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Total Solid-Phase Synthesis of Marine Cyclodepsipeptide IB-01212

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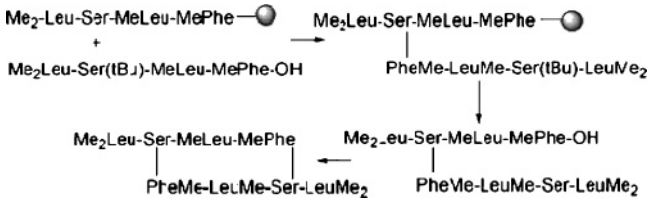

A suitable combination of synthetic design, orthogonal protecting groups and coupling reagents was used to complete the first known synthesis of the natural marine cyclodepsipeptide IB-01212. The cyclic, symmetric octapeptide contains two of each of the following residues: L-*N*,*N*-Me_2Leu, L-Ser, L-*N*-MeLeu and L-*N*-MePhe. IB-01212 also features two symmetric ester bonds between the hydroxyl group of Ser and the carboxyl function of the *N*-MePhe. Total solid-phase syntheses of the product was performed in parallel via three distinct routes: dimerization of heterodetic fragments, linear synthesis, and convergent synthesis. The convergent strategy gave the best results in terms of product yield and purity and is particularly suitable for the large-scale synthesis of IB-01212 and similar peptides.

Introduction

Marine sponges and tunicates, fungi, bacteria, and other lower animal forms are rich sources of structurally unusual, biologically active peptides.[1,2] These peptides exhibit a variety of activities, including insecticidal, antimicrobial, antiviral, antitumor, tumor promotive, antiinflammatory, and immunosuppressive actions.[1−3] Some of these compounds are in advanced clinical trials, and others have proven useful in studies directed toward the elucidation of biochemical pathways.[4] Their significant pharmacological variety is a function of peptide or depsipeptide structure and conformational diversity, specifically, rare residues such as D-amino acids, N- or C-alkylated amino acids, α,β-dehydro amino acids, hydroxyl acids and structurally elaborate amino acids such as the reverse prenyl (rPr) of two residues of Ser and Thr in trunkamide A.[5]

Solid-phase synthesis has proven especially utile in combinatorial chemistry and the synthesis of various non-peptide substrates.[6] Since the pioneering work of Merrifield,[7] this technique has become the standard for the preparation of certain families of natural oligomers, such as peptides,[8] nucleotides,[9] and oligosaccharides.[10] To date, no general solid-phase approach equivalent to that applied to peptides has been established for the preparation of depsides or depsipeptides, two structurally

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(1) Fusetani, N.; Matsunaga, S. *Chem. Rev.* **1993**, *93*, 1793.
(2) Davidson, B. S. *Chem. Rev.* **1993**, *93*, 1771.
(3) Fusetani, N., Ed.; *Drugs from the Sea*; S. Karger AG: Basel, Switzerland, 2000.
(4) Wipf, P. *Chem. Rev.* **1995**, *95*, 2115.
(5) Caba, J. M.; Rodríguez, I. M.; Manzanares, I.; Giralt, E.; Albericio, F. *J. Org. Chem.* **2001**, *66*, 7568.
(6) Kates, S. A., Albericio, F., Eds.; *Solid-Phase Synthesis. A Practical Guide*; Marcel Dekker: New York, 2000.
(7) (a) Merrifield, R. B. *J. Am. Chem. Soc.* **1963**, *85*, 2149. (b) Merrifield, R. B. *Angew. Chem., Int. Ed. Engl.* **1985**, *24*, 799. (c) Merrifield, R. B. *Science* **1986**, *232*, 341.
(8) (a) Stewart, J. M.; Young, J. D. *Solid-Phase Peptide Synthesis*, 2nd ed.; Pierce Chemical Co.: Rockford, IL, 1984. (b) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Chemical Approaches to the Synthesis of Peptides and Proteins*; CRC Press: Boca Raton, FL, 1997. (c) Fields, G. B.; Lauer-Fields, J. L.; Liu, R-q; Barany, G. In *Synthetic Peptides: A User's Guide*, 2nd ed.; Grant, G. A., Ed.; W. H. Freeman & Co.: New York, 2001; pp 93-219. (d) Goodman, M., Felix, A., Moroder, L. A., Toniolo, C., Eds.; *Houben-Weyl, Synthesis of Peptides and Peptidomimetics*; Georg Thieme Verlag: Stuttgart, Germany, 2002; Vol. E, pp 22a−e.
(9) Beaucage, S. L., Radhakrishnan, P. I. *Tetrahedron* **1992**, *48*, 2223.
(10) Seeberger, P. H.; Beebe, X.; Sukenick, G. D.; Pochapsky, S.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 491.

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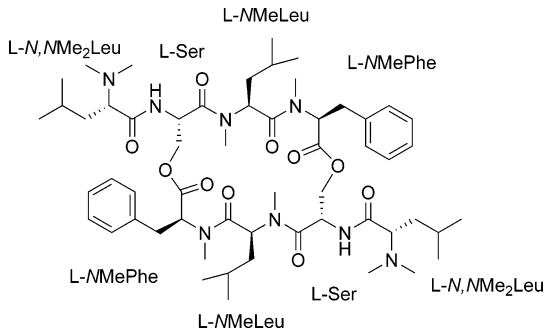


FIGURE 1. Structure of the cyclodepsipeptide IB-01212 isolated from the fermentation broth of a fungal strain *Clonostachys* sp. ESNA-A009.

related classes of compounds of primarily marine origin. Therefore, efficient synthetic routes to these molecules are needed, either for use in confirmation of the chemical structure of authentic samples or to ensure an adequate supply of homogeneous compounds for preclinical trials and SAR (structure−activity relationship) studies.[11,12]

