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Solid-Phase Synthesis of Aza-Kahalalide F Analogues: (2R,3R)-2-Amino-3-azidobutanoic Acid as Precursor of the Aza-Threonine

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The solid-phase synthesis of six novel analogues of Kahalalide F (KF), a natural product currently undergoing Phase II clinical trials, is reported. In all these compounds, amides were used as isosteres for the depsi bond. For two of these compounds, we performed an efficient synthesis of N-Fmoc-

Introduction

Kahalalide F (KF, 1, Figure 1) is a cyclodepsipeptide isolated from the marine mollusc Elvsia rufescens and its algae diet Bryopsis pennata.^[1,2] Preclinical studies have shown that KF has potent cytotoxic activity in vitro against cell lines from solid tumors, several with strong multidrug resistance, including prostate, breast, and colon carcinomas, neuroblastoma, and osteosarcoma.^[3] In particular, oncosis in human prostate and breast cancer cells has been observed.^[4] In contrast, nontumoral cell lines are five to forty times less sensitive to KF.^[4] Phase I clinical trials in adult patients with advanced solid tumors have identified the recommended dose for phase II clinical studies, which is limited by a transient elevation of aminotransferases.^[5] KF is a COMPARE (National Cancer Institute)-negative compound: among antitumor drugs it shows a unique mechanism of action. This mechanism is mostly unknown but may be related to the hydrophobic nature of the compound. Lysosomes are the intracellular targets of KF and, in fact, in HeLa cervical cancer cells and monkey COS-1 fibroblasts changes in lysosomal membranes have been ob-

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protected (2R,3R)-2-amino-3-azidobutanoic acid, precursor of the aza-threonine. This is the first example of the solid-phase reduction of an azide group in the preparation of aza-Thr-containing peptides in the solid phase.

served.^[6] However, the mechanism of cell death induction remains to be elucidated and KF is currently in phase II clinical trials.



Figure 1. Structure of Kahalalide F.

The head-to-side-chain cyclodepsipeptide structure of KF is a complex structure with 13 amino acids, including a rare (*Z*)-didehydro-(*R*)-aminobutyric acid (*Z*Dhb), and an aliphatic acid [5-methylhexanoic acid (5MeHex)] at the N-terminus.^[1,2] The cycle is formed between the carboxylic acid of L-Val(1) and the hydroxy group of D-*allo*-Thr(6). Stepwise solid-phase synthesis^[7] and a convergent methodology^[8] for the preparation of KF have been reported. Recently, structure–activity relationship studies on 132 novel KF analogues revealed that the biological activity of KF is highly sensitive to backbone stereotopic modification but not to modification of the length of the side-chain.^[9]

In a recent study by our laboratory addressing the search for new analogues of the natural marine product thiocoraline,^[10] a two-fold symmetric potent antitumor antibiotic isolated from *Micromonospora sp.* L-13-ACM2-092, the introduction of *N*-Me-amides, as synthetic surrogate for the depsi moiety, afforded derivatives with comparable activi-



ties and increased serum stabilities. The ester/carboxamide substitution was thus considered for other depsipeptides. Therefore, with the aim of finding new KF analogues with improved biological activity and to evaluate the effects of amide and *N*-Me-amide bonds as isosteres of the depsi bond, herein we report the solid-phase synthesis of five novel aza analogues of KF (2–6, Figure 2). The introduction of *N*-Me-amides allows the hydrogen-bonding map of the molecule to be preserved. Moreover, *N*-alkyl amino acids are quite common in bioactive natural peptides and have a considerable influence on pharmacological parameters: membrane permeability, proteolitic stability, and confor-



Figure 2. Novel analogues of Kahalalide F (2-6) and (2R,3R)-2-amino-3-azidobutanoic acid (7).

mational rigidity.^[11] All the analogues contained (4S)-MeHex instead of 5-MeHex because in previous studies its presence had been shown to enhance the efficacy of KF analogues against breast and prostate xenografts.^[9,12] First, the analogue containing (2S)-3-N-methyl-2,3-diaminopropanoic acid (3NMe-Dap-OH in 2) in place of D-allo-Thr-OH was prepared to validate the synthetic strategy by using the most economic L derivative. Then, analogues incorporating (2R)-3-N-methyl-2,3-diaminopropanoic acid (3NMe-D-Dap-OH in 3), (2R)-2,3-diaminopropanoic acid (D-Dap-OH in 4), (2R,3R)-3-N-methyl-2,3-diaminobutanoic acid (3NMe-D-Dab-OH in 5), and (2R,3R)-2,3-diaminobutanoic acid (D-Dab-OH in 6) were prepared. A key feature of the synthetic process for the preparation of analogues 5 and 6 was the use of Fmoc-protected (2R,3R)-2-amino-3-azidobutanoic acid (7, Figure 2) as precursor of the aza-threonine. Herein we also report an alternative efficient synthesis for the known compound 7.^[13]

Results and Discussion

The analogues 2-6 were synthesized following a strategy similar to that used for the first solid-phase synthesis of KF,^[7] with modifications for the introduction of the diamino acids and their further *N*-methylation.

