

## **Synthesis of IB-01212 by Multiple *N*-Methylations of Peptide Bonds**

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## ABSTRACT

There are many natural peptides with multiple *N*-methylamino acids that exhibit potent attractive biological activities. *N*-Methylation of a peptide bond(s) is also one of the standard approaches in medicinal chemistry of bioactive peptides, to improve the potency and physicochemical properties, especially membrane permeability. In this study, we investigated a facile synthesis process of *N*-methylated peptides via simultaneous *N*-methylation of several peptide bonds in the presence of peptide bonds that were not to be methylated. As a model study, we investigated the synthesis of the antiproliferative depsipeptide, IB-01212. We used a pseudoproline to protect the non-methylated peptide bond during a simultaneous *N*-methylation with MeI-Ag<sub>2</sub>O. Using further manipulations including a dimerization/cyclization process, IB-01212 and its derivatives were successfully synthesized. A preliminary structure-activity relationship study demonstrated that the symmetric structure contributed to the potent cytotoxic activity of IB-01212.

## KEYWORDS

depsipeptide, macrolactonization, *N*-methylamino acid, *N*-methylation

Abbreviations: Me<sub>2</sub>Leu, *N,N*-dimethylleucine; MeLeu, *N*-methylleucine; MePhe, *N*-methylphenylalanine; MSNT, 1-(mesitylene-2-sulfonyl)-3-nitro-1,2,4-triazole; NMI, 1-methylimidazole; DIEA, *N,N*-diisopropylethylamine; Cbz, benzyloxycarbonyl.

## 1. Introduction

A number of naturally occurring peptides with attractive biological activities contain multiple *N*-methylamino acids.<sup>1</sup> For example, cyclosporine A is an orally available immunosuppressive drug, which is currently used in the clinic.<sup>2</sup> Echinomycin shows antibiotic and antitumor activities by bifunctional DNA intercalation.<sup>3</sup> The *N*-methylamino acids in these nonribosomal peptides contribute to their improved membrane permeability, required for recruitment to their site of action. *N*-Methylation of a peptide bond(s) is also a promising approach for improving the biological activities, metabolic stability and physicochemical properties of bioactive peptides.<sup>1,4-7</sup> Membrane permeability and oral bioavailability by *N*-methylation of multiple amide bonds in cyclic peptides have been systematically investigated.<sup>8-13</sup>

Although these highly *N*-methylated peptides could be attractive pharmaceutical resources,<sup>14,15</sup> their synthesis is not straightforward in standard peptide synthesis. During the peptide elongation process, the coupling of an amino acid to the *N*-methylamino group is often inefficient and prone to cause racemization at the  $\alpha$ -position of activated amino acids, because of the steric hindrance.<sup>16</sup> In the process of the solid-phase or solution-phase synthesis of cyclosporine peptides<sup>17,18</sup> and omphalotin A,<sup>19</sup> the coupling reactions of consecutive *N*-methylamino acids were optimized using various coupling reagents. An alternative approach to prepare partially *N*-methylated cyclic peptides was provided by the on-resin or solution-phase *N*-methylation strategy, in which the solvent-accessible amide NH groups were subjected to *N*-methylation in a regioselective manner.<sup>10,20</sup> However, this approach is not suitable for synthesis of a target peptide(s), because the backbone conformations automatically determine the solvent-accessible positions for *N*-methylation. To overcome these problems, we designed a novel synthesis approach for *N*-methylated peptides via simultaneous *N*-methylation of all peptide bonds while protecting the non-methylated peptide bond(s).

As a synthesis target for a case study, we chose the antiproliferative depsipeptide, IB-01212 (**1**), which was isolated from the mycelium extract of *Clonostachys* sp. ESNA-A009 (Figure 1).<sup>21</sup> IB-01212 (**1**) shows potent cytotoxic activity against several cancer cell lines. The C2-symmetric octapeptide **1** containing a pair of Me<sub>2</sub>Leu, Ser, MeLeu and MePhe was previously prepared via the solid-phase peptide synthesis of linear tetrapeptide **4**,<sup>22</sup> in which the coupling reactions to the *N*-methylamino group were optimized using different coupling reagents (Figure 1). In this article, we report a new synthesis approach for IB-01212 (**1**) and the structure-activity relationships of IB-01212 (**1**) and its derivatives.

## 2. Results and discussion

### 2.1. Synthesis of the linear precursor of IB-01212 via site-selective *N*-methylation of peptide bonds

We planned to synthesize linear precursor **4** via simultaneous *N*-methylation of peptide bonds while protecting a peptide bond not to be methylated of tetrapeptide **2** (Scheme 1). Among the several potential protected amide congeners, the pseudoproline dipeptide motif, Leu-Ser( $\psi^{\text{Me,Me}}$ pro), which was originally designed to improve the sequence assembly by preventing the formation of a secondary structure(s),<sup>23</sup> was employed assuming that the non-methylated Leu-Ser peptide bond in IB-01212 (**1**) could be protected under simultaneous *N*-methylation.

The synthesis of linear substrate **4** began with the coupling of the commercially available Cbz-protected Leu-Ser( $\psi^{\text{Me,Me}}$ pro) unit to Leu-Phe dipeptide **5** (Scheme 2). The resulting oxazolidine-containing peptide **6** was subjected to *N*-methylations under MeI-Ag<sub>2</sub>O conditions.<sup>24</sup> During this process, the serine  $\alpha$ -amino and  $\beta$ -hydroxy groups were successfully protected from methylation. Removal of the Cbz group of **7** followed by additional *N*-methylation at the N-terminus, and deprotection by treatment with TFA provided the linear tetrapeptide **4**.

With the linear tetrapeptide **4** in hand, we proceeded to investigate the one-pot dimerization/macrocyclization process for the synthesis of IB-01212 (**1**) according to a previous report.<sup>22</sup> Simultaneous dimerization and macrocyclization using MSNT<sup>25</sup> gave the desired IB-01212 (**1**) with a yield of 4.1%. The low yield was due to the concomitant production of significant amounts of unexpected cyclic monomer, trimer and epimerized dimer during this process,<sup>22</sup> which prompted us to investigate the stepwise route.

