ORGANIC LETTERS

2012
Vol. 14, No. 2
612–615

Solid-Phase Synthesis of *N*Me-IB-01212, a Highly *N*-Methylated Cyclic Peptide

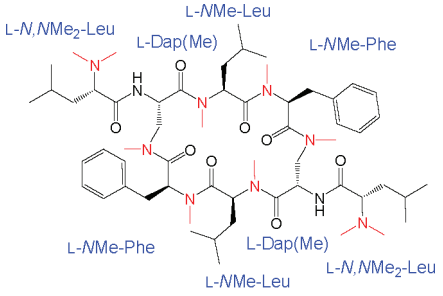
Eleonora Marcucci,[†,‡] Judit Tulla-Puche,*[,†,‡] and Fernando Albericio*[,†,‡]

Institute for Research in Biomedicine, Barcelona Science Park, Baldiri Reixac 10, 08028 Barcelona, Spain, and CIBER-BBN Networking Centre on Bioengineering, Biomaterials, and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028 Barcelona, Spain

judit.tulla@irbbarcelona.org; albericio@irbbarcelona.org

Received December 4, 2011

ABSTRACT



*N*Me-IB-01212, **1**

***N*-Methylation of peptides is an important synthetic tool in peptide-based medicinal chemistry. Herein, an optimized strategy for solid-phase synthesis of small but highly *N*-methylated cyclic peptides is described. The proposed route addresses several problems associated with the synthesis of peptides containing several sequential *N*-methyl-amino acids, such as in situ *N*-methylation, difficulty of acylation, epimerization, diketopiperazine formation, and stability at the *N*Me sites under trifluoroacetic acid exposure. The resulting *N*Me-IB-01212 exhibits micromolar activity and considerable stability.**

IB-01212 (Figure 1) is a cyclodepsipeptide isolated from the marine fungi *Clonostachys* sp. ESNA-A009.[1] The compound shows micromolar activity in various tumor cell lines, including LNCaP (prostate cancer), SK-BR-3 (breast cancer), HT-29 (colon cancer), and HELA (cervical cancer).[2] Like thiocoraline,[3] an antitumor cyclothiodepsipeptide belonging to the same chemical family, IB-01212 features a C2-symmetrical structure, but rather than the heterocyclic bisintercalator chromophores of the former, it contains *N*,*N*Me$_2$-Leu groups. Furthermore, IB-01212 contains two additional *N*Me-aa residues (Leu and Phe). In the course of our research on thiocoraline, we found that replacing the thioester moieties with bridged *N*Me amides confers an analogue (*N*Me-azathiocoraline) that is more stable yet retains the activity of the parent compound.[4] This finding is consistent with the work of Gilon, Kessler, and coworkers, who proposed multiple *N*-methylation of peptides as a new paradigm in peptide-based medicinal chemistry.[5]

Synthesis of peptides featuring several sequential *N*Me-amino acids is hindered by a limited number of cyclization points in the linear intermediates, poor acylation rates, a risk of epimerization, lability of the *N*Me-amide bond under acidic conditions, and formation of *C*-terminal and

(1) (a) Cruz, L. J.; Martínez Insua, M.; Pérez Baz, J.; Trujillo, M.; Rodriguez-Mias, R. A.; Oliveira, E.; Giralt, E.; Albericio, F.; Cañedo, L. M. *J. Org. Chem.* **2006**, *71*, 3335. (b) Cruz, L. J.; Cuevas, C.; Giralt, E.; Cañedo, L. M.; Albericio, F. *J. Org. Chem.* **2006**, *71*, 3339.

(2) Cruz, L. J.; Francesch, A.; Cuevas, C.; Albericio, F. *ChemMedChem* **2007**, *2*, 1076.

(3) (a) Romero, F.; Espliego, F.; Pérez-Baz, J.; García de Quesada, T.; Gravalos, D.; De la Calle, F.; Fernández-Puentes, J. L. *J. Antibiot.* **1997**, *50*, 734. (b) Pérez-Baz, J.; Cañedo, L. M.; Fernández-Puentes, J. L.; Silva Elipe, M. V. *J. Antibiot.* **1997**, *50*, 738.

(4) Tulla-Puche, J.; Marcucci, E.; Prats-Alfonso, E.; Bayó-Puxan, N.; Albericio, F. *J. Med. Chem.* **2009**, *52*, 834.

(5) Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H. *Acc. Chem. Res.* **2008**, *41*, 1331.