For the analogues 2–4 (Scheme 1), the approach involved the incorporation of Fmoc-Val-OH followed by the presynthesized dipeptide Alloc-Phe-(Z)-Dhb-OH on the amino group of the aza-serine after construction of the linear peptide sequence on a 2-chlorotrityl chloride resin (CITrt-Cl resin)^[14] using the Fmoc/*t*Bu strategy. When required, the *N*-methyl group was introduced onto the *o*-NBS-protected



Scheme 1. Synthetic strategies for the solid-phase synthesis of KF analogues **2–4**. Reagents and conditions: (a) Fmoc-D-Val-OH, DIEA, DCM; (b) piperidine/DMF (1:4); (c) Fmoc-aa-OH or (4*S*)MeHex acid, HATU, DIEA, DMF; (d) [Pd(PPh₃)₄], PhSiH₃, DCM; (e) *o*-NBS-Cl, DIEA, DCM; (f) PPh₃, MeOH, DIAD, THF; (g) HOCH₂CH₂SH, DBU, DMF; (h) Alloc-Phe-(*Z*)Dhb-OH, HATU, DIEA, DMF; (i) TFA/DCM (1:99); (l) DIPCDI, HOBt, DIEA, DCM; (m) TFA/H₂O (19:1). Alloc, allyloxycarbonyl; Boc, *tert*-butoxycarbonyl; Dap, diaminopropionic acid; Dhb, didehydro-(*R*)-aminobutyric acid; DIEA, *N*,*N*-diisopropylethylamine; DIPCDI, *N*,*N'*-diisopropylcarbodiim-ide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium hexafluorophos-phate 3-oxide; HOBt: 1-hydroxybenzotriazole; *o*-NBS, *o*-nosyl; TFA, trifluoroacetic acid.

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Figure 3. Structure of dimer 15.

amine under Mitsunobu conditions.^[11] The peptide was elongated by using HATU and DIPEA as coupling reagents. In all cases, single coupling with 4 equiv. of the Fmoc-amino acid and HATU and 8 equiv. of DIEA in DMF gave negative ninhydrin or chloranil tests.

The cyclization and final deprotection reactions were performed in solution: The use of DIPCDI/HOBt/DIEA prevented epimerization of D-Val(4) in the troublesome lactamization.^[9]

This approach afforded the crude cyclopeptides **2** and **4** in good yields and purity (74 and 89%, respectively).^[15] These compounds were then purified by using a semipreparative column to afford the pure compounds.^[15] Surprisingly, in the case of the analogue **3**,^[16] whereas the linear precursor **12** (Scheme 1) was prepared with a purity of >98%,^[15] in the last steps of the synthesis, the cyclization and deprotection of the side-chain protecting groups, a crude product containing a mixture of two compounds (47 and 32%, respectively) was obtained.^[15] These compounds were separated by using a semipreparative column to afford two pure compounds. By MALDI-TOF MS and ¹H NMR, TOCSY, and COSY analysis, the two compounds were identified as compound **3** and the dimer **15** (Figure 3).

For the preparation of the analogues 5 and 6, the D-azaallo-threonine was required. a, β-Diamino acids are common in natural products, as free amino acids, in peptides and β -lactam antibiotics. They can be prepared from β -hydroxy amino acids by azide displacement of the hydroxy group followed by azide reduction.^[17] This is a particularly attractive methodology for the synthesis of amines because both the "chiral pool" and contemporary asymmetric methodologies have made available a vast array of potential enantiomerically enriched azide substrates.^[18] In this work, the Fmoc-protected (2R,3R)-2-amino-3-azidobutanoic acid (7), prepared in solution, was introduced into the solidphase and after sequence elongation was completed and before cleavage from the resin the azide group was reduced on the solid phase. This strategy allowed us to avoid a formal protection of the amino group. Compound 7 was synthesized as shown in Scheme 2 and studies to reduce the azide group, incorporated into the linear sequence on the solid phase, were realized.^[19]

Boc-D-threonine (16), which has two stereogenic centers and is commercially available, was used as the starting material. The well-known stereospecific sequence, mesylation/ azide nucleophilic substitution, allowed the introduction of the desired configuration and, at the same time, the aminomasked group at C-3. Full deprotection of the functional



Scheme 2. Synthesis of Fmoc-protected (2R,3R)-2-amino-3-azidobutanoic acid (7). Reagents and conditions: (a) BOP-Cl, *tert*-butyl carbazate, DIEA; (b) MsCl, TEA, CH₂Cl₂, 65% for two steps; (c) NaN₃, DMF, 65 °C, 64%; (d) 6 N HCl, 110 °C; (e) Fmoc-OSu, NaHCO₃, dioxane/H₂O (1:1), 30% for two steps. BOP-Cl, bis(2oxo-3-oxazolidinyl)phosphinic chloride.

groups and reaction with Fmoc-OSu afforded the protected azide in five steps and 12% overall yield (the strategy was not optimized).

The linear peptide **19** (Scheme 3) was prepared with a purity of 92%. In this case elongation of the peptide was performed by using DIPCDI/HOAt as the coupling reagent in the presence of the azide group. In fact, partial β -elimination of the azide group, which gave a dehydro amino acid, was observed in the presence of HATU and DIPEA and milder conditions for the coupling reactions were required. In general, in all cases, single coupling with 3 equiv. of Fmoc-amino acid, DIPCDI, and HOAt in DMF gave negative ninhydrin or chloranil tests. It is remarkable that only 2 equiv. of the azido acid **7** were necessary for its coupling reaction.

