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Solid Phase-Mediated Cyclization of Head-to-Tail Peptides: Problems Associated with Side Chain Anchoring

Mari-Luz Valero, Ernest Giralt and David Andreu*

Department of Organic Chemistry, University of Barcelona, E-08028 Barcelona, Spain

Abstract: Solid-phase synthesis of cyclic head-to-tail peptides using Boc-based protection schemes and side chain anchoring via the β-carboxyl of Asp can lead to substantial levels of epimerization at the α-carbon of Asp. These problems arise largely from the chemistry used for the incorporation of Boc-Asp(OH)-OFm to the polymer. Several conditions for the attachment of this β-carboxyl to hydroxymethyl or bromomethyl resins have been evaluated, in an attempt to achieve effective, epimerization-free anchorings. The most successful routes involve the use of cesium or zinc salts of Boc-Asp(OH)-OFm. Copyright © 1996 Elsevier Science Ltd

Cyclic peptides have attracted the attention of chemists ever since peptide synthesis became a possibility. Structure-activity studies of natural cyclic peptides with antibiotic or toxic properties stimulated the synthetic demand for cyclic structures. More recently, the possibility of inducing prefered bioactive conformations in linear peptides through restricted mobility analogs has further increased the interest in peptide cyclizations[1]. Among the synthetic methodologies available for cyclic peptides, solid phase-mediated cyclizations have demonstrable advantages in that they reduce the number of isolation/purification steps required by conventional solution syntheses [2]. A particularly interesting application of solid phase-mediated cyclization is the use of side chain anchoring to prepare head-to-tail cyclic peptides. In a typical application, a residue of Asp or Glu suitably protected on both α-amino and α-carboxyl funtions is attached to the polymer support through its free side chain carboxyl group via a bifunctional handle (Figure 1). Selective deprotection

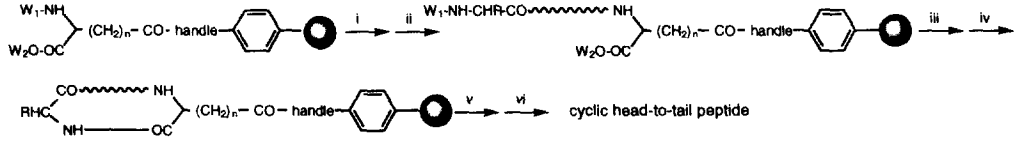


Figure 1. Solid phase-mediated synthesis of head-to-tail peptides using side chain anchoring via Asp/Asn (n=1) or Glu/Gln (n=2) residues. W_1 and W_2 are suitable protections for the N^{α} and α-carboxyl groups, respectively. The handle is a bifunctional spacer derived from either a benzyl alcohol (for Asp/Glu) or a benzhydrylamine (for Asn/Gln). Steps: i. N^{α}-deprotection; ii. Chain growth; iii. N^{α}-deprotection; iv. Carboxyl activation; v, vi. Side chain deprotection and cleavage from resin.

of the α-amino group allows solid phase synthesis to proceed until the desired sequence is assembled. Next, the terminal carboxyl and amino groups are deprotected, the former is activated and peptide bond formation takes place, ideally under pseudodilution conditions that favor the intramolecular product. Final deprotection and cleavage release the cyclic peptide into solution.

Among the orthogonal protection schemes compatible with this approach, those combining Boc[3] (α-NH_2)/OFm(α-COOH)/benzyl (side chains)[4,5] or Fmoc(α-NH_2)/OAll(α-COOH)/t-butyl(side chains)[2,5,6] have been mostly favored. In a recent study we compared their relative efficiency in the synthesis of *cyclo*(YTASARGDLAHLTTTU) (U= Gly or Cys), a model of antigenic site A of foot-and-mouth disease virus[5], using the single Asp residue for side chain anchoring. Although preliminary results[5] suggested that the first (Boc/OFm) protocol could be superior to the second (Fmoc/OAll), judging by the cleanliness (HPLC) of the final cyclic peptide product, the Boc/OFm approach suffered from low yields in the incorporation of the initial Asp residue to the polymer. This prompted us to reexamine the anchoring chemistry of the Boc/OFm strategy, which depends on an acid-resistant *p*-substituted methylphenylacetic acid[7] (Table 1) bifunctional handle. Several conditions for anchoring Boc-Asp(OH)-OFm to the solid support were explored (Table 1).