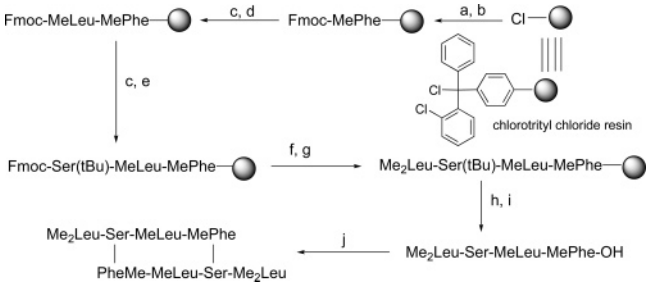
Recently, several biologically active cyclic peptides from marine sources have been synthesized on solid phase, including callipeltin B,[13] trunkamide A,[5] kahalalide B[14] and F,[15] phakellistatin 11,[16] and loloatins A−C.[17]

The cytotoxic cyclodepsipeptide IB-01212 was isolated from the mycelium extract of *Clonostachys* sp. ESNA-A009. Their structural determination was performed by NMR and MS/MS. The absolute configuration, in which all amino acids are in the L-configuration, was also determined by our group.[18] The cyclodepsipeptide IB-01212 is a symmetric octapeptide featuring a six-membered cyclic core, two each of L-*N*,*N*-Me_2Leu, L-Ser, L-*N*-MeLeu and L-*N*-MePhe. Herein several effective strategies for the solid-phase synthesis of cyclodepsipeptide IB-01212 in high yield and purity are discussed.

Results and Discussion

In the retrosynthetic analysis of macrocyclic peptides, ring disconnection is so strategically important that it can ultimately determine the success of a synthesis. Poor disconnections can lead to slow cyclization rates, facilitating side reactions such as oligomerization and/or epimerization of the C-terminal residue. The cyclization position should therefore be carefully chosen according to a number of simple guidelines.[19,20] However, designing a synthetic strategy for IB-01212 was challenging, as the compound does not contain any clear cyclization site. Retrosynthetic analysis of IB-01212 suggested that the only step that was not amenable to solid phase was the ester bond formation. Because of the typically low yield of esterifications on resin, we established that one of the two ester bonds in IB-01212 would be formed in solution.

SCHEME 1. Solid-Phase Strategy Developed for Preparation of Cyclodepsipeptide IB-01212 via Dimerization of Heterodetic Fragments[a]



[a] Reagents: (a) Fmoc-*N*-MePhe, DIEA, DCM; (b) MeOH; (c) piperidine/DMF (2:8) and piperidine/DBU/toluene/DMF (5:5:20:70); (d) Fmoc-*N*-MeLeu, PyBOP/HOAt/DIEA, DMF; (e) Fmoc-Ser(tBu)-OH/DIPCDI (2:1), DCM/DMF; (f) piperidine/DMF (2:8); (g) *N*,*N*-Me_2Leu, PyBOP/HOAt/DIEA, DMF; (h) TFA/DCM (1:99); (i) TFA/H_2O/TES (95:2.5:2.5); (j) MSNT/NMI/DIEA, DCM/DMF.

Strategy 1: Dimerization of Heterodetic Fragments. Thus, with the aim of preparing a sufficient quantity of IB-01212 for complete structural characterization and study of its biological activity, a simple and rapid method of synthesis was developed. Because of the symmetric character of the natural cyclodepsipeptide, it could be prepared by dimerization of heterodetic fragments as follows: (i) solid-phase elongation to the tetrapeptide, (ii) cleavage of the peptide from the resin, (iii) dimerization in solution of the symmetric fragment, and (iv) final purification. As depicted in Scheme 1, the synthesis of the tetrapeptide fragment was carried out using Fmoc/tBu chemistry on the super-acid-labile chlorotrityl chloride resin (ClTrt-Cl or Barlos resin).[21] A limited incorporation of the 9-fluorenylmethoxycarbonyl (Fmoc)-*N*-MePhe-OH on ClTrt-Cl resin was performed with *N*,*N*-diisopropylethylamine (DIEA).[22] The remaining resin chloride functions were capped with MeOH to avoid the formation of deletion sequences. Removal of the Fmoc protecting group was carried out with piperidine/DMF (1:4) and 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU)/piperidine/toluene/DMF (5:5:20:70) to cleave the Fmoc group completely. Fmoc-*N*-MeLeu-OH was then introduced with benzotriazol-1-yloxytris-(pyrrolidino)phosphonium hexafluorophosphate (PyBOP)/1-hydroxy-7-azabenzotriazole (3-hydroxy-3*H*-1,2,3-triazolo-[4,5-*b*]pyridine) (HOAt)/DIEA in DMF. This methodology is most effective for the coupling of Fmoc-aa-OH to NMe-amino acids.[23] However, if the crude product gave a positive result in the chloranil test for secondary amines, an elaboration of the 1-[bis-(dimethylamino)methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU) method should be provided. Otherwise, the process was continued by the incor-

(11) Xu, J. *J. Med. Chem.* **2002**, *45*, 5311.
(12) Nielsen, J. *Curr. Opin. Chem. Biol.* **2002**, *6*, 297−305.
(13) Lipton, M. A.; Hanshaw, N. T.; Benson, A. G.; Kowalski, J. A.; Kogut, E.; Ferguson, R. L.; Brunette, S. R.; Acevedo, C. M.; Alvira, E. *Abstracts of Papers*, 220th National Meeting of the American Chemical Society; American Chemical Society: Washington, DC, 2000; ORGN-038.
(14) López-Macià, À.; Jiménez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *Tetrahedron Lett.* **2000**, *41*, 9765.
(15) López-Macià, À.; Jiménez, J. C.; Royo, M.; Giralt, E.; Albericio, F. *J. Am. Chem. Soc.* **2001**, *123*, 11398.
(16) Pettit, G. R.; Lippert, J. W., III; Taylor, S. R.; Tan, R.; Williams, M. D. *J. Nat. Prod.* **2001**, *64*, 883.
(17) Scherkenbeck, J.; Chen, H.; Haynes, R. K. *Eur. J. Org. Chem.* **2002**, *14*, 2350.
(18) Cruz, L. J.; Martínez Insua, M.; Pérez Baz, J.; Trujillo, M.; Rodríguez-Mias, R. A.; Oliveira, E.; Giralt, E.; Albericio, F.; Cañedo, L. M. *J. Org. Chem.* **2005**, *70*, 3335.
(19) Anteunis, M. J. O.; Sharma, N. K. *Bull. Soc. Chim. Belg.* **1988**, *97*, 281.
(20) Kopple, K. D. J. *Pharm. Sci.* **1972**, *61*, 1345.
(21) (a) Barlos, K.; Gatos, D.; Schäfer, W. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 590. (b) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513.
(22) 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. Chiva, C.; Vilaseca, M.; Giralt, E,; Albericio, F. *J. Pept. Sci.* **1999**, *5*, 131.
(23) Albericio, F.; Cases, M.; Alsina, J.; Triolo, S. A.; Carpino, L. A.; Kates, S. A. *Tetrahedron Lett.* **1997**, *38*, 4853.