Once the linear precursor had been obtained, the feasibility of reducing the azide group on the CITrt-Cl resin was evaluated. In a first attempt, the Staudinger reaction^[20] was considered the most attractive method as a result of its mildness. In this reaction, triphenyl- or trimethylphosphane in THF was used to convert the azides into iminophosporanes, which were then easily hydrolyzed to afford the amine. Some examples of azide reduction on solid phases^[21] by using the Staudinger reaction have been reported in the literature. However, to the best of our knowledge, this is the first time that this reaction has been used for the preparation of aza-Thr-containing peptides on a solid phase.

The first step, namely the synthesis of iminophosphorane **20**, proved to be quite slow and even by using a higher concentration of triphenylphosphane (0.3 M in THF), the reaction was almost complete only after 48 h.^[15] Because long reaction times were considered to be impractical, the reaction was performed by using a 0.5 M solution of



Scheme 3. Solid-phase reduction of the azide group for the synthesis of KF analogues 5 and 6. Reagents and conditions: (a) piperidine/ DMF (2:8); (b) Fmoc-aa-OH or (4S)MeHex acid, DIPCDI, HOAt, DMF; (c) PPh₃, THF; (d) THF, H₂O, 75 °C; (e) SnCl₂, PhSH, DIEA, THF.

P(CH₃)₃ in THF. Under these conditions the synthesis of iminophosphorane is generally quicker.^[21] In this case, however, the reaction proved to be equally slow and a complex reaction mixture was observed after 12 h. Furthermore, the hydrolysis of 20 proved to be unexpectedly very difficult. Several attempts were made to hydrolyze 20 by using different ratios of THF/H₂O (4:1, 10:1, 1:4, 0:1), basic conditions (pyridine or NH4OH),[22] and microwave irradiation.^[23] In all cases, no amine 21 was obtained for short reaction times whereas for longer reaction times decomposition or the formation of a complex mixture was observed. Recently, some researchers reported the slow hydrolysis of iminophosphoranes, which was addressed by the addition of acid.^[24] However, this option was not compatible with our solid-phase system.

The steric hindrance and low accessibility of the high lipophilic peptide chain could explain the troubles with this step, as has also been pointed out by Vaultier et al., who reported different reactivities of iminophosphoranes depending on the character of the carbon (primary, secondary, or tertiary) bonded to the functional group.^[25] In a totally different context, Diederichsen and co-workers very recently reported that undesired azide reduction in a peptide depends on the position of the azide-containing moiety in the peptide.^[26] Moreover, even though a variety of reagents have been used for the reduction of azides, in terms of their practical applicability, most of these methods have certain disadvantages, especially for a solid support.^[18]

Bartra and Vilarrasa and co-workers reported that tin(II) complexes, prepared by treatment of $SnCl_2$ or $Sn(SR)_2$ with appropriate amounts of RSH and tertiary amines, are efficient reducing agents for the transformation of azides to amines.^[27] Some examples have been reported^[28] in which these conditions have been applied to azide reduction in the solid phase. However, to the best of our knowledge, they have not been used for peptide synthesis on the less robust linker of the chlorotrityl resin. To obtain the amine 21 (Scheme 3), we added a freshly prepared solution of 0.2 M SnCl₂, 0.8 M PhSH, and 1.0 M DIEA in THF to a suspension of the peptidyl resin in THF. After 1 h the reaction was not complete and after four treatments $(4 \times 3 h)$ the reaction proceeded no further and 66% of amine 21 and 34% of the starting material were detected by HPLC analysis.^[15] With regard to the difficulty of this step, the moderate yield was considered a good compromise and given that the reaction is relatively clean, the remaining steps for the synthesis of the KF analogues 5 and 6, shown in Scheme 4, were performed directly on the mixture. The methyl group, when required, was introduced into the o-nosyl-protected amine under Mitsunobu conditions but a longer reaction



Scheme 4. Solid-phase synthesis of KF analogues 5 and 6. Reagents and conditions: (a) o-NBS-Cl, DIEA, DCM; (b) PPh₃, MeOH, DIAD, THF; (c) HOCH₂CH₂SH, DBU, DMF; (d) Fmoc-aa-OH or Alloc-Phe-(Z)Dhb-OH, HATU, DIEA, DMF; (e) piperidine/DMF (2:8); (f) [Pd(PPh₃)₄], PhSiH₃, DCM; (g) TFA/DCM (1:99); (h) DIPCDI, HOBt, DIEA, DCM; (i) TFA/H₂O (19:1).

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time and more treatments were necessary to complete each step. The products were purified by using a semipreparative column to afford the two pure compounds.^[15] This approach afforded the crude cyclopeptides $5^{[16]}$ and 6 in satisfactory yields (23 and 59%, respectively) and purity.

Conclusions

Herein we have described the synthesis of six novel KF analogues of Kahalalide F (2–6 and 15) containing amide and *N*-Me-amide bonds in place of the depsi bond. Elongation of the peptide sequence and *N*-methylation, when required, were carried out in the solid phase. The aza-Thr derivatives, which are the most challenging derivatives from a synthetic point of view, were prepared by using (2R,3R)-2-amino-3-azidobutanoic acid as the aza-Thr precursor. Reduction of the azide to the amino function was performed in the solid phase. Although the scope of this reduction can be sequence-dependent, this method may be useful for the preparation of other aza-Thr-containing peptides. Aza-Kahalalide F showed stability in human serum for 72 h. Biological tests on tumor cell lines are currently in progress and will be reported in due course.