(6) (a) McDermott, J. R.; Benoiton, N. L. *Can. J. Chem.* **1973**, *51*, 2555. (b) McDermott, J. R.; Benoiton, N. L. *Can. J. Chem.* **1973**, *51*, 2562. (c) Humphrey, J. M.; Chamberlin, A. R. *Chem. Rev.* **1997**, *97*, 2243. (d) Teixidó, M; Albericio, F.; Giralt, E. *J. Peptide Res.* **2005**, *65*, 153–166.

10.1021/ol203231q
Published on Web 12/22/2011

internal diketopiperazines (DKPs).[6] Moreover, the lack of commercial availability for these *N*Me-amino acids means that there is a requirement for in situ methylation. Thus, the strategies reported here for the synthesis of *N*Me-IB-01212 could be extrapolated to the synthesis of other highly methylated peptides.

The synthesis of the natural IB-01212 was accomplished via a 3 + 3 fragment coupling on solid-phase through ester formation because the linear strategy gave very low yields due to the instability of the ester bond.[7]

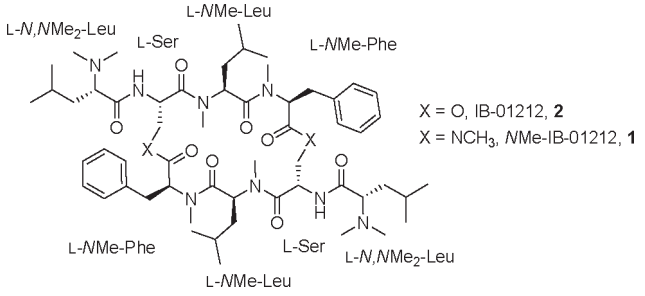


Figure 1. Structure of natural IB-01212 and *N*Me-IB-01212.

For the synthesis of *N*Me-IB-01212, as no clear point for cyclization exists (no Gly or Pro at the *C*-terminus is present, for example, that would avoid epimerization), the three different options were initially studied (Figure 2).

Strategy (a) was not pursued because we had previously observed that *N*-methylation of the side chain of the Dap residue, when it is directly anchored to the 2-chlorotrityl (2-CTC) resin,[8] results in a severe loss of peptide.[4] Strategy (b) has the advantage that the 6-membered ring DKP cannot be formed because the second residue, Dap, is elongated through its side chain; but, on the other hand, the cyclization between the bulky *N*Me-Phe and the β-branched *N*Me-Leu residue may prove complicated. In strategy (c), *N*Me-Phe has the drawback of DKP formation due to the consecutive *N*Me-amino acids, which was already observed during the earlier synthesis of natural IB-01212.[9]

The first attempts at preparing *N*Me-IB-01212 entailed a fragment approach[10] to avoid a double solid-phase *N*-methylation step, which could affect the purity of the final chain, and to circumvent the coupling of so many consecutive *N*Me-amino acids. Nevertheless, fragment couplings at

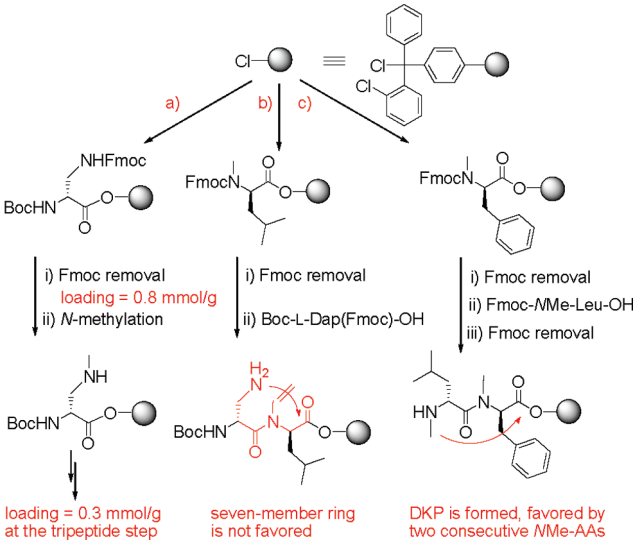


Figure 2. Starting points for the synthesis of *N*Me-IB-01212.