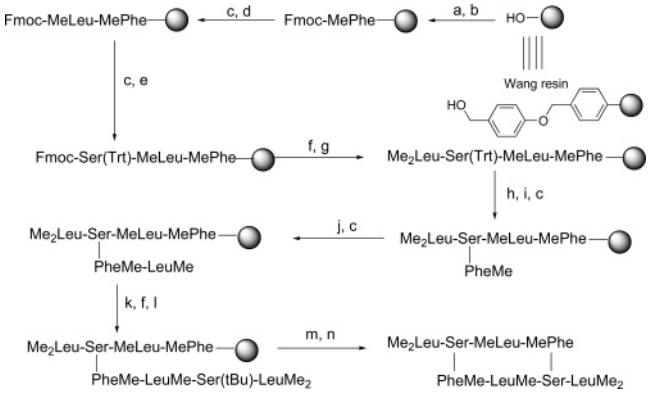
poration of Fmoc-Ser(tBu)-OH with substoichiometric amounts of *N*,*N*′-diisopropylcarbodiimide (DIPCDI) to form the asymmetric anhydride. The anhydride strategy combined with the use of Barlos resin minimized diketopiperazine formation.[24] Subsequently, Me_2Leu-OH prepared in solution using a catalytic reductive condensation with formaldehyde,[18] was coupled with PyBOP/HOAt/DIEA. The protected peptide fragment was cleaved from the resin by treatment with TFA/DCM (1:99). Finally, the tBu protecting group from the Ser was removed with TFA/H_2O/triethylsilane (TES) (95:2.5:2.5). The synthesis gave a good yield (78%) and the HPLC analysis of the crude product indicated a purity of 71%. The tetrapeptide fragment was characterized satisfactorily by mass spectrometry.

The dimer was prepared in solution via intermolecular cyclization of two ester bonds (between the hydroxyl group of Ser and the carboxyl group of *N*-MePhe) using 1-(mesitylene-2-sulfonyl)-3-nitro-1*H*-1,2,4-triazole (MSNT) and 1-methylimidazole (NMI) in DCM/DMF at a concentration of 5.6×10^{-3} M. The crude product was purified by medium pressure chromatography to give the title compound (2.3% yield), which showed a high purity by HPLC > 98% and was characterized satisfactorily by mass spectrometry (MALDI-TOF calcd 1032.66, found *m*/*z* 1034.33 $[M + H]^+$, 1056.28 $[M + Na]^+$, 1072.24 $[M + K]^+$). The low yield obtained for the cyclization was due to the formation of undesirable byproducts, which were isolated by reverse phase HPLC and identified by mass spectrometry as the cyclic monomer (MALDI-TOF calcd 516.33, found *m*/*z* 517.95 $[M + H]^+$, 539.91 $[M + Na]^+$, 555.89 $[M + K]^+$) and the cyclic trimer (MALDI-TOF calcd 1548.99, found *m*/*z* 1551.61 $[M + 1]^+$, 1573.56 $[M + Na]^+$, 1589.49 $[M + K]^+$).

Strategy 2: Linear Synthesis. The poor selectivity of the cyclization step prompted us to seek a new route to the dimer. The first alternative was a linear solid-phase approach with controlled formation of each ester bond. The hydroxyl side chain of the Ser was protected by two different groups: tBu and Trt, which can be removed on Wang resin without detaching the peptide from the resin. As observed in Scheme 2, this strategy implies formation of one of the ester bonds on solid-phase (between the hydroxyl and the carboxyl functions of Ser and *N*-MePhe, respectively). This feature is a drawback for the linear approach, as solid-phase esterifications are normally slower than those in solution.

The octapeptide was synthesized by Fmoc/tBu chemistry. Fmoc-MePhe was anchored on Wang resin by esterification with MSNT/NMI and DIEA. The remaining hydroxyl functions were acetylated with Ac_2O, NMI, and DIEA. To avoid diketopiperazine formation, dipeptide Fmoc-Ser(Trt)-*N*-MeLeu was incorporated directly with PyBOP/HOAt and DIEA. The dipeptide was obtained in solution by preparation of the pentafluorophenyl ester of the Fmoc-Ser(Trt) and subsequent reaction with free *N*-MeLeu using equimolar amounts of 1-hydroxybenzotriazole (HOBt), which increases the reaction rate.[25] The dipeptide was obtained in 76% yield, but with purity of only 18%. Purification of the crude product by semipreparative RP-HPLC afforded the desired dipeptide in a final yield of 14%. Taking into account the difficulties in the preparation of the dipeptide, further minimization of diketopiperazine formation was attempted by using the asymmetric anhydride method together with a shorter

SCHEME 2. Linear Approach (strategy 2) to Solid-Phase Synthesis of IB-01212[a]



[a] Reagents: (a) Fmoc-*N*-MePhe, MSNT/NMI/DIEA, DMF; (b) Ac_2O, NMI, DIEA; (c) piperidine/DMF (2:8) and piperidine/DBU/toluene/DMF (5:5:20:70); (d) Fmoc-*N*-MeLeu, PyBOP/HOAt/DIEA, DMF; (e) Fmoc-Ser(Trt)/DIPCDI (2:1), DCM/DMF; (f) piperidine/DMF (2:8); (g) *N*-Me_2Leu, PyBOP/HOAt/DIEA, DMF; (h) TFA/TES/DCM (1.5:5:93.5); (i) Fmoc-*N*-MePhe, MSNT/NMI/DIEA, DMF; (j) Fmoc-MeLeu-OH/DIPCDI (2:1), DCM/DMF; (k) Fmoc-Ser(tBu)/DIPCDI (2:1), DCM/DMF; (l) *N*-Me_2Leu/DIPCDI/HOAt, DMF; (m) TFA/H_2O/TES (95:2.5:2.5); (n) MSNT/NMI/DIEA, DCM/DMF.