## 2.2. Synthesis of IB-01212 via stepwise ester bond formations

The considerable epimerization in the dimerization/macrocyclization process may be derived from the first ester bond formation for dimerization or the second ester bond formation for macrocyclization. To identify the problematic process causing epimerization, we investigated an alternative stepwise process for the synthesis of **1**. TFA treatment of **7** followed by TBS-protection of the hydroxyl group afforded the carboxylic acid **8** (Scheme 3). On the other hand, BF<sub>3</sub>·Et<sub>2</sub>O treatment in MeOH of **7** gave the alcohol **9**. The esterification reaction between **8** and **9** using MSNT in DMF yielded the octadepsipeptide **10** with a yield of 69%. In this process, no concomitant production of the epimeric products at the MePhe position was observed. After the Cbz groups of **10** were removed, N-terminal methylation followed by TFA treatment yielded the linear octapeptide **11**.

MSNT-mediated macrolactonization of **11** gave the desired IB-01212 (**1**) with a yield of 7.5%. However, a significant formation of an additional product with an identical mass to IB-01212 (**1**) was observed. To identify this additional product, the macrolactonization process from the epimeric precursor **12** at the C-terminal MePhe was investigated. The same treatment of **12** with MSNT afforded the [D-MePhe]-epimer **13** more efficiently, with a yield of 15% without the formation of **1**, suggesting the cyclization from epimeric **12** is a more favorable process for providing cyclic products compared with that from **11** (Figure 2). Thus, we demonstrated that the epimerization during the simultaneous dimerization and macrocyclization for the synthesis of **1** occurred at the

final macrolactonization process. Of note, bis-cyclization to form the tetramers was not negligible, lowering the overall yields in both cases.

### 2.3. Synthesis and biological activity of IB-01212 derivatives.

Using the established synthesis approach for IB-01212 (**1**), a preliminary structure-activity relationship study was carried out to investigate the essential sequences for potent cytotoxicity of IB-01212 (**1**). For this purpose, two IB-01212 derivatives, **14** and **15**, were designed, in which the peptide sequence of the *C*<sub>2</sub>-symmetric component was arranged (Figure 3). These two peptides were synthesized via the simultaneous *N*-methylation process (Table 1). *N*-methylation by treatment with excess MeI smoothly proceeded independent of the sequences, producing the expected methylated products (**19** and **21**).

The symmetric derivatives **14** and **15** were evaluated for cytotoxic activity against A549 cells accompanied with IB-01212 (**1**) and the D-MePhe epimer **13**. D-MePhe epimer **13** exhibited 3.7-fold less potency compared with that of **1**, suggesting that the *C*<sub>2</sub>-symmetric scaffold of the cyclic peptides was favorable for potent bioactivity. Peptides **14** and **15** with the arranged sequences of the identical components showed slightly less potency compared with **1**, suggesting the effect of the amino acid sequences may be less significant than that of the *C*<sub>2</sub>-symmetry of the molecule.

### 3. Conclusions

In this study, we developed a novel synthesis approach for *N*-methylated peptides via simultaneous methylation of all the peptide bonds while protecting the peptide bond(s) not to be methylated. In a case study for the synthesis of the cytotoxic peptide, IB-01212 (**1**), a pseudoproline dipeptide component [Leu-Ser( $\psi^{\text{Me,Me}}$ pro)] was employed at the Me<sub>2</sub>Leu-Ser substructure to protect from MeI-mediated multiple *N*-methylations. The target, IB-01212 (**1**), was successfully synthesized using the resulting linear precursor. We also demonstrated by a control experiment using the stepwise

process that the epimerization during the one-pot dimerization/macrocyclization process of the synthesis of IB-01212 (**1**) occurred at the final macrolactonization step. The structure-activity relationship study on the resulting IB-01212 (**1**) and its derivatives suggested that the symmetric structure of IB-01212 (**1**) is more important for the cytotoxic activity than the amino acid sequence. Although the pseudoproline component used in this study is applicable only for protection of the Ser- or Thr-containing peptide bonds, a similar approach appears to be feasible using appropriate protecting groups for the other amino acids, and this attempt is in progress.

## **4. Experimental Section**

### **4.1. Synthesis**

#### **4.1.1. General Methods.**

Melting points were measured by a hot stage melting point apparatus and are uncorrected. <sup>1</sup>H NMR spectra were recorded using a JEOL ECA-500 spectrometer. Chemical shifts are reported in  $\delta$  (ppm) relative to residual peak or TMS (in CDCl<sub>3</sub> or CD<sub>3</sub>CN) as an internal standard. <sup>13</sup>C NMR spectra were referenced to the residual peak as an internal standard. Chemical shifts were reported in parts per million with the residual solvent peak used as an internal standard. <sup>1</sup>H NMR spectra are tabulated as follows: chemical shift, multiplicity (br = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet), number of protons, and coupling constant(s). Electrospray ionization mass spectrometry (ESI-MS) spectra were recorded using Quattro micro API (Waters). Infrared (IR) spectra were obtained on a JASCO FT/IR-4100 FT-IR spectrometer with JASCO ATR PRO410-S. Optical rotations were measured with a JASCO P-1020 polarimeter. For flash chromatography, Wakogel C-300E (Wako) was employed. For analytical HPLC, a COSMOSIL 5C18-ARII column (4.6  $\times$  250 mm, Nacalai Tesque) was employed with a linear gradient of CH<sub>3</sub>CN containing 0.1% (v/v) TFA aq. at a flow rate of 1 mL/min, and eluting products were detected by UV at 220 nm. Preparative HPLC was performed using a COSMOSIL 5C18-ARII column (20 or 10  $\times$  250 mm,

Nacalai Tesque) with a linear gradient of CH<sub>3</sub>CN containing 0.1% (v/v) TFA aq. at a flow rate of 8 or 4 mL/min, respectively.