Experimental Section

General: Details of materials, methods, HPLC profiles and complete NMR spectroscopic characterization of compounds **6** and **7** are reported in the Supporting Information.

Synthesis of 3 and 4

Synthesis of Resin 9: Cl-TrtCl resin (0.2 g, 1.54 mmol/g) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disc. The resin was then washed with CH_2Cl_2 (5×0.5 min) and a solution of Fmoc-D-Val-OH (67 mg, 0.2 mmol) and DIEA (0.050 mL, 0.31 mmol) in CH_2Cl_2 (1.0 mL) was added. The mixture was then stirred for 10 min. Further DIEA was then added (0.158 mL, 0.93 mmol) and the mixture was stirred for 50 min. The reaction was completed by the addition of MeOH (0.250 mL) after stirring for 10 min. The Fmoc-D-Val-O-TrtCl resin was subjected to washings/treatments with CH_2Cl_2 (3×0.5 min) and DMF (3×0.5 min) and was then incubated three times with 20% piperidine/DMF (3 mL, v/v) on a shaker platform for 2 min and 2×10 min followed by extensive washing with DMF (3×3 mL) and CH_2Cl_2 (3×3 mL). The loading was 0.77 mmol/g, as calculated by Fmoc determination.

Fmoc-D-*allo*-Ile-OH (217 mg, 0.62 mmol, 4 equiv.), Fmoc-D-Dap(Alloc)-OH (253 mg, 0.62 mmol, 4 equiv.), Fmoc-D-*allo*-Ile-OH (217 mg, 0.62 mmol, 4 equiv.), Fmoc-Orn(Boc)-OH (280 mg, 0.62 mmol, 4 equiv.), Fmoc-D-Pro-OH (207 mg, 0.62 mmol, 4 equiv.), and Fmoc-D-Val-OH (209 mg, 0.62 mmol, 4 equiv.), Fmoc-Val-OH (261 mg, 0.77 mmol, 5 equiv.), Fmoc-Thr(*t*Bu)-OH (0.306 mg, 0.77 mmol, 5 equiv.), Fmoc-D-Val-OH (209 mg, 0.62 mmol, 4 equiv.), and (4*S*)-MeHex-OH (80 mg, 0.62 mmol, 4 equiv.) were added sequentially to the H-D-Val-O-TrtCl resin described above using HATU (234 mg, for 0.62 mmol and 4 equiv.; 293 mg, for 0.77 mmol and 5 equiv.) and DIEA (210 μ L, for 1.24 mmol and 8 equiv.; 261 μ L, for 1.54 mmol and 10 equiv.) in DMF (1.0 mL). In all the cases, after 90 min of coupling, the ninhydrin or chloranil test, when required, were negative. Removal of

the Fmoc group and washings were carried out as described above. The Alloc group was removed with $[Pd(PPh_3)_4]$ (18 mg, 0.015 mmol, 0.1 equiv.) in the presence of PhSiH₃ (190 µL, 1.5 mmol, 10 equiv.) under Ar followed by extensive washing with CH₂Cl₂ (3×3 mL), 0.02 M sodium diethyldithiodicarbamate in DMF (3×3 mL), DMF (3×3 mL), and CH₂Cl₂ (3×3 mL). An aliquot of the peptidyl resin was treated with 1% TFA in CH₂Cl₂ and HPLC analysis of the crude **9** obtained after evaporation showed a purity of 94%. HPLC: $t_R = 5.6 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 50 to 100; column: SunFireTM C₁₈, 3.5 µm, 4.6×100 mm; detection 220 nm. MS (ES): calcd. for C₆₅H₁₁₈N₁₂O₁₅ 1307.7; found 1308.9 [M + H]⁺. At this point the resin was divided into two equal portions.

Synthesis of 3: A solution of o-NBS-Cl (51 mg, 0.23 mmol) and 2,4,6-collidine (0.050 µL, 0.38 mmol) in CH₂Cl₂ was added to the resin and the mixture was stirred for 60 min. This reaction was repeated under the same conditions three times. After filtration and washing with CH₂Cl₂ (1×0.5 min), DMF (3×0.5 min), and CH_2Cl_2 (3 × 0.5 min), a solution of PPh₃ (101 mg, 0.38 mmol) and MeOH (31 µL, 0.77 mmol) in THF was added to the resin and stirred for 1 min and then a solution of DIAD (76 µL, 0.38 mmol) in THF was added to the peptidyl resin. After stirring the resin for 20 min, it was washed with CH_2Cl_2 (1×0.5 min), DMF $(2 \times 0.5 \text{ min})$, and CH₂Cl₂ $(3 \times 0.5 \text{ min})$. This reaction was repeated under the same conditions three times. After treatment $(2 \times 15 \text{ min})$ with DBU (115 µL, 0.77 mmol) and 2-mercaptoethanol (108 µL, 1.54 mmol) in DMF (1.5 mL), the resin was washed with DMF $(3 \times 0.5 \text{ min})$, CH₂Cl₂ $(3 \times 0.5 \text{ min})$, and DMF $(3 \times 0.5 \text{ min})$. Fmoc-Val-OH (130 mg, 0.77 mmol, 5 equiv.) and Alloc-Phe-(Z)-Dhb-OH (205 mg, 0.62 mmol, 4 equiv.) were added sequentially using HATU (117 mg, for 0.31 mmol and 4 equiv.; 146 mg, for 0.38 mmol and 5 equiv.) and DIEA (105 µL, for 0.62 mmol and 8 equiv.; 130 µL, for 0.77 mmol and 10 equiv.) in DMF (1.0 mL). After 90 min of coupling, the chloranil and ninhydrin tests were negative. Removal of the Fmoc and Alloc groups and washings were carried out as reported before. The protected peptide was cleaved from the resin by TFA/CH₂Cl₂ (1:99) (8×2 s), the filtrate was collected in H₂O (70 mL) and H₂O was partially removed under reduced pressure. A light-yellow precipitate appeared in the remaining water. Acetonitrile was then added to dissolve this solid until the solution turned clear. The mixture was then lyophilized to give 125 mg (0.071 mmol) of the linear protected peptide 12 with a purity of >98%, as shown by HPLC [$t_R = 6.9 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 40 to 100, at room temperature; $t_{\rm R}$ = 5.5 min, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 50 to 100 at 40 °C; column: SunFireTM C₁₈, $3.5 \,\mu\text{m}, 4.6 \times 100 \,\text{mm}; \text{detection } 220 \,\text{nm}].$