reaction time Fmoc removal. An excess of the third residue, Fmoc-Ser(Trt), was then incorporated by the asymmetric anhydride method. The *N*-Me_2Leu was introduced with PyBOP/HOAt and DIEA. To continue the linear chain elongation, Trt group was selectively removed with TFA/DCM (1.5:98.5), which does not affect the peptide−Wang resin bond. Esterification of Fmoc-*N*-MePhe with the free hydroxyl group of Ser was performed with MSNT/NMI and DIEA for 3 h. A second coupling was then performed. The Fmoc-*N*-MeLeu was coupled via asymmetric anhydride to avoid using DIEA, which could affect the stability of the ester bond. The incorporation of Fmoc-Ser(tBu) was carried via the asymmetric anhydride. The *N*,*N*-Me_2Leu was coupled under neutral conditions with DIPCDI and HOAt, and the resulting octapeptide was subsequently cleaved from the Wang resin with TFA/DCM (19:1). After precipitation and lyophilization, the peptide was obtained in 62% yield, with a purity of 28% as shown by HPLC.

The macrocyclization was carried out at a concentration of 1.05×10^{-3} M with MSNT and NMI to reduce the risk of enantiomerization. Purification of the crude product by semipreparative HPLC gave IB-01212 in 4% yield, and with an excellent purity of 97.5% as shown by HPLC.

These results reveal two main disadvantages of the linear strategy: double diketopiperazine formation, which was detected by HPLC-MS after the third and seventh couplings, and the low stability of the ester bond between the hydroxyl group of the Ser and carboxyl function of the *N*-MePhe. Nevertheless, the linear strategy is superior to the dimerization of heterodetic fragments because of the higher yield and selectivity.

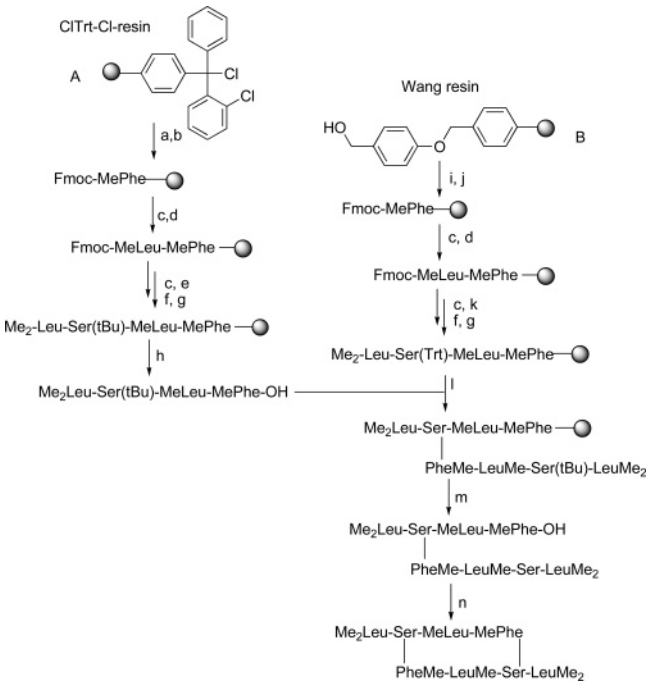
Strategy 3. Convergent Synthesis. The last alternative approach to IB-01212 that we explored was a convergent (4 + 4) synthesis, in which two tetrapeptide fragments were prepared on two different resins and then coupled together (Scheme 3). ClTrt-Cl resin[26] allowed selective cleavage of the protected

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

(25) (a) Simmonds, R. *Int. J. Pept. Protein Res.* **1996**, *47*, 36. (b) Albericio, F.; Chinchilla, R.; Dodsworth, D.; Nájera, C. *Org. Prep. Proc. Int.* **2001**, *33*, 203.

(26) The SASRIN resin is also compatible with this strategy. Using this resin, the Fmoc-*N*-MePhe was incorporated by esterification with MSNT, NMI, and DIEA. The remaining hydroxyl functions were acetylated with Ac_2O, NMI, and DIEA to avoid formation of deletion sequences. The

SCHEME 3. Convergent (4 + 4) Solid-Phase Synthesis of IB-01212[a]



[a] Reagents: (a) Fmoc-*N*-MePhe, DIEA, DCM; (b) MeOH; (c) piperidine/DMF (2:8) and piperidine/DBU/toluene/DMF (5:5:20:70); (d) Fmoc-*N*-MeLeu, PyBOP/HOAt/DIEA, DMF; (e) Fmoc-Ser(tBu) (8 equiv), DIPCDI (4 equiv), DCM/DMF; (f) piperidine/DMF (2:8); (g) *N*,*N*-Me_2Leu, PyBOP/HOAt/DIEA, DMF; (h) TFA/DCM (1:99); (i) Fmoc-*N*MePhe, MSNT/NMI/DIEA, DMF; (j) Ac_2O, NMI, DIEA; (k) Fmoc-Ser(Trt)/DIPCDI (2:1); (l) MSNT/NMI/DMF; (m) TFA/H_2O/TES (95:2.5:2.5); (n) MSNT/NMI/DIEA, DCM/DMF.

tetrapeptide Me_2Leu-Ser(tBu)-MeLeu-MePhe-OH, and Wang resin was stable to the removal of highly acid-labile side chain protecting groups. The Me_2Leu-Ser(tBu)-MeLeu-MePhe-OH fragment was then coupled to the Me_2Leu-Ser(Trt)-MeLeu-MePhe-Wang resin. The central ring of the resulting octapeptide was ultimately formed in solution via ester bond formation.

Both syntheses employed standard Fmoc/tBu chemistry. The synthesis of fragment A began with incorporation of Fmoc-*N*-MePhe onto ClTrt-Cl resin through ester bond formation using DIEA. The remaining Cl sites were capped by addition of MeOH. The tetrapeptide was assembled following the experimental procedure described in Scheme 3. The protected peptide fragment was obtained by cleavage with TFA/DCM (1:99).

