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Solid-Phase Synthesis of a Cyclodepsipeptide: Cotransin

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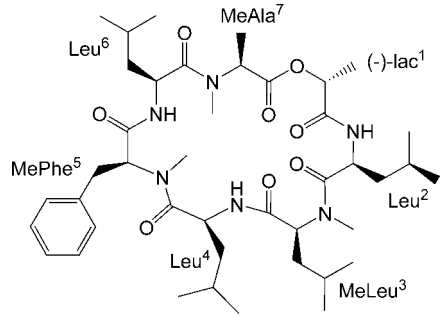
ABSTRACT



The first solid-phase synthesis of cotransin—a cyclic depsipeptide having high pharmacological potential—was achieved, by a proper choice of coupling reagents and use of either TBAF or DBU for Fmoc removal to suppress the otherwise dominating, sequence-derived diketopiperazine formation. Starting the assembly from C-terminal lactic acid allowed fast and epimerization-free cyclization in solution. Novel conditions for orthogonal use of the Fmoc/Bsmoc-protection system were discovered, and an unexpected nucleophilic behavior of DBU was observed.

Cotransin is a cyclic heptadepsipeptide structurally derived from the fungal product HUN-7293.[1] Like HUN-7293, cotransin is able to repress, selectively and reversibly, the expression of a subset of secreted and membrane proteins in mammalian cells, being particularly efficient in inhibiting cell adhesion molecule expression.[2] Due to the crucial role played by membrane proteins and adhesion molecules in the context of a variety of diseases, cotransin and analogous peptides have a high therapeutic potential. Up to now, the synthesis of these compounds has been carried out in solution, by applying the strategy first developed by Boger and co-workers,[3] which requires the separate assembly of two segments, their condensation, and subsequent macrocyclization at the MeLeu^3-Leu^4 position. Although even a small library of analogues has been prepared by using this laborious procedure,[3b] a synthesis method based on a solid-phase strategy would provide a more efficient way to prepare much more analogues in a short time, thus allowing for extended structure−function relationship studies. To synthesize cotransin via SPPS is not an easy task: the peptide contains three *N*-methylamino acids, which are difficult to be acylated, and a lactic acid residue, which requires the formation of an ester bond. In addition, the conformational effects induced by the presence of N-alkylated residues favor

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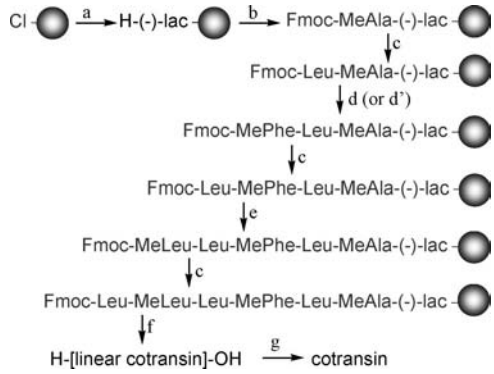
the formation of diketopiperazine (DKP) during removal of the temporary N-protection.[4] In particular, the Leu[6]-MeAla[7] sequence following N-terminally the ester bond gives an extraordinary extent of DKP formation during deblocking of Leu[6], thus making cotransin particularly suitable for searching for appropriate conditions to suppress DKP formation during stepwise synthesis of depsipeptides in general.[5] With the prospect of having a method in hand for preparing a lot of analogues, we have aimed for using the convenient Fmoc chemistry instead of the more harmful Boc chemistry, although the latter can be advantageous with respect to suppression of DKP formation.[6] For depsipeptides bearing a single ester bond, DKP formation could in principle be avoided if the ring is disconnected at the N-terminal position following the ester bond.[7] In the case of cotransin, such a strategy would be not appropriate because in this case the cyclization should be performed at an N-methylated residue (Leu[6] to MeAla[7]), thus giving reason to a slow reaction and occurrence of epimerization.

We describe here a simple strategy to synthesize cotransin using Fmoc chemistry on solid phase, which relies on the suppression of DKP formation by the use of an appropriate base for Fmoc removal and on the choice of a disconnection position that enables fast and epimerization-free ring closure in solution.