each starting point (Figure 2b,c) led to substantial degrees of epimerization,[11] thereby making it impossible to continue in this direction (see the Supporting Information). In contrast, the successful strategy to reach *N*Me-IB-01212 is depicted in Scheme 1, and it involves a completely stepwise approach. The starting point for the synthesis was the Fmoc-*N*Me-Phe-OH residue, which was anchored onto 2-CTC resin (**3**). Here, we have faced the challenge of coupling Fmoc-*N*Me-Leu-OH as the second amino acid.[12] In a first attempt using 1-[bis(dimethylamino)-methylene]-1*H*-1,2,3-triazolo-[4,5-*b*]pyridinium hexafluorophosphate 3-oxide (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), and diisopropylethylamine (DIEA) in DMF as coupling reagent, significant deletion was observed, reaching 40%.[13] DKP formation was also detected. To further optimize the preparation of the initial tripeptide, several changes were introduced: the synthesis was performed on 2-CTC resin, but the loading was lowered to ca. 0.5 mmol/g in order to favor coupling of *N*Me-Leu; second, the less-hindered Alloc-*N*Me-Leu-OH was used with the double objective of facilitating the coupling and simultaneously preventing DKP formation due to the rather neutral conditions to remove the Alloc group; and finally, the coupling system used was changed to (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate

(7) Cruz, L. J.; Cuevas, C.; Cañedo, L. M.; Giralt, E.; Albericio, F. *J. Org. Chem.* **2006**, *71*, 3339.

(8) Barlos, K.; Gatos, D.; Kallitsis, J.; Papaphotiu, G.; Sotiriu, P.; Yao, W.; Schaefer, W. *Tetrahedron Lett.* **1989**, *30*, 3943.

(9) Although our group (Chiva, C.; Vilaseca, M.; Giralt, E. Albericio, F. *J. Peptide Sci.* **1999**, *5*, 131.) and others (Rovero, P.; Vigano, S.; Pegoraro, S.; Quartara, L. *Lett. Peptide Sci.* **1996**, *2*, 319.) have shown that CTC resins avoid DKP formation during synthesis of *C*-terminal Pro peptides, CTC resin can not prevent DKP formation in sequences that are highly prone to this side reaction.

(10) The tripeptide, which constitutes half of the molecule, was assembled on solid-phase. At this point, one-third of the resin-bound peptide was deprotected, and the remaining two-thirds was cleaved from the resin. After lyophilization, the 3 + 3 fragment coupling was carried out on solid phase.

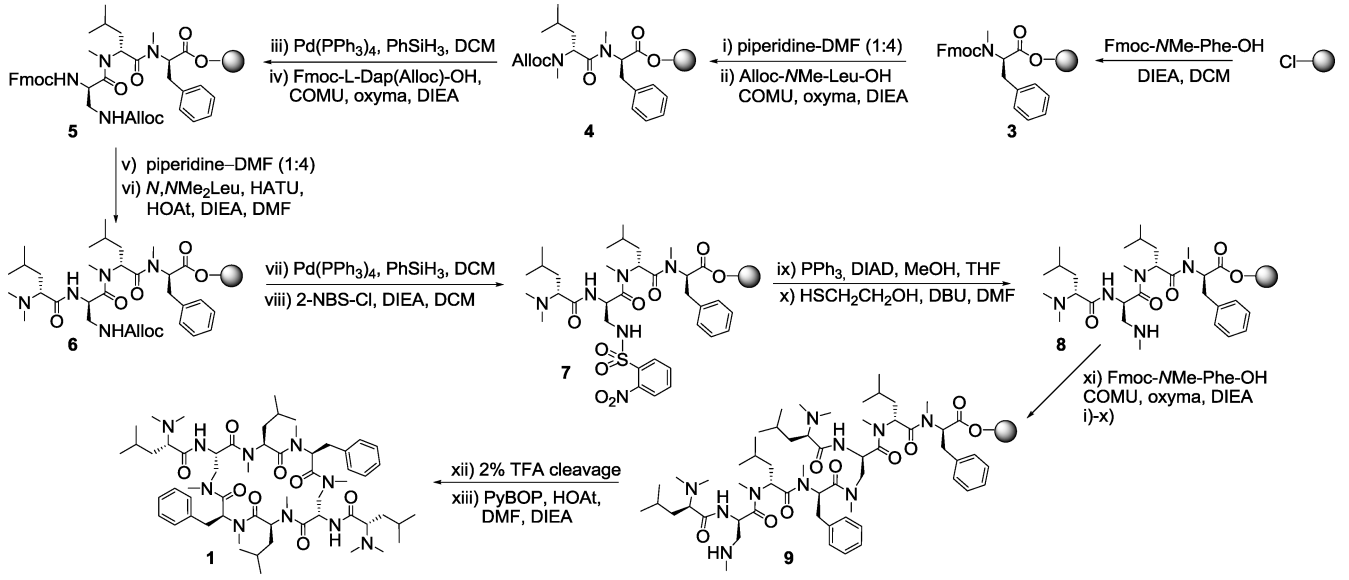
(11) With *N*Me-Leu (b) at the *C*-terminus, the epimerization ratio was 50:50, whereas with *N*Me-Leu (c) at the same position, the ratio was 65:35.