The synthesis of fragment B began with anchoring of Fmoc-*N*-MePhe onto Wang resin by esterification with MSNT, NMI, and DIEA. The remaining hydroxyl functions were acetylated with Ac_2O, NMI, and DIEA. The tetrapeptide was assembled according to the procedure described above, except for the third residue, for which Fmoc-Ser(tBu) was replaced with Fmoc-Ser(Trt). Selective removal of the Trt group with TFA/DCM (1.5:98.5) was required to avoid cleaving the peptide−resin ester bond.

The free terminal carboxyl of the protected fragment (Me_2-Leu-Ser(tBu)-MeLeu-MePhe-OH) obtained from ClTrt-Cl resin was coupled to the free hydroxyl group of the Ser of Me_2Leu-Ser-MeLeu-MePhe-O-Wang resin using MSNT/NMI/DIEA or 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/HOAt/DIEA for 24 h. Simultaneous removal of the tBu group and cleavage of the octapeptide from the Wang resin was achieved with TFA/H_2O/TES (95:2.5:2.5) for 2 h. The crude peptide was obtained in 48% yield with a purity of 48% as shown by HPLC analysis.

The cyclization step was carried out with MSNT, NMI, and DIEA in DCM/DMF as described in Scheme 2. The crude cyclopeptide was purified by semipreparative RP-HPLC to give the target product in 14% yield and 96% purity. The pure cyclodepsipeptide was characterized satisfactorily by MS and ^{1}H and ^{13}C NMR spectra (500 MHz, $CDCl_3$), ^{1}H, ^{13}C, TOCSY (70 ms), GHSQC, GHMBC (see preceding manuscript).[18]

The cyclodepsipeptide IB-01212 has been synthesized by three new solid-phase routes: a dimerization of heterodetic fragments, a linear synthesis, and a convergent synthesis. The convergent method was determined to be the best strategy, as it provides IB-01212 in higher yields than the other methods and permits full characterization of the intermediates during synthesis. In contrast, the linear approach was low-yielding, primarily as a result of diketopiperazine formation, and the dimerization suffered from low selectivity in the cyclization step. Diketopiperazine formation in the linear approach could be further reduced by minimizing the basicity of the reaction conditions, which would imply the use of Fmoc in conjunction with an orthogonal protecting group such as Alloc that can be removed in neutral conditions, or by using Boc (i.e., acid-labile) chemistry.[27] The present work clearly demonstrates the feasibility of synthesizing this class of cyclodepsipeptides via convergent solid-phase synthesis.[28] The convergent method developed in this work should prove amenable to the synthesis of IB-01212 analogues.

Experimental Procedure

General Methods. *N*,*N*-Me_2Leu was synthesized as described in the preceding manuscript.[18] CLTrt-Cl-resin, protected Fmoc-amino acid derivatives, HOAt, PyBOP, and MSNT were purchased from different sources as well as DIEA, DIPCDI, piperidine, DMF, DCM, methanol, TFA, and CH_3CN (HPLC grade). 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.

Symmetry octadecylsilica (C_{18}) reverse-phase HPLC columns were 4.6 mm × 150 mm, 5 μm. Analytical HPLC was carried out on a instrument with photodiode array detectors equipped with a separation module and a software. UV detection was performed at 220 nm, and linear gradients of CH_3CN (+0.036% TFA) into H_2O (+0.045% TFA) were run at 1.0 mL/min flow rate from the following: (condition A) 5% to 100% over 20 min; (condition B) 20−50% over 40 min.

HPLC-MS analyses were performed on a system coupled to a double wavelength UV detector and a mass spectromet. UV detection was performed at 220 nm, and linear gradients from 5% to 100% of CH_3CN (+0.07% formic acid) into H_2O (+0.1% formic acid) were run at 1.0 mL/min flow rate. The peptides were purified by semipreparative RP-HPLC with a C_{18} reverse-phase column, 5 μm, 30 mm × 100 mm.

Vibration frequencies into the IR spectra are expressed in cm^{-1}. MALDI-TOF and ES (+)-MS analyses of peptide samples were

protected peptide fragment was obtained from this resin by treatment with TFA/DCM (2:98). The yield and quality of the synthetic process on the ClTrt-Cl resin were better than those obtained on SASRIN resin (data not shown). This may be due to greater diketopiperazine formation for the case of the SASRIN resin.

(27) Albericio, F. *Biopolymers* **2000**, *55*, 123.
(28) Lloyd-Williams, P.; Albericio, F.; Giralt, E. *Tetrahedron* **1993**, *49*, 11065.

performed using 2,5-dihydroxybenzoic acid (DHB). ^{1}H, ^{13}C, TOCSY, ROESY, GHMBC, and GHSQC spectra were recorded on either 400 and 500 MHz. ^{1}H and ^{13}C NMR chemical shifts are expressed in parts per million (ppm) downfield from tetramethylsilyl chloride (TMS) as internal reference. The coupling constants are expressed in Hertz and the multiplicities of the signals are indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet) and m (multiplet).

Solid-phase syntheses were carried out in polypropylene syringes (10−20 mL) fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by vacuum. Removal of the Fmoc group was carried out with piperidine/DMF (1 × 1 min, 2 × 10 min) and DBU/piperidine/DMF/toluene (5:5:70:20) (1 × 1 min, 2 × 5 min). Washings between deprotection, coupling, and again, deprotection steps were carried out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL solvent/g resin per treatment. Peptide syntheses and washes were performed at 25 °C. Solid-phase syntheses were monitored by RP-HPLC of the intermediates obtained from cleavage of small aliquots of peptidyl resin.

Fmoc-Ser(Trt)-OPfp. Fmoc-Ser(Trt)-OPfp was prepared as described by Packman et al.[29] To a solution of Fmoc-Ser(Trt)-OH (2.63 mmol, 1.5 g) in DCM/DMF (9:1) (10 mL) was added pentafluorophenol (2.63 mmol, 0.48 g) and DCC (3 mmol, 0.62 g) at 0 °C. Upon reaction completion (2 h), the dicyclohexylurea was filtered off. The filtrate was evaporated, and the residue was resuspended in EtOAc. The organic phase was washed with 10% aqueous Na_2CO_3 and H_2O. The organic phase was dried over $MgSO_4$ and evaporated to yield the desired Fmoc-Ser(Trt)-OPfp (1.7 g, 89% yield) with a purity of 97.5% as determined by HPLC (t_R = 17.59 min, condition A). TLC of the crude product indicated only one major component, which was UV positive. MALDI-TOF calcd for $C_{43}H_{30}F_5NO_5$ 735.20, found *m*/*z* 757.96 $[M + Na]^+$, 773.86 $[M + K]^+$.