The protected peptide (125 mg, 0.071 mmol) was dissolved in CH2Cl2 (71 mL, 1 mM). HOBt (43 mg, 0.284 mmol, 4 equiv.) dissolved in the minimum volume of DMF, DIEA (28 µL, 0.163 mmol, 2.3 equiv.), and DIPCDI (44 µL, 0.284 mmol, 4 equiv.) were progressively added. The mixture was stirred on a shaker platform overnight. Then the solvent was removed by evaporation under reduced pressure. The protected cyclic peptide was dissolved in TFA/H₂O (19:1; 11.4:0.6 mL) and the mixture was stirred for 1 h. Most of the solvent was removed by evaporation under reduced pressure. The residue was then added dropwise to cold diethyl ether. A white precipitate appeared that was centrifuged and washed with cold diethyl ether three times, dissolved in CH₃CN/H₂O (1:1), and lyophilized to afford 114 mg of the crude product. HPLC showed a mixture containing two main products $[t_{\rm R} = 16.0 \, \text{min} \ (3, 47\%) \text{ and } 26.0 \, \text{min} \ (15, 32\%); \ CH_3CN$ (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to 60; column:

Symmetry C_{18} , 5 µm, 7.8 × 100 mm, detection at 220 nm]. 66 mg of the crude product was purified by HPLC under the same conditions to afford 5.6 mg of pure **3** and 8 mg of pure **15**.

3: MS (MALDI-TOF): calcd. for $C_{75}H_{125}N_{15}O_{15}$ 1475.95; found 1476.95 [M + H]⁺, 1499.02 [M + Na]⁺, 1515.0 [M + K]⁺.

15: MS (MALDI-TOF): calcd. for $C_{150}H_{250}N_{30}O_{30}$ 2951.90; found 2952.91 [M + H]⁺, 2974.89 [M + Na]⁺, 2990.86 [M + K]⁺.

Synthesis of 4: Sequence elongation, cyclization, and deprotection of the side-chains were realized as described. After deprotection, no precipitation was observed. The crude (206 mg) was recovered after lyophilization and analyzed by HPLC [$t_R = 4.5 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to 100; column: SunFireTM C₁₈, 3.5 µm, 4.6 × 100 mm; detection at 220 nm]. 20 mg of the crude product was purified by HPLC [$t_R = 15.3 \text{ min CH}_3$ CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to 60; column: Symmetry C₁₈, 5 µm, 7.8 × 100 mm, detection at 220 nm] to afford 2.0 mg of pure **4**.

4: MS (MALDI-TOF): calcd. for $C_{75}H_{125}N_{15}O_{15}$ 1475.95; found 1476.95 [M + H]⁺, 1499.02 [M + Na]⁺, 1515.0 [M + K]⁺.

Synthesis of Fmoc-Protected (2*R*,3*R*)-2-Amino-3-azidobutanoic Acid 7

Synthesis of 17: tert-Butyl carbazate (1.8 g, 13.7 mmol), BOP-Cl (4.6 g, 17.9 mmol), and DIEA (4.8 mL, 27.4 mmol) were added to a solution of Boc-D-threonine (16; 3.0 g, 13.7 mmol) in dry CH₂Cl₂ (70 mL) at 0 °C over 10 min. The mixture was warmed to room temperature and after 6 h was concentrated, dissolved in EtOAc, and washed with a solution of 1 N NaHCO₃, 1 N H₂SO₄, and a saturated aqueous solution of NaCl. The combined organic phases were concentrated and dried (MgSO₄) to give the crude product (4.50 g), which was used without further purification in the next step. TEA (3.8 mL, 2.70 mmol) and MsCl (1.9 mL, 2.43 mmol) were added to the crude product (4.5 g, 13.5 mmol) in dry CH₂Cl₂ (110 mL) at 0 °C over 10 min. The mixture was warmed to room temperature and after 1 h a saturated aqueous solution of NaCl was added and the mixture was extracted with CH₂Cl₂ $(2 \times 100 \text{ mL})$. The combined organic phases were concentrated and dried (MgSO₄) to give the crude product, which was purified by flash chromatography (Hex/EtOAc from 70:30 to 20:80) to give 17 as a white amorphous solid (3.63 g, 65% for two steps).