The stepwise assembly of the linear peptide starts with linkage of nonprotected (−)-lactic acid onto ClTrt-Cl polystyrene resin in the presence of DIEA (see Scheme 1). The choice of this residue as C-terminal is based on the consideration that lactic acid cannot form oxazolone when activated and is therefore expected to provide ideal conditions for the final cyclization.[8] Next, the α-hydroxyl group is esterified with Fmoc-MeAla-OH using carbodiimide (DIC) activation in the presence of *N*-methylimidazole (NMI) as catalyst.[9] After standard Fmoc removal with piperidine (20% in DMF, 2 × 5 min), quantitative coupling of Fmoc-Leu-OH onto the *N*-methyl alanine residue was achieved using HATU activation, which gave here the best result in comparison to PyBOP activation and acid fluoride coupling.[10]

Scheme 1. Scheme for the Synthesis of Cotransin[a]



[a]Steps: (a) *H*-(−)-lac-OH/DIEA/DCM, (b) esterification via DIC/NMI, (c) Fmoc removal and coupling via HATU, (d) (*i*) TBAF 0.15 M in DMF, 2 × 1 min, (*ii*) MeOH, 15 s, (*iii*) DCM, 15 s, (*iv*) Fmoc-MePhe-OH/HBTU/DIEA preactivated, DMF, 20 min; (d′) (*i*) DBU 10% in DMF, 10 s, (*ii*) HOBt 0.2 M in DMF, 20 s, (*iii*) Fmoc-MePhe-OH/HBTU/DIEA preactivated, DMF, 20 min, (e) Fmoc removal and coupling via HBTU, (f) acidic deprotection, (g) via HATU/DIEA in DCM (0.5 mM). More details are reported in the Supporting Information.

By applying the standard conditions for Fmoc removal to deprotect Fmoc-Leu[6]-MeAla[7]-(−)-lac-P (P: polymer support), rapid DKP formation took place, leading to complete loss of the Leu-MeAla-DKP from the lactic acid residue. Even by reducing the time of piperidine treatment to the point of incomplete deblocking, we still observed almost quantitative DKP formation. It has been reported[5b] that DKP can be efficiently suppressed by using instead of the Fmoc group the more base-labile Bsmoc group,[11] Bsmoc being deprotected under less basic conditions than Fmoc. Nevertheless, no recovery of the target peptide was achieved even after fast removal of the Bsmoc group from Bsmoc-Leu[6]-MeAla[7]-(−)-lac-P with diluted piperidine (2% in DMF, 3 × 1 min).

Deprotection by piperidine seems to be slower than the DKP formation, so that all of the target peptide is consumed. Thus, a more rapid deprotection should be advantageous for diminishing DKP formation. DBU is a strong base ($pK_a \sim 12$), which is able to remove Fmoc very quickly.[12] Indeed, a short-time DBU treatment led to a complete Fmoc removal from Fmoc-Leu[6]-MeAla[7]-(−)-lac-P with a reduced extent of DKP formation. The best result was obtained by performing a kind of "flash" treatment, in which the peptide-resin was simply washed once with a solution of DBU 10% in DMF (10 s), rapidly neutralized with a HOBt solution (20 s), and immediately acylated with the next amino acid (preactivated Fmoc-MePhe-OH). By this, for the deblocking−coupling step resulting in Fmoc-MePhe-Leu-MeAla-(−)-lac-P, a 30% yield was obtained, according to the resin loading estimated by the amount of dibenzofulvene (dbf) formed during the next deblocking step.

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(12) DBU: Diaza(1,3)bicyclo[5.4.0]undecane: (a) Chang, C. D.; Waki, M.; Ahmad, M.; Meienhofer, J.; Lundell, E. O.; Haug, J. D. *Int. J. Pept. Protein Res.* **1980**, *15*, 59. (b) Wade, J.; Bedford, J.; Sheppard, R.; Tregear, G. *Pept. Res.* **1991**, *4*, 194.

An alternative route for reducing DKP formation was found to be the use of TBAF.[13] By deblocking Fmoc-Leu-MeAla-(−)-lac-P with TBAF (0.15 M in DMF, 2 min), although Fmoc removal was slower than in the case of DBU, DKP formation was even more reduced (∼ 50%). Interestingly, after the TBAF treatment and DMF washings, the coupling of the next activated amino acid (Fmoc-MePhe-OH) was incomplete. Significant amounts (∼ 40%) of the nonacylated peptide H-Leu-MeAla-(−)-lac-P were found, as determined by LC-MS of the crude cleaved from the peptide resin. By performing a washing with methanol (15 s) after deblocking,[13] followed by reswelling of the peptide-resin in DCM (15 s), coupling of the next amino acid occurred quantitatively, the yield of the deblocking−coupling step being ∼ 45% (determined by quantification of liberated dbf during the following deblocking step).

Thus, by using TBAF for Fmoc removal at this sensitive position and HATU activation for couplings onto *N*-methyl amino acid residues, stepwise assembly of the linear cotransin was smoothly accomplished, as described in Scheme 1.

Crude linear cotransin was obtained in very good quality and showed a single peak in LC-MS analysis. As the sole impurity, the lactic acid as the product of DKP formation was coeluted with the injection peak. Lactic acid was easily removed by a preparative HPLC run (in principle, it could also be simply washed out), and linear cotransin was obtained in a high yield of 34% (based on the loading at the level of Fmoc-MeAla-(−)-lac-P).