#### 4.1.2. Cbz-Leu-Ser( $\psi^{\text{Me,Me}}$ pro)-Leu-Phe-Ot-Bu (6)

To a stirred solution of H-Leu-Phe-Ot-Bu **5**<sup>26</sup> (2.33 g, 7.0 mmol) in DMF (35 mL) at room temperature were added Cbz-Leu-Ser( $\psi^{\text{Me,Me}}$ pro)-OH (2.87 g, 7.3 mmol), Et<sub>3</sub>N (1.01 mL, 7.3 mmol), HOBT·H<sub>2</sub>O (1.23 g, 8.0 mmol) and EDCI·HCl (1.40 g, 7.3 mmol). After being stirred at room temperature overnight, the solvent was evaporated. The residue was dissolved in EtOAc (150 mL), and the solution was washed with sat. citric acid, brine, 5 % NaHCO<sub>3</sub> and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Purification by flash column chromatography over silica gel (*n*-hexane:EtOAc = 3:2) gave the compound **6** as a colorless solid (4.16 g, 84%): mp 75–77 °C;  $[\alpha]_{\text{D}}^{28}$  –24.0° (*c* 2.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>)  $\delta$  0.89–0.96 (m, 12H), 1.27–1.38 (m, 10H), 1.57–1.67 (m, 5H), 1.68–1.86 (m, 6H), 2.99 (m, 2H), 4.10 (dd, *J* = 9.9, 6.4 Hz, 1H), 4.22–4.37 (m, 3H), 4.52 (d, *J* = 8.1 Hz, 1H), 4.69 (m, 1H), 4.81 (d, *J* = 11.6 Hz, 1H), 4.97 (d, *J* = 11.6 Hz, 1H), 5.30 (d, *J* = 6.4 Hz, 1H), 6.91 (d, *J* = 7.5 Hz, 1H), 7.10–7.46 (m, 10H), 7.61 (d, *J* = 7.5 Hz, 1H). <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$  21.5, 21.6, 22.4, 22.9, 23.5, 24.8, 24.9, 26.1, 27.8, 38.1, 40.4, 53.1, 53.6, 54.2, 59.9, 67.2, 68.0, 81.8, 96.9, 126.7, 127.9, 128.1, 128.4, 129.6, 135.8, 136.5, 157.5, 169.7, 170.3, 170.4, 171.4; IR (neat) 3321 (br), 2961, 2253, 1665, 1514, 1369, 1264, 1154, 905, 726 cm<sup>–1</sup>; MS(ESI+) 710.1 [M+H]<sup>+</sup>.

#### 4.1.3. Cbz-MeLeu-Ser( $\psi^{\text{Me,Me}}$ pro)-MeLeu-MePhe-Ot-Bu (7)

MeI (8.79 mL, 141 mmol) was added dropwise to a stirred suspension of **6** (2.50 g, 3.5 mmol) and Ag<sub>2</sub>O (16.3 g, 71 mmol) in dry DMF (12 mL). After being stirred in dark at room temperature overnight, the reaction was quenched with MeOH (6.85 mL, 169 mmol). The whole was extracted with EtOAc (220 mL), and the solution was washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Purification by flash chromatography over silica gel (*n*-hexane:EtOAc = 3:1) gave the compound **7**



as a colorless solid (2.50 g, 94%): mp 54–56 °C;  $[\alpha]_{\text{D}}^{25} -164.7^\circ$  ( $c$  1.0,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (400 MHz,  $\text{CDCl}_3$ , mixture of rotamers)  $\delta$  0.61–1.03 (m, 13H), 1.18–1.87 (m, 19H), 2.05 (m, 1H), [2.19 (s), 2.34 (s), *all sum to* 3H], [2.68 (s), 2.72 (s), 2.77 (s), 2.83 (s), 2.86 (s), 3.01 (s), *all sum to* 6H], [2.96 (m), 3.43 (m), 3.95 (dd,  $J = 8.1, 5.8$  Hz), *all sum to* 4H], [4.67–4.82 (m), 4.89 (m), 5.14 (m), 5.31 (m), 5.39 (m), 5.54 (m), *all sum to* 6H], 7.08–7.52 (m, 10H).  $^{13}\text{C}$  NMR (100 MHz,  $\text{CDCl}_3$ )  $\delta$  21.7, 22.1, 23.2, 23.5, 24.2, 24.8, 25.1, 27.8, 29.4, 29.6, 31.3, 34.4, 37.1, 37.3, 37.8, 51.8, 56.4, 57.9, 58.4, 67.33, 67.37, 82.0, 97.3, 126.7, 127.2, 127.6, 127.93, 127.96, 128.4, 128.5, 128.76, 129.86, 128.93, 136.3, 137.4, 156.5, 167.1, 168.3, 169.5, 170.0; IR (neat) 2958, 1650, 1455, 1395, 1368, 1307, 1155, 909,  $730\text{ cm}^{-1}$ ; MS(ESI+) 774.2  $[\text{M}+\text{Na}]^+$ , 751.2  $[\text{M}+\text{H}]^+$ .

#### 4.1.4. Me<sub>2</sub>Leu-Ser-MeLeu-MePhe-OH (4)

10% Pd-C (13.3 mg) was added to a stirred solution of **7** (200 mg, 0.27 mmol) in EtOH (2.7 mL). The suspension was stirred under H<sub>2</sub> balloon at room temperature for 16 h. After the reaction reached completion, 10% Pd-C (73.4 mg) and formalin (0.300 mL) were added to the reaction mixture. The solution was stirred under H<sub>2</sub> atmosphere at room temperature for 6 h. The catalyst was filtered off through Celite and the filtrate was concentrated. The residue was dissolved in TFA/H<sub>2</sub>O (95:5, 5.3 mL), and the solution was stirred at room temperature for 2 h. The solvent was evaporated, and the residue was purified by preparative HPLC with a linear CH<sub>3</sub>CN gradient (20 to 50% over 60 min) in 0.1% TFA aq. to give **4** as a colorless powder (111 mg, 78%):  $[\alpha]_{\text{D}}^{24} -128.7^\circ$  ( $c$  0.89,  $\text{CH}_3\text{CN}$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{CN}$ , mixture of rotamers)  $\delta$  [0.61 (d,  $J = 6.3$  Hz), 0.68 (d,  $J = 6.9$  Hz), *all sum to* 3H], [0.79 (m), 1.00 (m), *all sum to* 1H], 0.84 (m, 3H), 0.88–0.96 (m, 6H), [1.29 (m), 1.44 (m), *all sum to* 3H], 1.57 (m, 1H), 1.87 (m, 1H), [2.32 (s), 2.34 (s), 2.71 (s), 2.79 (s), 2.80 (s), 2.82 (s), 2.83 (s), *all sum to* 12H], 3.00 (m, 1H), 3.32 (m, 1H), [3.50 (m), 3.61 (m), 3.74 (m), *all sum to* 2H], 3.81 (m, 1H), [4.76 (m), 4.89 (m), *all sum to* 1H], [4.85 (m), 5.23 (m), 5.30 (m), *all sum to* 2H], 7.22 (m, 3H), 7.29 (m, 2H), 8.04 (m, 1H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  21.4, 21.5, 22.5, 22.6, 23.3, 23.72,