$$\label{eq:alpha} \begin{split} & [a]_{\rm D} = -10.9 \ (c = 1.0, \ {\rm CHCl_3}). \ ^1{\rm H} \ {\rm NMR} \ (400 \ {\rm MHz}, \ {\rm CDCl_3}): \ \delta = \\ & 8.11 \ ({\rm br. s}, 1 \ {\rm H}, \ {\rm NH}), \ 6.39 \ ({\rm br. s}, 1 \ {\rm H}, \ {\rm NH}), \ 5.47 \ ({\rm br. d}, \ J = 8.6 \ {\rm Hz}, \\ & 1 \ {\rm H}, \ {\rm NH}), \ 5.29 \ ({\rm m}, 1 \ {\rm H}, \ 3-{\rm H}), \ 4.48 \ ({\rm m}, 1 \ {\rm H}, \ 2-{\rm H}), \ 3.03 \ ({\rm br. s}, 3 \ {\rm H}, \\ & {\rm CH_3SO_2}), \ 1.52-1.44 \ [{\rm m}, \ 21 \ {\rm H}, \ {\rm overlapped}, \ {\rm C(CH_3)_3} \ {\rm and} \ 4-{\rm H}] \ {\rm ppm}. \\ & {\rm ^{13}C} \ {\rm NMR} \ (100 \ {\rm MHz}, \ {\rm CDCl_3}): \ \delta = 168.9, \ 155.7, \ 149.7, \ 86.4, \ 82.2, \\ & 78.2, \ 57.0, \ 38.3, \ 28.0 \ (\times 6), \ 18.0 \ {\rm ppm}. \ {\rm MS} \ ({\rm ES}): \ {\rm calcd.} \ {\rm for} \\ & {\rm C_{15}H_{29}N_3O_8S} \ 411.2; \ {\rm found} \ 412.2 \ [{\rm M} + {\rm H}]^+. \end{split}$$

Synthesis of 18: NaN₃ (3.6 g, 55.4 mmol), was added to a solution of **17** (5.7 g, 13.8 mmol) in dry DMF (180 mL) at room temperature. The mixture was heated at 65 °C for 24 h and after the addition of water (150 mL) was extracted with hexane/Et₂O (1:1, 300×3). The combined organic phases were washed with water (3 × 150 mL) and a saturated aqueous solution of NaCl (3 × 150 mL) and then were dried (Na₂SO₄) and concentrated in vacuo to give crude **18** (3.0 g, 64%) as a white amorphous solid. This product was shown to be quite pure by HPLC analysis and was used in the next step without further purification. HPLC: t_R = 10.9 min, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 0 to 100; column: XBridgeTM C₁₈, 5 µm, 4.6 × 150 mm; detection 220 nm]. MS (ES): calcd. for C₁₄H₂₆N₆O₅ 358.39; found 359.2 [M + H]⁺.

Synthesis of 7: A 6 N HCl solution (34 mL) was added to **18** (1.6 g, 4.5 mmol). The mixture was initially warmed to 65 °C and the starting material slowly dissolved. The temperature was then increased to 110 °C. The reaction was checked by HPLC and after

24 h was stopped, concentrating the reaction mixture under reduced pressure. This crude product was used in the next step without further purification. HPLC: $t_{\rm R} = 2.3 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 0 to 100; column: XBridgeTM C₁₈, 5 µm, 4.6 × 150 mm; detection 220 nm]. MS (ES): calcd. for C₄H₈N₄O₂ 144.0; found 144.9 [M + H]⁺.

Fmoc-OSu (1.95 g, 5.8 mmol) and NaHCO₃ (2.4 g, 2.8 mmol) were added to a solution of the crude product (4.5 mmol) in a mixture of dioxane/H₂O (1:1, 38 mL) at 0 °C. The mixture was allowed to react at room temperature for 5 h under controlled pH (8–9). H₂O (30 mL) was added to the mixture, which was extracted twice with EtOAc (80 mL each). Water was acidified to give pH 3–4 and extracted with EtOAc (3×200 mL). The combined organic phases were washed with H₂O (2×100 mL) and brine (100 mL), dried (Na₂SO₄), and concentrated in vacuo to give 7 (0.477 g, 30% for two steps) as a white amorphous solid. This product was quite pure, as shown by HPLC analysis [$t_R = 12.2$ min, CH₃CN (plus 0.036% TFA) in H₂O (plus 0.045% TFA): from 0 to 100; column: XBridgeTM C₁₈, 5 µm, 4.6 × 150 mm; detection 220 nm].