As expected, peptide cyclization by activating the C-terminal lactic acid proceeded smoothly using HATU/DIEA in DCM and was complete within less than 30 min.[14] The LC-MS analysis of the cyclic product showed a single peak, having the mass value (*m*/*z*) calculated for cotransin, with the only impurity observed being the cyclodimer of cotransin, the content of which was less than 5% when the cyclization was carried out at low concentration (0.5 mM). NMR data (see Supporting Information) of the product isolated via preparative HPLC assessed its identity and did not show the presence of epimers. Cotransin was obtained in a total yield of 23% based on the loading of Fmoc-MeAla-(−)-lac-P and exhibited in a preliminary biological test the expected activity.[15]

Although the synthesis of cotransin following our protocol enables the synthesis of quite pure crude cyclodepsipeptide, the effective exploitation of the resin capacity is still limited by the extent of DKP formation. An approach to further reduce DKP formation using the Fmoc chemistry may result from developing a simultaneous deprotection−coupling procedure (tandem deprotection/coupling),[16] by which the removal of the Fmoc group from the N-terminus is carried out in the presence of an activated amino acid, orthogonally protected at the Nα, which acylates in situ the free amino terminus as soon as liberated.

The Fmoc and Bsmoc groups have quite different sensitivity toward bases, the first being deprotected through a β-elimination reaction promoted by abstraction of the acidic proton at the 9-position of the fluorene ring[17] and the second through a Michael-type addition process.[11] First of all, we tested the stability of the two groups toward different bases possibly compatible with the presence of an activated species (Table 1, SI): the acylation catalysts NMI and DMAP, TBAF, and the tertiary amine DBU. Both amino protecting groups were stable in the presence of NMI, whereas DMAP (0.2−0.4 M) provided no sufficient selectivity in the removal. At the concentrations tested, good selectivity was found for TBAF[18] (0.1−0.2 M) and diluted DBU (0.2−0.5%, corresponding to 0.013−0.033 M) in DMF, with Fmoc being easily removed and Bsmoc being substantially stable (see Table 1, SI).

On the basis of these results, the use of TBAF and DBU in combination with a Nα-Bsmoc-protected activated amino acid seemed to be conceivable for a one-pot approach. However, we observed that TBAF causes fast hydrolysis of activated species (amino acid fluorides, OPfp, OSu, OpNp esters) due to the unavoidable presence of water in the system,[19] so that its use in this context had to be discarded. By adding DBU (0.013−0.033 M) to Fmoc-peptides (0.04 M) in the presence of an activated Nα-Bsmoc-protected amino acid (0.2 M), we observed no Fmoc removal. Surprisingly, this was found to be due to acylation of DBU by the activated species (Scheme 2). The reaction is so fast that the base is acylated before the abstraction of the fluorenylic proton from Fmoc can occur. In fact, even by treating an activated Fmoc-amino acid with up to 1 equiv of DBU, almost no Fmoc removal was observed, but only acylation of DBU took place.

Although DBU is traditionally considered a non-nucleophilic base,[20] several examples of its nucleophilic properties are reported in recent literature.[21] Our finding about the rapid acylation of DBU gives a rationale to a recent report of Han and co-workers,[22] who studied the catalytic activity of bicyclic amidines in acylation reactions and detected no catalysis operated by DBU. The acyl-imidinium formed by DBU is in fact a low reactive species: it was stable several

(13) TBAF: tetrabutylammonium fluoride: Ueki, M.; Amemiya, M. *Tetrahedron Lett.* **1987**, *28*, 6617.

(14) Ehrlich, A.; Heyne, H. U.; Winter, R.; Beyermann, M.; Haber, H.; Carpino, L. A.; Bienert, M. *J. Org. Chem.* **1996**, *61*, 8831.

(15) Cotransin-mediated decrease in Corticotropin Releasing Factor Receptor expression was investigated in the research group of Ralf Schülein at the FMP, Berlin. Further experiments are in progress.

(16) The first example of coupling in situ during deprotection of Z is reported by: Shute, R. E.; Rich, D. H. *J. Chem. Soc., Chem. Commun.* **1987**, 1155. Use of Alloc for tandem deprotection/coupling reaction: Thieriet, N.; Alsina, J.; Giralt, E.; Guibé, F.; Albericio, F. *Tetrahedron Lett.* **1997**, *38*, 7275. Thieriet, N.; Gomez-Martinez, P.; Guibé, F. *Tetrahedron Lett.* **1999**, *40*, 2505. Zorn, C.; Gnad, F.; Salmen, S.; Herpin, T.; Reiser, O. *Tetrahedron Lett.* **2001**, *42*, 7049.

(17) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404.