(12) In previous work with the 2-CTC resin, which contains bulky trityl groups, we observed that when the first amino acid coupled to the resin is sterically hindered, the coupling of the second amino acid is very difficult.

(13) To test a less hindered solid support, the same tripeptide was elongated on Wang resin; however, the deletion of *N*Me-Leu was still present. Moreover and as expected, a greater amount of DKP was formed.

(14) Subirós-Funosas, R.; Prohens, R.; Barbas, R.; El-Faham, A.; Albericio, F. *Chem.—Eur. J.* **2009**, *15*, 9394.

Scheme 1. Final Approach to Assemble *N*Me-IB-01212



(COMU),[14] ethyl (hydroxyimino)cyanoacetate (oxyma),[15] and DIEA in DMF. The excellent solubility in DMF of this coupling system over HATU and HOAt allows the use of several equivalents of amino acid in minimal solvent, resulting in a far better coupling yield. Therefore, after perfoming the coupling with Alloc-*N*Me-Leu-OH (3 equiv), COMU (3 equiv), oxyma (3 equiv), and DIEA (6 equiv) for one hour and stirring one further hour after adding COMU (1 equiv) and oxyma 1 (equiv), the protected dipeptide (**4**) was obtained in 93% purity.

After removing the Alloc group from *N*Me-Leu with $Pd(PPh_3)_4$ and $PhSiH_3$ in DCM, the Dap residue was introduced, using Fmoc-L-Dap(Alloc)-OH (4 equiv) in the presence of COMU (4 equiv), oxyma (4 equiv), and DIEA (8 equiv) in DMF for 1 h (**5**).[16] After removing the Fmoc group with piperidine, *N*,*N*Me_2-Leu was coupled over the free α-amino functionality of the Dap group with HATU/HOAt/DIEA in DMF for 1 h (**6**).

The Alloc group was again removed using the same conditions described above, and the side chain of the Dap residue was *N*-methylated on solid-phase using Kessler's conditions:[17] first, the free amino functionality was reprotected with the 2-nitrobenzenesulfonyl (2-NBS) group (**7**), then methylated via a Mitsunobu reaction [PPh_3, MeOH, diisopropyl azodicarboxylate (DIAD)], and finally deprotected (removal of 2-NBS) by treating the resin with 2-mercaptoethanol and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in DMF. With the tetrapeptide (**8**) in hand, the same set of reactions was repeated in order to achieve the complete linear skeleton of *N*Me-IB-01212. The octapeptide (**9**) was cleaved from the resin with a TFA–DCM solution (2:98, 5 × 1 min) and then lyophilized. The crude peptide was obtained in 52% yield with a purity of 56% as shown by HPLC analysis.

In earlier work using the hexapeptide {[Boc-L-Dap(Me&1)-*N*Me-Leu-*N*Me-Phe&2][Boc-L-Dap(Me&2)-*N*Me-Leu-*N*Me-Phe&1]}, various cyclization conditions were investigated that would imply minimal epimerization (Table 1).

Table 1. Cyclization Studies

entry	coupling reagent	base	solvent	pH	conv	diastereomeric ratio (D:L)
1	PyBOP/HOAt	DIEA	DMF–DCM	8	low	15:85
2	PyBOP/HOAt	collidine	DMF–DCM	8	low	67:33
3	PyBOP/HOAt	DIEA	DMF	8	high	13:87
4	DIPCDI/HOAt		DMF–DCM	7		

Cyclization under neutral conditions (entry 4) did not take place and only proceeded when performed at pH 8. The other three experiments were designed to examine the influence of the solvent and base on the epimerization side-reaction. When using the milder base 2,4,6-collidine (entry 2), low conversion was observed, whereas a high degree of epimerization was detected. The combination of DIEA as base and DMF as solvent gave the highest conversion and the lowest epimerization percentage (13%, entry 3). Therefore, the octapeptide precursor was dissolved

(15) El-Faham, A.; Subirós-Funosas, R.; Prohens, R.; Albericio, F. *Chem.—Eur. J.* **2009**, *15*, 9404.