23.74, 23.8, 24.5, 24.9, 25.5, 25.6, 30.1, 30.4, 30.6, 33.2, 34.8, 35.6, 36.4, 36.8, 38.0, 38.2, 41.6, 41.7, 51.3, 52.4, 53.08, 53.12, 59.6, 61.6, 62.0, 62.1, 66.8, 66.9, 127.5, 127.6, 129.4, 129.6, 129.9, 138.6, 138.7, 166.9, 167.0, 169.9, 170.1, 171.0, 171.4, 172.5; IR (neat) 3328, 3250, 2959, 1735, 1674, 1633, 1471, 1408, 1369, 1201, 1179, 1134, 1055, 846, 800, 663  $\text{cm}^{-1}$ ; MS(ESI+) 557.5  $[\text{M}+\text{Na}]^+$ , 535.5  $[\text{M}+\text{H}]^+$ .

#### 4.1.5. Cbz-MeLeu-Ser(TBS)-MeLeu-MePhe-OH (8)

Compound **7** (540 mg, 0.72 mmol) was dissolved in TFA/ $\text{H}_2\text{O}$  (95:5, 14 mL), and the solution was stirred at room temperature for 2 h. The solvent was evaporated. To the solution of the residue in dry DMF (3.2 mL) were added  $\text{Et}_3\text{N}$  (0.892 mL, 6.5 mmol) and TBSCl (217 mg, 1.4 mmol) at 0 °C. After being stirred at room temperature for 22 h, the reaction was quenched by addition of  $\text{H}_2\text{O}$  (3 mL). The whole was extracted with EtOAc (70 mL), and the organic phase was washed with water, and dried over  $\text{Na}_2\text{SO}_4$ . Purification by flash column chromatography over silica gel ( $\text{CHCl}_3$ :MeOH = 9:1) gave the compound **8** as a colorless oil (451 mg, 82%):  $[\alpha]_{\text{D}}^{26} = -115.2^\circ$  ( $c$  1.2,  $\text{CHCl}_3$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ , mixture of rotamers)  $\delta$  [−0.02 (s), −0.01 (s), 0.00 (s), *all sum to 6H*], [0.54 (m), 0.64 (m), 0.81 (m), 0.83 (s), 0.86 (m), *all sum to 21H*], 1.26 (m, 1H), 1.40 (m, 2H), 1.61 (m, 2H), 1.69 (m, 1H), [2.43 (s), 2.72 (s), 2.81 (s), 2.82 (s), 2.86 (s), 2.88 (s), 2.94 (s), 3.01 (s), *all sum to 9H*], [3.05 (m), 3.24 (m), 3.38 (dd,  $J = 15.0, 4.6$  Hz), *all sum to 2H*], 3.52–3.76 (m, 2H), [4.61 (m), 4.78 (m), 4.89 (m), *all sum to 2H*], 4.96–5.24 (m, 3H), 5.30 (m, 1H), 6.75 (m, 1H), 7.13–7.32 (m, 10H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  −5.6 −5.4, 18.2, 21.2, 21.4, 21.8, 22.7, 23.1, 24.5, 24.7, 25.8, 29.7, 29.9, 33.6, 34.3, 37.0, 37.5, 51.2, 52.2, 56.9, 59.9, 62.8, 67.5, 67.7, 126.9, 127.1, 127.7, 128.0, 128.1, 128.5, 128.6, 128.9, 129.1, 129.2, 136.4, 137.1, 156.9, 169.5, 170.8, 171.8, 173.9; IR (neat) 2957, 2869, 2253, 1694, 1644, 1470, 1403, 1316, 1259, 1163, 1110, 905, 727  $\text{cm}^{-1}$ ; MS(ESI+) 791.8  $[\text{M}+\text{Na}]^+$ , 769.6  $[\text{M}+\text{H}]^+$ .

#### 4.1.6. Cbz-MeLeu-Ser-MeLeu-MePhe-Ot-Bu (9)

BF<sub>3</sub>·Et<sub>2</sub>O (0.113 mL, 0.90 mmol) was added to a stirred solution of **7** (675 mg, 0.90 mmol) in MeOH (3 mL) at room temperature. The solution was stirred under argon at room temperature for 12 h. The reaction was quenched by addition of Et<sub>3</sub>N (1.24 mL, 9.0 mmol) at 0 °C, and the solvent was evaporated. The residue was dissolved in EtOAc (50 mL) and washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Purification by flash column chromatography over silica gel (*n*-hexane:EtOAc = 1:1) gave the compound **9** as a colorless oil (519 mg, 81%): [α]<sub>D</sub><sup>26</sup> −131.2° (*c* 1.17, CHCl<sub>3</sub>); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>, mixture of rotamers) δ 0.59 (m, 1H), 0.69 (m, 1H), 0.72–1.04 (m, 10H), 1.25–1.75 (m, 15H), 1.92 (m, 1H), [2.42 (s), 2.46 (s), 2.80 (s), 2.86 (s), 2.88 (s), 2.92 (s), *all sum to* 9H], [2.97 (m), 3.27 (dd, *J* = 15.0, 4.0 Hz), 3.37 (dd, *J* = 15.0, 4.0 Hz), *all sum to* 2H], 3.46–3.77 (m, 2H), [4.58–4.77 (m), 4.88 (m), *all sum to* 2H], [5.02 (m), 5.34 (m), *all sum to* 2H], 5.16 (s, 2H), [6.72 (m), 6.85 (m), 7.00 (m), *all sum to* 1H], 7.18–7.36 (m, 10H). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>) δ 21.8, 22.5, 23.0, 23.3, 24.0, 24.7, 28.0, 29.8, 30.0, 30.3, 32.0, 34.5, 35.1, 36.8, 37.0, 37.3, 51.4, 51.5, 57.3, 58.7, 61.8, 63.2, 67.6, 82.0, 126.7, 126.9, 127.7, 128.0, 128.2, 128.5, 128.8, 128.9, 129.1, 136.7, 137.2, 157.2, 169.5, 169.6, 170.4, 170.5; IR (neat) 2956, 2253, 1641, 1456, 1404, 1369, 1311, 1156, 908, 725 cm<sup>−1</sup>; MS(ESI+) 733.7 [M+Na]<sup>+</sup>.