Table 1. Anchoring of Boc-Asp(OH)-OFm to hydroxy- or bromomethylphenylacetic acid-MBHA

Entry	Esterification method	Yield (%)[a]	Epimerization (%)
	X=Y=OH		
1	amino acid / DIPCDI / DMAP (4:4: 0.1 eq), DMF,rt, 3x1 h	4	n.d.
2	amino acid / CDI (4:4 eq), DMF, rt, overnight	1	n.d.
3	amino acid / DEAD / PPh_3 (5:10:10 eq), DMF, rt, 2 h	4	n.d.
4	amino acid / DIPCDI / DMAP (4:4: 0.5 eq), DMF, rt, 3x1 h	10	17
	X=O-metal, Y=Br		
5	amino acid / $NaHCO_3$ (6:12 eq), DMF, 50°C, 24 h	11	3-4
6	amino acid (6eq) / $CsHCO_3$ pH 7, DMF, 50°C, overnight	100	2-3
7	amino acid/$ZnCO_3$ (6:3 eq), DMF, rt, 45 min, ultrasonic bath	80	<2

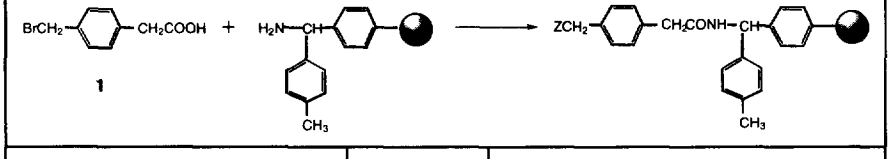
[a] Determined by acid hydrolysis (propionic acid/HCl, 1:1, 130°C, 6 h) and AAA of aminoacyl-resin.

The conventional procedure used in Fmoc/OAll chemistry (amino acid and DIPCDI, 4 equiv each + 0.1 equiv DMAP, 3 x 1 h, rt) gave very low incorporation yields. Several other anchoring protocols, including Mitsunobu conditions, failed also to provide adequate substitution levels. Increasing the amount of the DMAP catalyst to 0.5 equiv (entry 4, Table 1) raised the yield to a modest 10%; however, chiral GC-MS amino acid analysis[8] of the final cyclic peptide revealed substantial epimerization (21%) at the Asp residue. In this type of synthetic schemes, epimerization can take place at either the anchoring step itself, or at the repetitive deprotection-neutralization-coupling cycles during chain growth, or at the α-COOH activation prior to macrocyclization. For entry 4, Table 1, the relative contribution of the anchoring step to total epimerization could be evaluated by HPLC analysis of the diastereomeric mixture resulting from reaction of Boc-L-Leu-OSu[9] with the crude from HF cleavage of Boc-Asp(O-resin)-OFm. This showed most of the epimerization (17% over 21%) to take place at the esterification reaction, not during the chain growth or macrocyclization steps. In a recent report[10], high levels of epimerization were found in the synthesis of conformationally restricted cyclic peptide libraries by the Boc/OFm approach, using Mitsunobu chemistry for the anchoring

step. In this case, based on the assumption that Mitsunobu chemistry preserves chiral integrity[11], epimerization was attributed exclusively to premature α-carboxyl deprotection and ensuing activation.

In search for more effective, ideally epimerization-free anchoring schemes, we turned our attention to methods based on nucleophilic substitution of bromomethylphenylacetic handles (Table 1, Y=Br) by the Boc-Asp(OH)-OFm carboxylate salts of mono and divalent metals. Use of the sodium salt[12] (entry 5) did not improve significantly the incorporation yield, but decreased the epimerization of the anchoring step to 3-4%. More satisfactory results were obtained with either the cesium[13] or the zinc[14] salts (entries 6-7), with high substitution levels (80-100%) and minimal epimerization (ca. 2%). *A note of caution* is needed regarding the attachment of the bromomethylphenylacetic acid handle (**1**) to the MBHA resin (Table 2). When carboxyl activation was done with HOBt-derived reagents such as PyBOP[15] or TBTU[16], attempts to anchor Boc-Asp(O-metal)-OFm were unsuccessful, i.e., did not lead to polymers with any detectable loading of the amino acid by AAA. Analysis of these unreactive polymers by IR and gel-phase ^{13}C-NMR[17] suggested the replacement of the benzylic Br by an hydroxybenzotriazol-1-yl group (Table 2). This was further confirmed by ES (CI, NH_3) of the residue resulting from acidolysis (HF-anisole, 9:1 (v/v), 1 h, 0°C) of the polymers. In contrast, DCC-mediated incorporation of the bromoacid to MBHA resin gave the expected product.