(18) Fresh prepared solutions of TBAF·3H_2O in DMF dried over molecular sieves were used. Bsmoc was stable toward TBAF only when using dried DMF, whereas by using commercial DMF as such unidentified products of degradation of the Bsmoc protected substrates were formed.

(19) No improvement resulted from attempts to remove water from the system, neither by prolonged storage of the TBAF solution on molecular sieves nor by working in the presence of *N*,*O*-bis(trimethylsilyl)acetamide, nor by using other fluoride sources like TASF [tris(dimethylamino)sulfonium difluorotrimethylsilicate] or CsF (the latter used alone or in the presence of 18-crown-6 ether).

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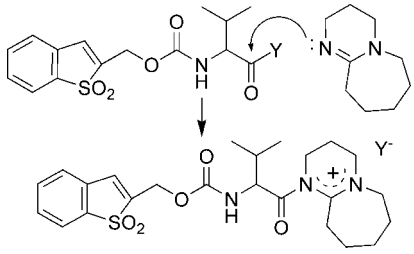
(21) Nucleophilic catalysis of DBU in Baylis−Hillman reactions: Aggarwal, V. K.; Mereu, A. *Chem. Commun.* **1999**, 2311. Aza-Michael addition promoted by DBU: Yeom, C.-E.; Kim, M. J.; Kim, B. M. *Tetrahedron* **2007**, *63*, 904. See also: Ghosh, N. *Synlett* **2004**, 574.

(22) Birman, V. B.; Li, X.; Han, Z. *Org. Lett.* **2007**, *9*, 37.

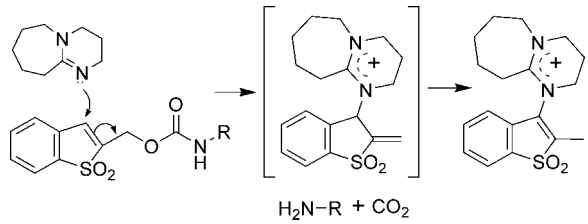
Scheme 2. Reaction between Bsmoc-Val-Y (Y = F, OPfp, OSu, OpNp) and DBU[a]



[a] The product was isolated via HPLC and identified via LC-MS and NMR (see also Supporting Information).

days in aqueous solutions, DMSO, and MeOH, and it did not react with nucleophiles (H-Xaa-OR). In the presence of pyridine, Bsmoc-Val-DBU$^+$ in MeOH gave Bsmoc-Val-OMe ($\sim$ 30% yield in 3 h).[23] At increased concentration of DBU, its nucleophilic behavior becomes apparent by a Michael-like attack at the dioxobenzothiophene system of Bsmoc, in the same way which is described for the removal operated by piperidine[11] (Scheme 3, see also SI for discussion about the structure of the resulting product).

Scheme 3. Bsmoc Removal Operated by DBU[a]



[a] The adduct was isolated via HPLC and identified via LC-MS and NMR.

Taken together, our results identify new conditions for the selective removal of Fmoc in the presence of Bsmoc, for a case in which both groups are used as Nα-protection, but show on the other hand that bases tested are not suited to be used for a tandem deprotection−coupling system.

In conclusion, we described here an efficient protocol to synthesize the cycloheptadepsipeptide cotransin in high purity, using Fmoc chemistry on the solid phase. By substituting either DBU or TBAF for piperidine, DKP formation during Fmoc removal at the second position following the ester bond has been reduced to an extent that makes the synthesis of cotransin achievable in good yields using Fmoc chemistry, without the need for implementation of other protecting groups and chemistries (like for instance the use of the Alloc[24] group).[16] The choice of an appropriate disconnection position has also been of particular importance. In fact, when we tried to assemble cotransin using MeLeu^3-Leu^4 as the disconnection point, cyclization using PyBOP occurred slowly and gave rise to extensive epimerization ($\sim$ 20%). For this cyclization, reduced epimerization was reported[3] by the use of a low activation method (azide activation[25]), but this requires a long reaction time (up to several days) and gives mostly lower yields.[26] Our results show that activation of lactic acid at the C-terminus of linear cotransin allows for a fast and epimerization-free cyclization, a method that can be adopted as a guideline principle for the cyclization of substrates containing α-hydroxy acids,[27] in perfect accordance with the known advantages given by the use of C-terminal depsipeptide units for segment coupling.[28]

Our protocol for the synthesis of cotransin may represent a general strategy for the synthesis of cyclodepsipeptides.

Acknowledgment. We are indebted to the Deutsche Forschungsgemeinschaft (Be 1434/5-2) for financial support.

Supporting Information Available: Materials and methods, experimental procedures, and NMR spectra. LC-MS analysis of the final product. Table 1. Discussion about the adduct formed from Bsmoc removal via DBU. This material is available free of charge via the Internet at http://pubs.acs.org.

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