#### 4.1.7. Cbz-MeLeu-Ser[Cbz-MeLeu-Ser(TBS)-MeLeu-MePhe]-MeLeu-MePhe-Ot-Bu (10)

To a stirred solution of **9** (252 mg, 0.36 mmol) in DMF (1.9 mL) were successively added **8** (300 mg, 0.39 mmol), DIEA (0.247 mL, 1.4 mmol), NMI (0.056 mL, 0.71 mmol) and MSNT (210 mg, 0.71 mmol). The solution was stirred under argon at room temperature for 16 h. The whole was extracted with EtOAc (50 mL), and the solution was washed with water and brine, and dried over Na<sub>2</sub>SO<sub>4</sub>. Purification by flash column chromatography over silica gel (*n*-hexane:EtOAc = 2:1) gave the compound **10** as a colorless solid (358 mg, 69%): mp 61–63 °C; [α]<sub>D</sub><sup>27</sup> −111.5° (*c* 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>, mixture of rotamers) δ [0.03 (s), 0.04 (s), *all sum to* 6H], 0.59–0.91 (m,

33H), 1.31 (m, 2H), 1.37–1.52 (m, 13H), 1.69 (m, 6H), [2.20 (s), 2.24 (s), 2.40 (s), 2.49 (s), 2.79 (s), 2.83 (s), 2.84 (s), 2.86 (s), 2.95 (s), *all sum to 18H*], 2.98 (m, 2H), 3.34 (m, 2H), [3.53 (m), 3.58 (m), 3.69 (m), 3.78 (m), 4.18 (dd,  $J = 10.9, 2.9$  Hz), 4.51 (dd,  $J = 11.5, 3.4$  Hz), *all sum to 4H*], [4.64 (m), 4.74 (m), *all sum to 3H*], 4.92–5.27 (m, 5H), 5.30–5.40 (m, 2H), 5.44 (m, 1H), 5.58 (m, 1H), 6.58 (m, 1H), 6.79 (m, 1H), 7.21–7.30 (m, 20H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CDCl}_3$ )  $\delta$  –5.5, –5.3, 18.2, 21.7, 21.8, 22.5, 22.7, 23.1, 24.5, 24.6, 24.7, 24.8, 25.8, 28.0, 29.5, 29.7, 29.8, 30.1, 31.5, 31.8, 34.2, 36.9, 37.0, 37.4, 37.5, 48.7, 51.3, 51.6, 52.2, 56.9, 57.2, 57.6, 58.0, 62.5, 62.6, 64.8, 64.9, 67.5, 67.8, 82.0, 126.9, 127.6, 127.8, 128.0, 128.2, 128.5, 128.6, 128.7, 128.9, 129.1, 136.2, 136.5, 136.9, 137.1, 156.0, 157.0, 169.0, 169.6, 170.1, 170.2, 170.5, 170.8, 171.1, 171.4; IR (neat) 3307, 2958, 2932, 2870, 2290, 1737, 1694, 1648, 1472, 1405, 1368, 1314, 1256, 1219, 1160, 1110, 926, 839, 739, 699  $\text{cm}^{-1}$ ; MS(ESI+) 1484.9  $[\text{M}+\text{Na}]^+$ , 1462.9  $[\text{M}+\text{H}]^+$ .

#### 4.1.8. Me<sub>2</sub>Leu-Ser(Me<sub>2</sub>Leu-Ser-MeLeu-MePhe)-MeLeu-MePhe-OH (**11**)

10% Pd-C (20.9 mg) was added to a stirred solution of **10** (102 mg, 0.070 mmol) in EtOH (0.700 mL). The suspension was stirred under H<sub>2</sub> balloon at room temperature for 9 h. After the reaction reached completion, 10% Pd-C (13.9 mg) and formalin (0.0785 mL) were added to the reaction mixture. The solution was stirred under H<sub>2</sub> atmosphere at room temperature for 16 h. The catalyst was filtered off through Celite and the filtrate was concentrated. The residue was dissolved in TFA/DCM (1:1, 2.8 mL), and the solution was stirred at room temperature for 4 h. The solvent was evaporated, and the residue was purified by preparative HPLC with a linear CH<sub>3</sub>CN gradient (30 to 55% over 50 min) in 0.1% TFA aq. to yield the compound **11** as a colorless powder (41.3 mg, 56%):  $[\alpha]_{\text{D}}^{25} -111.8^\circ$  ( $c$  1.0, CH<sub>3</sub>CN);  $^1\text{H}$  NMR (500 MHz, CD<sub>3</sub>CN, mixture of rotamers)  $\delta$  0.57–0.72 (m, 6H), 0.77–1.07 (m, 20H), 1.27–1.67 (m, 8H), 1.89 (m, 2H), [2.50 (s), 2.53 (s), 2.72 (s), 2.76 (s), 2.77 (s), 2.82 (s), 2.84 (s), 2.86 (s), 2.87 (s), *all sum to 24H*], 3.05 (m, 2H), 3.32 (m, 2H), [3.60 (m), 3.70 (m), *all sum to 2H*], [3.84 (m), 3.90 (m), 3.96 (m), 4.04 (m), 4.23 (m), 4.37 (m), 4.58 (m), *all sum to*

4H], [4.80 (m), 4.86 (m), 4.91 (m), 5.03 (m), 5.13 (m), *all sum to 5H*], 5.33 (m, 1H), 7.15–7.33 (m, 10H), [8.06 (m), 8.20 (m), 8.34 (m), 8.53 (m), *all sum to 2H*].  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  21.8, 21.9, 22.7, 23.4, 23.6, 23.8, 23.9, 24.9, 25.0, 25.27, 25.34, 25.8, 25.9, 30.7, 31.0, 33.7, 34.4, 35.1, 35.2, 35.9, 36.1, 36.7, 37.6, 37.7, 38.3, 38.5, 38.6, 41.8, 41.9, 50.3, 50.5, 50.9, 51.8, 52.1, 52.4, 52.8, 53.0, 53.1, 53.5, 61.0, 61.3, 61.7, 61.9, 62.1, 62.5, 64.8, 65.7, 67.1, 67.2, 127.9, 128.1, 129.61, 129.67, 129.70, 129.86, 129.94, 129.97, 130.13, 130.16, 130.21, 130.3, 138.6, 138.7, 139.0, 139.3, 161.9, 167.2, 167.5, 168.0, 168.6, 168.8, 169.0, 170.6, 170.7, 171.0, 171.2, 171.4, 171.5, 171.7, 172.6, 173.9; IR (neat) 3388, 2959, 2872, 1735, 1671, 1645, 1473, 1413, 1201, 1129, 836, 800, 721, 704  $\text{cm}^{-1}$ ; MS(ESI+) 1074.4  $[\text{M}+\text{Na}]^+$ , 1052.2  $[\text{M}+\text{H}]^+$ .