(16) Alternatively, when we used Boc-L-Dap(Fmoc)-OH to construct the hexapeptide skeleton and then removed the Boc groups in solution after cyclization to later introduce the *N*,*N*Me_2-Leu residues, use of TFA–DCM (1:1) caused the peptide to break at its *N*Me sites (as evidenced by a complex chromatogram). Other conditions that were tested, such as lowering the percentage of TFA, minimizing the time of exposure, or using HCl instead of TFA, did not offer any improvements; therefore, this strategy was abandoned (see the Supporting Information).

(17) Biron, E.; Chatterjee, J.; Kessler, H. *J. Peptide Sci.* **2006**, *12*, 213 and references cited therein.

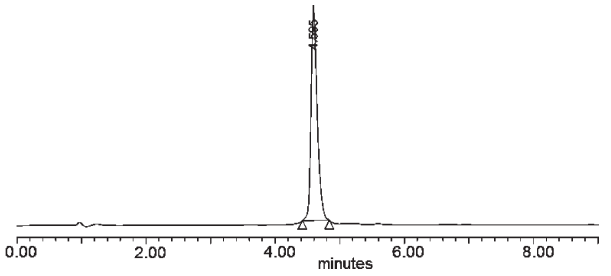


Figure 3. Analytical HPLC of pure *N*Me-IB-01212. Linear gradient from 30:70 to 50:50 (0.036% TFA in ACN/0.045% TFA in H_2O) in 8 min.

in DMF and cyclized with PyBOP and HOAt by maintaining the pH at 8 with the addition of DIEA. The cyclization proceeded smoothly despite the rigidity imposed by the high number of *N*-methyl amino acids and was complete after 3 h. A low degree of epimerization (9%) was observed.

The crude cyclic peptide was purified by semipreparative HPLC (linear gradient from 30:70 to 40:60 (0.036% TFA in ACN/0.045% TFA in H_2O) to afford the pure *N*Me-IB-01212 (99% purity, 4% overall yield, Figure 3). The pure peptide showed cytotoxic activity in four cell lines (HT-29 5.8 μM; SK-BR-3 15 μM; A-549 23 μM; HeLa 11.3 μM), as well as high stability in human serum, with a half-life time of 60 h (see the Supporting Information), much higher than that of *N*Me-azathiocoraline.

In summary, a highly *N*-methylated cyclopeptide, which showed micromolar activity in four cell lines and considerable stability in human serum, was assembled by optimized stepwise solid-phase mode.

The synthetic strategy described herein could be exploited for the preparation of other highly *N*-methylated cyclic peptides.[5,17,18] The principal features to consider are (i) the use of an orthogonal protecting group scheme would avoid the use of Boc/tBu groups, which can cause breakage at *N*Me sites;[19] (ii) a low loading of the resin is required to obtain satisfactory purities of the linear chain; (iii) the use of the Alloc group to mask the α-amino functionality of the second amino acid provides higher yields in the coupling step and minimizes DKP formation; and (iv) COMU and oxyma allow carrying out the demanding couplings with a higher concentration when compared to other coupling reagents and additives, thus favoring high-yielding couplings.

Acknowledgment. We thank Drs. José Pastor and Miriam Royo from the Barcelona Science Park for kindly performing the biological assays. This work was partially supported by CICYT (CTQ2009-07758), the Generalitat de Catalunya (2009SGR 1024), the Institute for Research in Biomedicine, and the Barcelona Science Park. E.M. and J.T.-P. thank MICINN for a Torres y Quevedo and a Juan de la Cierva contract, respectively.

Supporting Information Available. Experimental procedures, characterization, data, and cytotoxic and stability assays. This material is available free of charge via the Internet at http://pubs.acs.org

(18) (a) Plaza, A.; Bifulco, G.; Masullo, M.; Lloyd, J. R.; Keffer, J. L.; Colin, P. L.; Hooper, J. N. A.; Bell, L. J.; Bewley, C. A. *J. Org. Chem.* **2010**, *75*, 4344. (b) Mas-Moruno, C.; Beck, J. G.; Doedens, L.; Frank, A. O.; Marinelli, L.; Cosconati, S.; Novellino, E.; Kessler, H. *Angew. Chem.* **2011**, *50*, 9496.

(19) Peptide skeletons that contain *N*Me-amino acids tend to be unstable, depending of the number of consecutive *N*Me-amino acids and/or the rigidity of the peptide structure, as demostrated in this case, whereby a relatively smaller cycle (compared with the thiocoraline family) cannot tolerate even a moderate amount of acid.