 $\begin{bmatrix} a \end{bmatrix}_{\rm D} = -57.5 \ (c = 0.3, \ {\rm CHCl_3}). \ ^1{\rm H} \ {\rm NMR} \ (400 \ {\rm MHz}, \ {\rm CDCl_3}): \delta = 7.76 \ ({\rm m}, 2 \ {\rm H}, \ {\rm ArH}), \ 7.59 \ ({\rm m}, 2 \ {\rm H}, \ {\rm ArH}), \ 7.40 \ ({\rm m}, 2 \ {\rm H}, \ {\rm ArH}), \ 7.31 \ ({\rm m}, 2 \ {\rm H}, \ {\rm ArH}), \ 5.60 \ ({\rm d}, \ J = 8.3 \ {\rm Hz}, \ 1 \ {\rm H}, \ {\rm NH}), \ 4.51 \ ({\rm dd}, \ J = 8.0, \ 3.2 \ {\rm Hz}, \ 1 \ {\rm H}, \ 2-{\rm H}), \ 4.43 \ ({\rm m}, \ 2 \ {\rm H}, \ {\rm CH_2-Fmoc}), \ 4.23 \ ({\rm t-like}, \ J = 6.8 \ {\rm Hz}, \ 1 \ {\rm H}, \ {\rm CH-Fmoc}), \ 3.95 \ ({\rm m}, \ 1 \ {\rm H}, \ 3-{\rm H}), \ 1.42 \ ({\rm d}, \ J = 6.6 \ {\rm Hz}, \ 3 \ {\rm H}, \ 4-{\rm H}) \ {\rm ppm}. \ \ ^{13}{\rm C} \ {\rm NMR} \ (100 \ {\rm MHz}, \ {\rm CDCl_3}): \ \delta = 173.0, \ 155.9, \ 143.6, \ 143.5, \ 141.0 \ (\times 2), \ 127.8 \ (\times 2), \ 127.1 \ (\times 2), \ 125.0 \ (\times 2), \ 120.0 \ (\times 2), \ 67.4, \ 58.6, \ 57.6, \ 47.1, \ 15.6 \ {\rm ppm}. \ {\rm MS} \ ({\rm ES}): \ {\rm calcd.} \ {\rm for} \ {\rm C_{19}H_{18}N_4O_4 \ 366.37; \ {\rm found} \ 367.2 \ [{\rm M} + {\rm H}]^+. \ \ {\rm Her}$

Synthesis of 5 and 6

Synthesis of 19: Cl-TrtCl resin (0.300 g, 1.54 mmol/g) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disc. The resin was then washed with CH_2Cl_2 (5×0.5 min) and a solution of Fmoc-D-Val-OH (100 mg, 0.269 mmol) and DIEA (0.078 mL, 0.462 mmol) in CH₂Cl₂ (0.5 mL) was added. The mixture was then stirred for 10 min. Further DIEA was then added (0.235 mL, 1.39 mmol) and the mixture was stirred for 50 min. MeOH (0.24 mL) was added and the reaction was completed after 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) and DMF (3×0.5 min), and the resin was then incubated three times with 20% piperidine/DMF (3 mL, v/v) on a shaker platform for 2 min and 2×7 min followed by extensive washing with DMF $(3 \times 3 \text{ mL})$ and CH₂Cl₂ $(3 \times 3 \text{ mL})$. The loading was 0.84 mmol/g, as calculated by Fmoc determination. Fmoc-D-allo-Ile-OH (268 mg, 0.76 mmol, 3 equiv.), 7 (185 mg, 0.50 mmol, 2 equiv.), Fmoc-D-allo-Ile-OH (273 mg, 0.76 mmol, 3 equiv.), Fmoc-Orn(Boc)-OH (344 mg, 0.76 mmol, 3 equiv.), Fmoc-D-Pro-OH (256 mg, 0.76 mmol, 3 equiv.), and Fmoc-D-Val-OH (258 mg, 0.76 mmol, 3 equiv.), Fmoc-Val-OH (258, mg, 0.76 mmol, 3 equiv.), Fmoc-Thr(tBu)-OH (302 mg, 0.77 mmol, 3 equiv.), Fmoc-D-Val-OH (258 mg, 0.76 mmol, 3 equiv.), and (4S)-MeHex-OH (99 mg, 0.76 mmol, 3 equiv.) were added sequentially to the H-D-Val-O-TrtCl resin described above using DIPCDI (117 µL, for 0.76 mmol and 3 equiv.; 78 µL, for 0.50 mmol and 2 equiv.), HOAt (103 mg, for 0.76 mmol and 3 equiv.; 69 mg, for 0.50 mmol and 2 equiv.) in DMF (1.2 mL). In all cases, after 90 min of coupling, the ninhydrin and chloranil tests were negative. Removal of the Fmoc group and washings were carried out as described above. An aliquot of the peptidyl resin was treated with 1% TFA in CH₂Cl₂ and HPLC analysis of the crude product obtained after evaporation showed a purity of 91% [$t_{\rm R}$ = 6.1 min CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 80 to 100; column: SunFireTM C₁₈, 3.5 µm, 4.6 × 100 mm, detection 220 nm]. MS (ES): calcd. C₆₆H₁₁₈N₁₄O₁₅ 1346.89; found 1347.5 [M + H]⁺.

Synthesis of 21. Bartra and Vilarrasa Reduction: A freshly prepared solution containing 0.2 M of SnCl₂, 0.8 M of PhSH, and 1 M of DIEA (6.3 mL) in dry THF was added to a suspension of the peptidyl resin **19** in dry THF (180 μ L). The suspension was stirred on a shaker platform for 1 h. This treatment was repeated under the same conditions three times. An aliquot of the peptidyl resin was treated with 1% TFA in CH₂Cl₂ and HPLC analysis of the crude product obtained after evaporation showed a purity of 66%. HPLC [$t_{\rm R} = 7.5 \text{ min CH}_3\text{CN}$ (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 5 to 100; column: SunFireTM C₁₈, 3.5 μ m, 4.6 × 100 mm, detection 220 nm]. MS (ES): calcd. for C₆₆H₁₂₀N₁₂O₁₅ 1321.7; found 1322.9 [M + H]⁺.