#### 4.1.9. Synthesis of IB-01212 (1) from linear octapeptide precursor 11

To a stirred solution of **11** (19 mg, 0.018 mmol) in DCM/DMF/NMI (90:8:2, 18 mL) were added DIEA (0.0757 mL, 0.43 mmol) and MSNT (42.8 mg, 0.15 mmol). The solution was stirred at room temperature for 24 h. The solvent was evaporated, and the residue was purified by preparative HPLC with a linear  $\text{CH}_3\text{CN}$  gradient (30% to 60% over 60 min) to give IB-01212 (**1**) as a colorless powder (1.4 mg, 7.5%). Analytical data were found to be identical to those reported in the literature:<sup>21,22</sup>  $[\alpha]_{\text{D}}^{26} -104.2^\circ$  (*c* 0.25,  $\text{CH}_3\text{CN}$ );  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{CN}$ , mixture of rotamers, TFA salt)  $\delta$  [0.51–0.62 (m), 0.74–0.95 (m), *all sum to 24H*], [0.97 (m), 1.11–1.64 (m), 1.84 (m), *all sum to 12H*], [2.41 (s), 2.75 (s), 2.76 (s), 2.81 (s), 2.84 (s), 2.91 (s), *all sum to 24H*], [3.20 (m), 3.48 (dd,  $J = 14.5$ , 4.1 Hz), *all sum to 4H*], [3.79 (m), 3.89 (m), 4.12 (dd,  $J = 12.2$ , 2.9 Hz), 4.42 (m), *all sum to 6H*], 4.52–4.67 (m, 3H), 5.05 (m, 2H), 5.24 (m, 1H), 7.05–7.35 (m, 10H), 8.32 (m, 2H).  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{CN}$ )  $\delta$  21.4, 21.5, 22.7, 22.8, 23.1, 23.5, 23.7, 24.6, 25.0, 25.4, 25.7, 30.7, 30.95, 30.99, 34.6, 35.7, 36.8, 36.1, 37.3, 37.9, 38.6, 41.2, 48.1, 49.4, 51.9, 52.8, 62.0, 64.2, 65.9, 66.0, 66.1, 127.5, 128.0, 129.4, 129.7, 129.9, 130.3, 137.6, 138.8, 168.4, 168.6, 170.5, 170.8, 171.2; IR (neat) 3352,

2959, 1747, 1647, 1470, 1412, 1255, 1202, 1130, 836, 800, 721  $\text{cm}^{-1}$ ; MS(ESI+) 1056.5  $[\text{M}+\text{Na}]^+$ , 1034.4  $[\text{M}+\text{H}]^+$ .

#### **4.2. Growth inhibition assay.**

Growth inhibition assays were performed using A549 cells as described previously.<sup>27</sup> A549 cells were cultured in DMEM medium (Sigma) supplemented with 10% (v/v) FBS at 37 °C in a 5% CO<sub>2</sub> incubator. Growth inhibition assays using these cells were performed in 96-well plates (BD Falcon). A549 cells were seeded at 500 cells/well in 50  $\mu\text{L}$  of culture media and placed for 6 h. Chemicals in DMSO were diluted 250-fold with the culture medium in advance. Following the addition of 40  $\mu\text{L}$  of the fresh culture medium, 30  $\mu\text{L}$  of the chemical diluents was also added to the cell cultures. The final volume of DMSO in the medium was equal to 0.1% (v/v). The cells under chemical treatment were incubated for a further 72 h. The wells in the plates were washed twice with DMEM medium without phenol red (GIBCO). After 1 hour incubation with 100  $\mu\text{L}$  of the medium, the cell culture in each well was supplemented with 20  $\mu\text{L}$  of the MTS reagent (Promega), followed by incubation for an additional 40 min. Absorbance at 490 nm of each well was measured using a Wallac 1420 ARVO SX multilabel counter (Perkin Elmer).

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#### **Supplementary data**

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2014.08.036>.

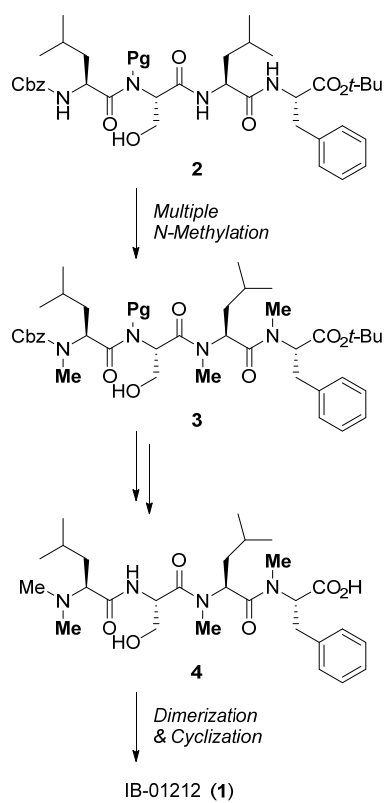
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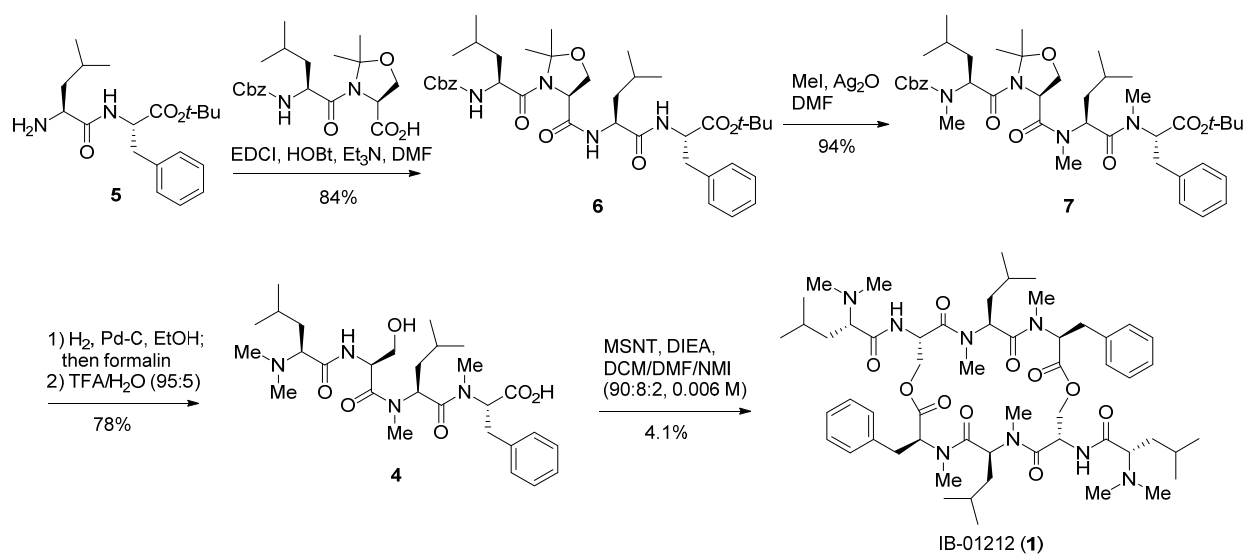