Synthesis of Fmoc-Ser(Trt)-MeLeu-OH. To a solution of Fmoc-Ser(Trt)-OPfp (0.95 mmol, 0.7 g) in DCM (5 mL) was added *N*-MeLeu (1.5 mmol, 0.22 g) dissolved in DCM/DMF (1:1). The mixture was treated with DIEA (0.95 mmol, 162 μL) and a catalytic amount of HOBt. The mixture was left to stir at room temperature and the reaction was monitored by TLC with UV−visualization and RP-HPLC. Upon reaction completion, the solvent was removed by evaporation under reduced pressure, and the resulting solid was dissolved in EtOAc. The solution was washed with 10% citric acid, saturated brine, and H_2O. The organic phase was dried over Na_2SO_4, filtered, and evaporated to dryness to give Fmoc-Ser(Trt)-*N*-MeLeu-OH (0.5 g, 76%) with a purity of 18% as determined by HPLC (t_R = 16.18 min, condition A). The product was purified by semipreparative RP-HPLC using a gradient from 50% to 100% of CH_3CN (+1% TFA) to H_2O (+0.5% TFA). The pure product was obtained with a 99.3% purity and a 14% global yield. MALDI-TOF calcd for $C_{44}H_{44}N_2O_6$ 696.32, found *m*/*z* 719.24 $[M + Na]^+$, 735.21 $[M + K]^+$.

Strategy 1. Dimerization of Heterodetic Fragments. ClTrt-Cl resin (0.6 g, 1.6 mmol/g) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk and then resin washed with DCM (5 × 2 min), DMF (5 × 2 min), and DCM (5 × 2 min). A solution of Fmoc-*N*-MePhe-OH (240 mg, 0.6 mmol) and DIEA (162 μL, 1 equiv) in DCM (2 mL) was added, and the mixture was stirred for 5 min. DIEA (326 μL, 1.92 mmol, 2 equiv) was added, and the mixture was stirred for 1 h. The reaction quenched by the addition of MeOH (400 μL) with stirring for 15 min. The Fmoc-*N*-MePhe-O-Trt-Cl-resin was then washed with DCM (3 × 1 min) and DMF (3 × 1 min) and treated with piperidine/DMF (1 × 1 min, 2 × 10 min) and DBU/piperidine/toluene/DMF (5:5:20:70, 1 × 1 min, 2 × 5 min). The loading calculated by Fmoc determination was 0.7 mmol/g. The Fmoc-*N*-MeLeu (705 mg, 1.92 mmol, 2 equiv) was added using PyBOP (999.2 mg, 1.6 mmol, 2 equiv), HOAt (261.4 mg, 1.92 mmol, 2 equiv), and DIEA (978 μL, 5.8 mmol, 6 equiv) in DMF. After 1 h of coupling, the reaction gave a negative result for the chroranil test. The Fmoc group was removed as described above, and Fmoc-Ser(tBu) (2.94 g, 7.7 mmol, 8 equiv) was coupled sequentially with DIPCDI (588 μL, 3.8 mmol, 4 equiv) using the asymmetric anhydride method. After 3 h of coupling, the reaction gave a negative result for the chroranil test. The Fmoc group was removed by treatment with piperidine/DMF (1 × 1 min, 2 × 10 min). Next, the *N*,*N*-Me$_2$Leu-OH (307 mg, 1.92 mmol, 2 equiv) was coupled with PyBOP (999.2 mg, 1.92 mmol, 2 equiv), HOAt (261 mg, 1.92 mmol, 2 equiv), and DIEA (978 μL, 5.8 mmol, 6 equiv) for 2 h. The reaction was verified by the ninhydrin test.[30]

Once the tetrapeptide was synthesized, it was cleaved from the resin with TFA/DCM (1:99) (5 × 1 min). The combined filtrates were evaporated to dryness under reduced pressure. The residue was resuspended in TFA/H_2O/TES (95:2.5:2.5) and stirred for 2 h to eliminate the tBu group completely. The TFA was evaporated under reduced pressure, and the residue was dissolved in CH_3CN/H_2O (1:1) and then lyophilized to give the target compound (333 mg, 78% yield) with a purity of 71% as determined by HPLC (t_R 7.8 min, condition A). MALDI-TOF calcd for $C_{28}H_{46}N_4O_6$ 534.34, found *m*/*z* 535.25 $[M + H]^+$, 557.26 $[M + Na]^+$, 573.24 $[M + K]^+$).

To a solution of the crude tetrapeptide (30 mg, 0.056 mmol) in DCM/DMF/NMI (9:0.8:0.2, 10 mL) were added MSNT (60 mg, 0.152 mmol, 4 equiv) and DIEA (117 μL, 0.672 mmol, 12 equiv). The mixture was stirred, and the cyclization was monitored by HPLC-MS. Upon completion of the reaction, the solvent was removed by evaporation under reduced pressure. The cyclic peptide was dissolved in CH_3CN/H_2O (1:1) and purified by semipreparative RP-HPLC using a linear gradient from 15% to 70% of CH_3CN (+0.05% TFA) in H_2O (+0.1% TFA) for 30 min, 20 mL/min, detection at 220 nm, to give the title product (0.7 mg, 0.7 μmol, 2.3% yield, 98% purity). HPLC analysis (t_R = 29.1 min, condition B) and MALDI-TOF calcd for $C_{56}H_{88}N_8O_{10}$ 1032.66, found *m*/*z* 1034.33 $[M + H]^+$, 1056.28 $[M + Na]^+$, 1072.24 $[M + K]^+$.