Synthesis of 5: Methylation, sequence elongation, and cleavage from the resin were performed as described above with some exceptions on two-thirds of the peptidyl resin 21. This time nosylation and methylation proved to be more difficult and the reactions were repeated four and five times, respectively. The protected peptide was cleaved from the resin by TFA/CH₂Cl₂ (1:99) (8×2 min), the filtrate was collected in H₂O (135 mL), and the H₂O was partially removed under reduced pressure. A light-yellow precipitate appeared in the remaining water. Acetonitrile was then added to dissolve this solid until the solution turned clear. The mixture was then lyophilized to give a crude product (218 mg) containing the title compound [25%, as shown by HPLC analysis: $t_{\rm R}$ = 7.2 min, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to 100 at 40 °C; column: SunFireTM C₁₈, 3.5 μ m, 4.6 × 100 mm; detection 220 nm]. Cyclization and deprotection of the side-chains were realized as described above. The protected cyclic peptide was dissolved in TFA/H₂O (19:1; 22.8:1.2 mL) and the mixture was stirred for 1.5 h. Most of the solvent was removed by evaporation under reduced pressure and then the residue was added dropwise to cold diethyl ether. A white precipitate appeared and was centrifuged and washed with cold diethyl ether three times, dissolved in CH₃CN/ H₂O (1:1), and lyophilized to afford 206 mg of the crude product, which was then analyzed by HPLC [5: $t_R = 6.0 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 40 to 60; column: SunFireTM C₁₈, 3.5 μ m, 4.6 × 100 mm; detection at 220 nm]. 57 mg of the crude product was purified by HPLC [$t_{\rm R}$ = 6.0 CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 45 to 60, column: Symmetry C₁₈, 5 μ m, 7.8 × 100 mm, detection at 220 nm] to afford 5.8 mg of 5.

5: MS (MALDI-TOF): calcd. for $C_{76}H_{127}N_{15}O_{15}$ 1489.96; found 1513.02 [M + Na]⁺, 1529.0 [M + K]⁺. A complete description of the chemical shifts and NMR spectroscopic characterization data for compound **5** are reported in the Supporting Information.

Synthesis of 6: Sequence elongation was performed on one-third of the peptidyl resin 21 as described before. Fmoc-Val-OH (114 mg, 0.34 mmol, 4 equiv.) and Alloc-Phe-(*Z*)DhbOH (112 mg, 0.34 mmol, 4 equiv.) were added sequentially using HATU (129 mg, 0.34 mmol, 4 equiv.) and DIEA (114 μ L, 0.67 mmol, 4 equiv.) in DMF (1.0 mL). After 90 min the ninhydrin test was negative. The Alloc group was removed with [Pd(PPh_3)_4] (10 mg, 0.0084 mmol, 0.1 equiv.) in the presence of PhSiH₃ (91 μ L, 0.84 mmol, 10 equiv.) under Ar followed by extensive washing with CH₂Cl₂ (3 × 3 mL), 0.02 M sodium diethyldithiodicarbamate in DMF (3 × 3 mL), DMF (3 × 3 mL), and CH₂Cl₂ (3 × 3 mL). The

protected peptide was cleaved from the resin by TFA/CH₂Cl₂ (1:99; 8×2 min). The filtrate was collected in H₂O (130 mL) and the H₂O was partially removed under reduced pressure. A light-yellow precipitate appeared in the remaining water. Acetonitrile was then added to dissolve this solid until the solution turned clear. Lyophilization afforded 112 mg of the linear protected peptide 26 with a purity of 57% as shown by HPLC [$t_R = 1.9 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 80 to 100; column: SunFireTM C₁₈, 3.5 µm, 4.6 × 100 mm; detection 220 nm]. Cyclization and deprotection of the side-chains were realized as described above. The protected cyclic peptide was dissolved in TFA/H₂O (19:1; 11.4:0.6 mL) and the mixture was stirred for 1.5 h. Most of the solvent was removed by evaporation under reduced pressure and then the residue was added dropwise to cold diethyl ether. A white precipitate appeared which was centrifuged and washed with cold diethyl ether three times, dissolved in CH₃CN/H₂O (1:1), and lyophilized to afford 90 mg of crude product which had a purity of 59%, as shown by HPLC analysis [$t_R = 5.0 \text{ min}$, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to 100; column: SunFireTM C₁₈, 3.5 μ m, 4.6 × 100 mm; detection at 220 nm. 50 mg of the crude product was purified by HPLC [$t_{\rm R}$ = 5.0, CH₃CN (+ 0.036% TFA) in H₂O (+ 0.045% TFA): from 30 to100; column: Symmetry C_{18} , 5 µm, 7.8 × 100 mm, detection at 220 nm] to afford 7.9 mg of 6.

6: MS (MALDI-TOF): calcd. for $C_{75}H_{125}N_{15}O_{15}$ 1475.95; found 1499.10 [M + Na]⁺. A complete description of the chemical shifts and NMR spectroscopic characterization for compound **6** are reported in the Supporting Information.

Supporting Information (see also the footnote on the first page of this article): General procedures, detailed experimental for the azide reduction reaction, and characterization data (HPLC and NMR).

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