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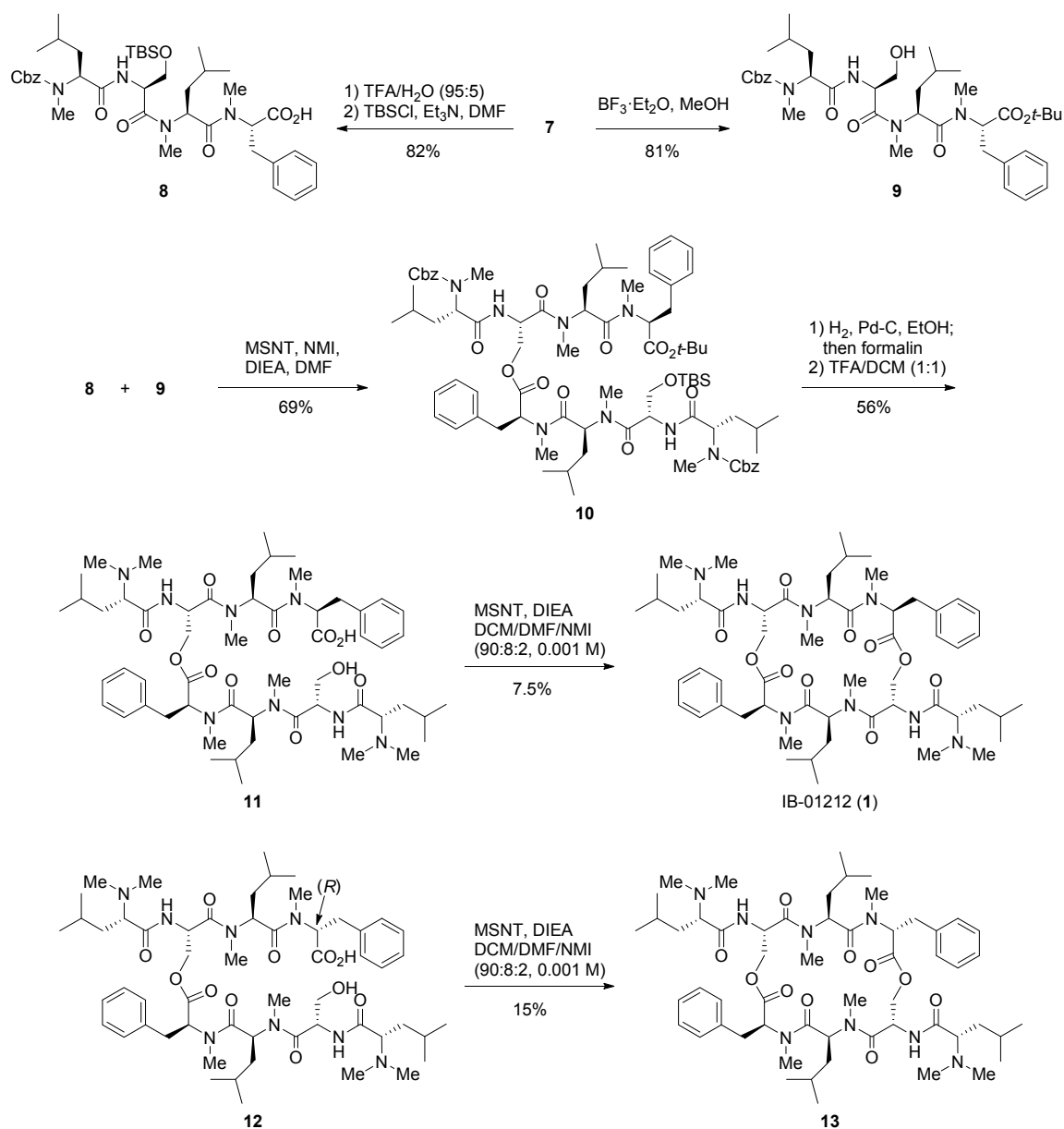
**Scheme 1.** Synthesis strategy for IB-01212 via multiple *N*-methylations. Pg = protecting group.



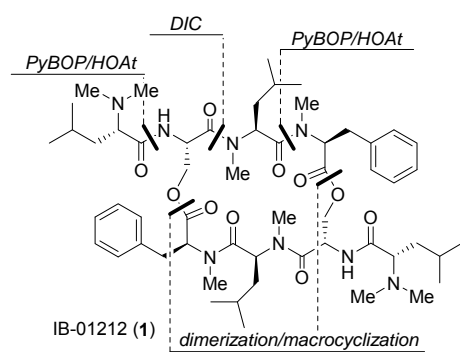
**Scheme 2.** Synthesis of IB-01212 via site-selective *N*-methylation(s) of peptide bonds.