Strategy 2. Linear Synthesis. Wang resin (1 g, 0.82 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. After washing of the resin, a solution of Fmoc-*N*-MePhe (658.3 mg, 1.64 mmol, 2 equiv), MSNT (486 mg, 1.64 mmol, 2 equiv), NMI (130 μL, 1.64 mmol, 2 equiv), and DIEA (855 μL, 4.92 mmol, 6 equiv) was added. The mixture was stirred for 3 h. The remaining hydroxyl functions were acetylated by treatment with Ac_2O/NMI/DIEA/DMF (2:1:1:6) for 30 min. The Fmoc group was removed as described above. Next, Fmoc-*N*-MeLeu (603 mg, 1.64 mmol, 2 equiv) was coupled using PyBOP (853.5 mg, 1.64 mmol, 2 equiv), HOAt (223.2 mg, 1.64 mmol, 2 equiv) and DIEA (570 μL, 3.28 mmol, 4 equiv) in DMF. After Fmoc removal and washing, Fmoc-Ser(tBu)-OH (2.515 g, 6.56 mmol, 8 equiv) was coupled with DIPCDI (508 μL, 3.28 mmol, 4 equiv) using the asymmetric anhydride method. After 3 h of coupling, the Fmoc group was removed and *N*,*N*-Me$_2$Leu-OH (262 mg, 1.64 mmol, 2 equiv) was incorporated with DIPCDI (254 μL, 1.64 mmol, 2 equiv) and HOAt (223 mg, 1.64 mmol, 2 equiv). The Trt protecting group was removed completely by treatment with TFA/TES/DCM (1.5:5:93.5) for 2 h. The TFA salt was neutralized by washing with DIEA/DCM (1:19). Fmoc-*N*-MePhe (1.316 g, 3.28 mmol, 4 equiv) was then esterified with MSNT (972 mg, 3.28 mmol, 4 equiv), NMI (260 μL, 3.28 mmol, 4 equiv) and DIEA (1.140 mL, 6.56 mmol, 8 equiv) for 3 h. A second coupling using similar conditions was carried out. Fmoc-*N*-MeLeu (1.205 g, 3.28 mmol, 4 equiv) was coupled with DIPCDI (254 μL, 1.64 mmol, 2 equiv) overnight. Similarly, Fmoc-Ser(tBu)-OH (2.515 g, 6.56 mmol, 8 equiv) was coupled with DIPCDI (508 μL, 3.28 mmol, 4 equiv) using the asymmetric anhydride method. Finally, *N*,*N*-Me$_2$Leu (262 mg, 1.64 mmol, 2 equiv) was incorporated with

(29) Packman, L. C.; Quibell, M.; Johnson, T. *Pept. Res*. **1994**, *7*, 125.

(30) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. I. *Anal. Biochem*. **1970**, *34*, 595.

DIPCDI (254 μL, 1.64 mmol, 2 equiv) and HOAt (223 mg, 1.64 mmol, 2 equiv). Simultaneous cleavage of the crude peptide from the Wang resin and removal of the tBu side chain protecting group was accomplished by treatment with TFA/TES/H_2O (95:2.5:2.5, 10 mL) for 2 h. The cleavage solution was filtered into 50 mL centrifuge tubes containing 30 mL of cold ether. After centrifugation (5 min at 4000 rpm) and decantation, the precipitate was washed two times by addition of cold ether. Finally, the peptide was dissolved in CH_3CN/H_2O (20 mL, 1:1, v:v) and lyophilized, to give the linear peptide (534.8 mg, 508 μmol, 62% yield). HPLC analysis of the crude product showed a purity of 28%. ES-MS calcd 1050.67 for $C_{56}H_{90}N_8O_{11}$, found *m*/*z* 1052.13.

Cyclization of the crude peptide (100 mg, 0.095 mmol) was carried out with MSNT (112.6 mg, 0.38 mmol, 4 equiv) and DIEA (198 μL, 1.14 mmol, 12 equiv) in DCM/DMF/NMI (9:0.8:0.2, 100 mL). The mixture was allowed to stir and the cyclization was monitored by HPLC-MS. Upon reaction completion, the solvent was removed by evaporation under reduced pressure. The cyclic peptide was dissolved in CH_3CN/H_2O (1:1) and purified by semipreparative RP-HPLC using the same conditions described above, to afford the target compound (4 mg, 4% yield, 97.5% purity). MALDI-TOF calcd for $C_{56}H_{88}N_8O_{10}$ 1032.66; found *m*/*z* 1034.54 $[M + H]^+$, 1056.87 $[M + Na]^+$, 1072.40 $[M + K]^+$).

Strategy 3. Convergent Synthesis. Synthesis of Fragment A (*N*,*N*-Me$_2$Leu-Ser(tBu)-*N*-MeLeu-*N*-MePhe-OH) using SASRIN or ClTrt-Cl Resin. The synthesis of the tetrapeptide (fragment A) on ClTrt-Cl resin was performed as described above, except that the amount of resin was doubled. SASRIN (1 g, 0.92 mmol/g) was placed in a 20 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). A solution of Fmoc-*N*-MePhe-OH (747 mg, 1.84 mmol, 2 equiv), MSNT (551 mg, 1.84 mmol, 2 equiv), NMI (146 μL, 1.84 mmol, 2 equiv), and DIEA (640 μL, 3.68 mmol, 4 equiv) in DMF was then added. The mixture was stirred for 3 h, at which point the remaining hydroxyl functions were acetylated with Ac_2O/NMI/DIEA/DMF (2:1:1:6) for 30 min. After washing three times with DMF for 5 min, the Fmoc protecting group was removed with piperidine/DMF (1 × 1 min, 2 × 10 min) and DBU/piperidine/toluene/DMF (5:5:20:70, 1 × 1 min, 2 × 5 min). Quantitative Fmoc-determination indicated a loading of 0.6−0.8 mmol per gram of dry resin. Next, Fmoc-*N*MeLeu (676 mg, 1.84 mmol, 2 equiv) was added using PyBOP (959 mg, 1.84 mmol, 2 equiv), HOAt (250 mg, 1.84 mmol, 2 equiv), and DIEA (937 μL, 5.52 mmol, 6 equiv) in DMF. After 1 h of coupling, the reaction gave a negative chroranil test result. Removal of the Fmoc protecting group and washes were carried out as described above. Fmoc-Ser(tBu) (1.342 g, 3.68 mmol, 4 equiv) was coupled with DIPCDI (270 μL, 1.84 mmol, 2 equiv) employing the asymmetric anhydride method. After 3 h of coupling, the reaction gave a negative result for the ninhydrin test. The last amino acid, *N*,*N*-Me$_2$Leu-OH (589 mg, 3.68 mmol, 4 equiv), was coupled using DIPCDI (570 μL, 3.68 mmol, 4 equiv) and HOAt (500 mg, 3.68 mmol, 4 equiv). The protected peptide fragment was cleaved from SASRIN resin with TFA−DCM (2:98). The crude obtained after evaporation was analyzed by RP-HPLC (t_R = 8.8 min, condition A) MALDI calcd 590.40, found *m*/*z* 591.57/613.27. Finally, the peptide was dissolved in CH_3CN/H_2O (1:1, 20 mL) and lyophilized to give 830 mg, 1.4 mmol, 76.5% yield.