Table 2. Coupling of bromomethylphenylacetic acid to MBHA resin



Coupling method	Z	Comments
1 / PyBOP / DIEA (4:4:8 eq), DMF, rt, overnight		**IR** (KBr, cm^{-1}): 3500-3300 (two bands, similar intensity); 1700 (broad); **^{13}C-NMR** (δ ppm; 300 MHz, CD_2Cl_2): 43.6 (C_6H_4-$\underline{C}H_2$-CO), 82.2 (Z-$\underline{C}H_2$), 108.5, 120 (oxybenzotriazole); **MS** (CI, NH_3): m/z 120 (benzotriazole); 137 (N-hydroxybenzotriazole); 300 (benzotriazole-oxy-CH_2-C_6H_4-CH_2-$CONH_2$)
1 / TBTU / DIEA (4:4:8 eq), DMF, rt, overnight	as above	as above
1 / DCC (4:4 eq), DMF, rt, overnight	Br	**IR** (KBr, cm^{-1}): 3350 (strong); 1620 (sharp); **^{13}C-NMR** (δ ppm; 300 MHz, CD_2Cl_2): 43.6 (C_6H_4-$\underline{C}H_2$-CO), 44.4 (Br-$\underline{C}H_2$)

Although both Cs and Zn salt procedures can be considered comparable in terms of anchoring yields and low epimerization, the former procedure[13] involves prior neutralization of Boc-Asp(OH)-OFm with aqueous $CsHCO_3$, an operation that requires careful control of pH to avoid premature loss of the OFm group. The Zn procedure uses solid $ZnCO_3$ and is relatively simpler. Any of the two methods can be satisfactorily applied to prepare head-to-tail cycles using Boc chemistry. As an example, the syntheses of the cyclic peptides *cyclo* (YTASRGDLAHLTTTGCG), *cyclo* (YTASRGDLAHLTTTGGC), and *cyclo* (YTASRGDLAHLTTTGC) are

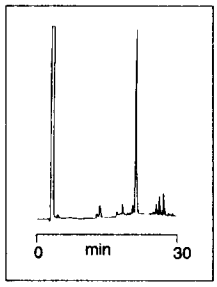


Fig. 2. HPLC profile of the HF crude of *cyclo* (YTASRGDLAHLTTT-GGC).

briefly described. The linear precursors were assembled on Boc-Asp(O-resin)-OFm prepared by the Cs salt method (substitution 0.17 mmol/g; epimerization 2%). Boc derivatives with the usual side chain protections[5] were coupled via DCC, except Boc-Arg(Tos), which was coupled as the HOBt active ester. After chain assembly, the peptide-resins were treated with DBU (2% in DMF, 1 min+2x5 min) to remove the α-OFm protection from Asp, then with TFA/DCM (40% v/v) to deprotect the α-NH_2. Solid-phase cyclizations were performed by reaction with BOP/DIEA (5:10 eq, 2 x 2h, rt). His(Dnp) deprotection was performed with PhSH/DIEA/DMF (3:3:4 v/v, 4 x 30 min)[18] and final cleavage/deprotection with HF/anisole (9:1, 0°C, 1 h). The crude products were very clean by HPLC (Fig. 2) and were purified to the target peptides, with satisfactory AAA and MALDI-TOF mass spectra.

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3. Abbreviations used in this paper for amino acids and peptides follow the rules of the IUPAC-IUB Comission of Biochemical Nomenclature in *Eur. J. Biochem.*, **1984**, *138*, 9-37 and *J. Biol. Chem.*, **1989**, *264*, 633-673. The following additional abbreviations are used: AAA, amino acid analysis; All, allyl; Boc, *tert*-butoxycarbonyl; BOP, benzotriazole-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate; Bzl, benzyl; CI, chemical ionization; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIEA, N,N-diisopropylethylamine; DIPCDI, 1,3-diisopropylcarbodiimide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Dnp, 2,4-dinitrophenyl; Fm, 9-fluorenylmethyl; Fmoc, 9-fluorenylmethyloxycarbonyl; GC-MS, gas chromatography-mass spectrometry; HOBt, 1-hydroxy-benzotriazole; HPLC, high performance liquid chromatography; IR, infrared spectroscopy; MALDI-TOF, matrix-assisted laser desorption ionization/time-of-flight, MBHA, *p*-methylbenzhydrylamine; NMR, nuclear magnetic resonance, PyBOP, benzotriazole-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate; Su, succinimidyl; TBTU, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; Tos, *p*-toluenesulfonyl. The amino acid symbols used denote the L configuration.
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18. Partial deprotection of His takes place during the DBU treatment. The presence of low levels of free His during the cyclization step does not seem to affect significantly the quality of the final product.

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