6O_{10}$ 760.44; found *m*/*z* 762.3 $[M + H]^+$.

Cyclization was carried out as for compound **6**, and the Boc was then removed with TFA−H_2O (19:1) (1 h). The solvent was removed under reduced pressure, and dioxane was added (245 μL). The solvent was removed by evaporation under reduced pressure (the process was repeated three times), and then H_2O (1 mL) was added and lyophilized. The crude product was purified by HPLC (Symmetry C8 5 μm, 30 mm × 100 mm), isocratic 30% MeCN (+0.05% TFA) in water (+0.05% TFA), 20 mL/h, detection at 220 nm, to give the title product (50.2 mg, 67.6 μmol, 90% yield).

The product was characterized by HPLC (R_t 5.32 min, Column A) and by ES-MS: calcd $C_{33}H_{61}N_6O_9$ 642.37; found *m*/*z* 642.035.

***C*-Component 3: MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-D-*allo*-Ile-OH.** Experimental procedures as for the obtention of *C*-Component 1, except that the peptide synthesis was initiated by incorporation of Fmoc-D-*allo*-Ile-OH to the Cl-TrtCl-resin. The product was characterized by HPLC (R_t 10.25 min, Column A) and by MALDI-TOF-MS: calcd for $C_{51}H_{92}N_8O_{12}$ 1,008.68; found *m*/*z* 1,009.8.

Kahalalide F: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-D-*allo*-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val& from *C*- and *N*-Components 3. Experimental procedures were as described for the other syntheses of Kahalalide F. The HPLC of the final product showed the presence of the epimeric peptide MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-Ile-D-*allo*-Thr(&)-D-*allo*Ile-D-Val-Phe-*Z*Dhb-Val& (Epimer 3), which indicates racemization during the coupling step between the two protected peptides. The product was characterized by HPLC (R_t 7.92 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,478.5 $[M + H]^+$ 1,501.4 $[M + Na]^+$, 1,517.6 $[M + K]^+$.

Epimer 3: MeHex-D-Val-Thr-Val-D-Val-D-Pro-Orn-Ile-D-*allo*-Thr(&)-D-*allo*-Ile-D-Val-Phe-*Z*Dhb-Val&. Experimental procedures were as described for the stepwise synthesis of Kahalaide F, except that Fmoc-Ile-OH was used instead of Fmoc-D-*allo*-Ile-OH.[8] MeHex-D-Val-Thr(*t*Bu)-Val-D-Val-D-Pro-Orn(Boc)-Ile-OH: (R_t 10.25 min, Column A and MALDI-TOF-MS calcd for $C_{51}H_{92}N_8O_{12}$ 1,008.68; found *m*/*z* 1,009.5). The final product was characterized by HPLC (R_t 8.02 min, Column A). MALDI-TOF-MS calcd for $C_{75}H_{124}N_{14}O_{16}$, 1,476.93; found *m*/*z* 1,478.2 $[M + H]^+$ 1,501.1 $[M + Na]^+$, 1,517.3 $[M + K]^+$.

Acknowledgment. This study was partially supported by CICYT (BQU 2003-00089), the *Generalitat of* Catalunya, and the Barcelona Science Park. AI thanks the *DURSI*, *Generalitat de Catalunya* and European Social Funds for a predoctoral fellowship.

Supporting Information Available: HR-MS and ^1H NMR characterization of *C*- and *N*-Components 1, 2, and 3, as well as ^1H NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

JO060976F