Fragment B Synthesis: Me$_2$Leu-Ser-MeLeu-MePhe-O-Wang Resin. Wang resin (0.5 g, 0.82 mmol/g) was placed in a 10 mL polypropylene syringe fitted with a polyethylene filter disk. The resin was washed with DMF (5 × 1 min) and DCM (5 × 1 min). The hydroxyl groups (Wang resin) were esterified with a solution of Fmoc-*N*-MePhe (374 mg, 0.82 mmol, 2 equiv), MSNT (276 mg, 0.82 mmol, 2 equiv), NMI (65 μL, 0.82 mmol, 2 equiv), and DIEA (285 μL, 1.64 mmol, 4 equiv) in DMF. The remaining hydroxyl functions were acetylated as described above. The loading calculated by Fmoc determination was ca. 0.6 mmol per gram of dried resin. Next, Fmoc-*N*-MeLeu (342 mg, 0.82 mmol, 2 equiv) was added using PyBOP (484 mg, 0.82 mmol, 2 equiv), HOAt (127 mg, 0.82 mmol, 2 equiv), and DIEA (418 mg, 2.46 mmol, 6 equiv) in DMF. After 1 h of coupling, the reaction gave a negative result for the chroranil test. The Fmoc group was removed, and Fmoc-Ser(Trt)-OH (1.6 g, 1.64 mmol, 4 equiv) was coupled with DIPCDI (190 μL, 0.82 mmol, 2 equiv) employing the asymmetric anhydride method. After 3 h of coupling, the reaction gave a negative result for the ninhydrin test. The last amino acid, Me$_2$Leu-OH (262 mg, 1.64 mmol, 4 equiv), was coupled with DIPCDI (254 μL, 1.64 mmol, 4 equiv) and HOAt (223 mg, 1.64 mmol, 4 equiv). Washings between deprotection and coupling were carried out with DMF (5 × 1 min) and DCM (5 × 1 min) using 10 mL solvent/g resin for each treatment. Finally, the Trt protecting group was removed completely by treatment with TFA/TES/DCM (1.5:5:93.5) for 2 h. The TFA salt was neutralized by washing it with DIEA/DCM (81:19). An aliquot of the peptidyl resin was treated with TFA/TES/H_2O (95:2.5:2.5) and analyzed by RP-HPLC (t_R = 7.9 min, condition A) and MS (MALDI-TOF calcd for $C_{28}H_{46}N_4O_6$ 534.34, found *m*/*z* 535.25 $[M + H]^+$, 557.26 $[M + Na]^+$, 573.24 $[M + K]^+$).

Convergent Synthesis of Fragments A and B. The crude peptide (fragment A, *N*,*N*-Me$_2$Leu-Ser(tBu)-*N*-MeLeu-*N*-MePhe-OH, 485 mg, 0.82 mmol, 2 equiv) obtained from step 1 was coupled to the free hydroxyl group of the Ser (fragment B, *N*,*N*-Me$_2$Leu-Ser-*N*-MeLeu-*N*-MePhe-O-Wang resin, 500 mg, 0.410 mmol, 1 equiv) using MSNT (486 mg, 1.64 mmol, 4 equiv), NMI (130 μL, 1.64 mmol, 4 equiv), and DIEA (570 μL, 3.28 mmol, 8 equiv) in DMF. The reaction was monitored by taking an aliquot of the peptide-resin every 4 h, cleaving it with TFA, and analyzing the resulting crude product by RP-HPLC. Simultaneous cleavage of the peptide from the resin and deprotection of side chain protecting group was accomplished with TFA/TES/H_2O (95:2.5:2.5, 10 mL) for 2 h. The cleavage solution was filtered into 50 mL centrifuge tubes containing 30 mL of cold *tert*-butyl methyl ether. After centrifugation (5 min at 4000 rpm) and decantation, the precipitates were washed four times by addition of cold ether. The peptide was dissolved in CH_3CN/H_2O (1:1, 20 mL) and lyophilized, to give 206 mg, 195 umol, 48% yield. HPLC analysis of the crude linear peptide showed a 48% purity. ES-MS calcd for $C_{56}H_{90}N_8O_{11}$ 1050.67, found *m*/*z* 1052.13 $[M + H]^+$.

For the cyclization, the crude linear peptide (40 mg, 0.038 mmol) was dissolved in DCM/DMF/NMI (9:0.8:0.2, 40 mL), and MSNT (45 mg, 0.152 mmol, 4 equiv) and DIEA (79 μL, 0.456 mmol, 12 equiv) were added. The mixture was allowed to stir until the cyclization was shown by RP-HPLC to be complete. The solvent was removed by evaporation under reduced pressure. The cyclic peptide was dissolved in CH_3CN/H_2O (1:1) and purified by semipreparative RP-HPLC with a linear gradient from 15% to 70% of CH_3CN (+0.05% TFA) in H_2O (+0.1% TFA) for 30 min, 20 mL/min, detection at 220 nm, to give the title product (5.4 mg, 5.2 μmmol, 14% yield and 95% purity). MALDI-TOF calcd for $C_{56}H_{88}N_8O_{10}$ 1032.66, found *m*/*z* 1033.56 $[M + H]^+$.

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