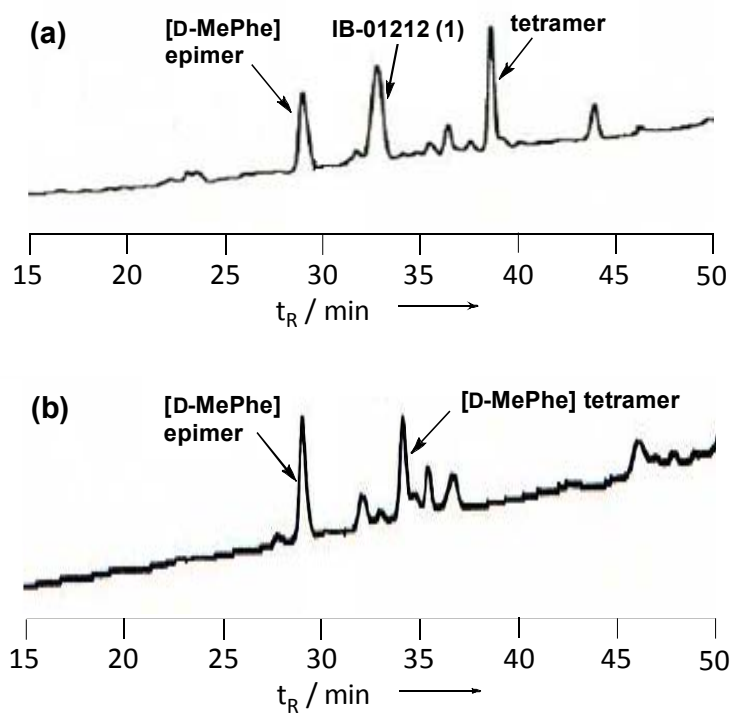
**Scheme 3.** Synthesis of IB-01212 via stepwise ester bond formations.



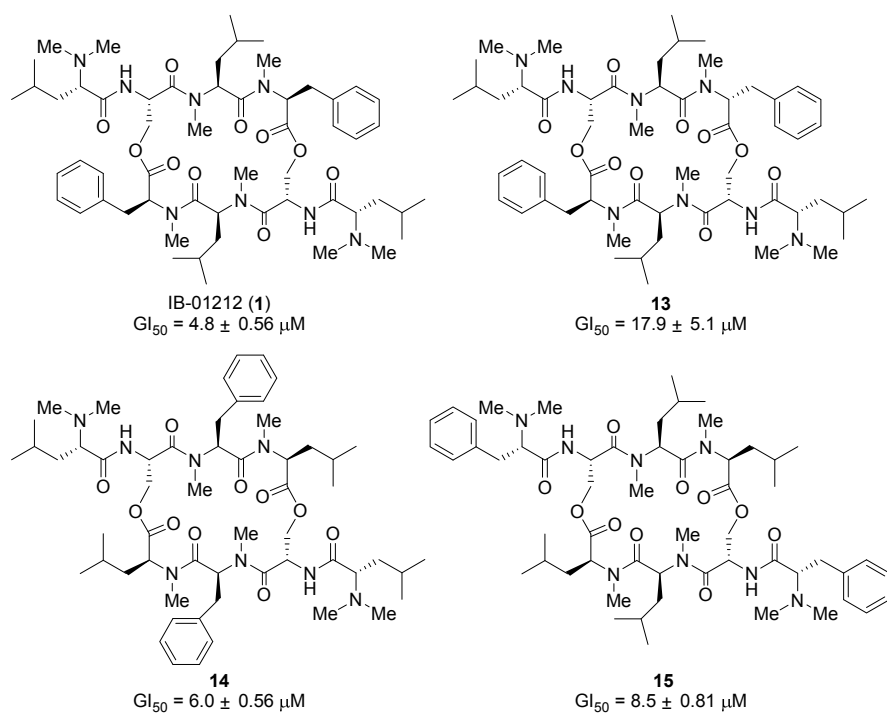
**Figure 1.** Structure of IB-01212 and the previous synthesis.



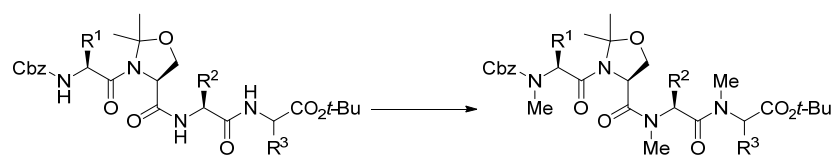
**Figure 2.** HPLC analysis of the macrolactonization process in the synthesis via stepwise ester bond formations. (a) From [L-MePhe] linear precursor **11**; (b) from [D-MePhe] linear precursor **12**. HPLC conditions: a linear CH<sub>3</sub>CN gradient (30 to 55% over 50 min) in 0.1% TFA aq.



**Figure 3.** Cytotoxicity of IB-01212 and its derivatives in A549 cells.



**Table 1.** Site-selective *N*-methylation for the synthesis of IB-01212 derivatives.



Substrate	Product	Yield (%)
Cbz-Leu-Ser( $\psi^{\text{Me,Me}}$ pro)-Leu-Phe- <i>Ot</i> -Bu ( <b>6</b> )	Cbz-MeLeu-Ser( $\psi^{\text{Me,Me}}$ pro)-MeLeu-MePhe- <i>Ot</i> -Bu ( <b>7</b> )	94
Cbz-Leu-Ser( $\psi^{\text{Me,Me}}$ pro)-Leu-D-Phe- <i>Ot</i> -Bu ( <b>16</b> )	Cbz-MeLeu-Ser( $\psi^{\text{Me,Me}}$ pro)-MeLeu-D-MePhe- <i>Ot</i> -Bu ( <b>17</b> )	98
Cbz-Leu-Ser( $\psi^{\text{Me,Me}}$ pro)-Phe-Leu- <i>Ot</i> -Bu ( <b>18</b> )	Cbz-MeLeu-Ser( $\psi^{\text{Me,Me}}$ pro)-MePhe-MeLeu- <i>Ot</i> -Bu ( <b>19</b> )	97
Cbz-Phe-Ser( $\psi^{\text{Me,Me}}$ pro)-Leu-Leu- <i>Ot</i> -Bu ( <b>20</b> )	Cbz-MePhe-Ser( $\psi^{\text{Me,Me}}$ pro)-MeLeu-MeLeu- <i>Ot</i> -Bu ( <b>21</b> )	93