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Comparative Studies of the Coupling of N-Methylated, Sterically Hindered Amino Acids During Solid-Phase Peptide Synthesis

Yvonne M. Angell, Carlos García-Echeverría, and Daniel H. Rich*

School of Pharmacy and Department of Chemistry, University of Wisconsin-Madison
425 N. Charter St., Madison, WI 53706

Abstract: Comparison of different coupling reagents for effective coupling of N-methylated, sterically hindered amino acids under solid-phase peptide synthesis (SPPS) conditions is described. Superior results were obtained with the coupling additive 1-hydroxy-7-azabenzotriazole (HOAt), as well as its uronium salt derivative (HATU), which both produced quantitative couplings. Application of these reagents to the synthesis of the 2-7 sequence found in cyclosporin is reported.

Cyclosporin (CsA) (Figure 1), a lipophilic, cyclic undecapeptide with important and diverse biological properties[1-7], is distinguished by the presence of seven sterically hindered, N-methylated amino acids including MeLeu, MeVal and the unique amino acid, (4R)-4-[(E)-butenyl]-4,N-dimethyl-L-threonine (MeBmt).[8] Although efficient methods for the total synthesis of CsA and analogues in solution have been available for several years[9-11], the synthesis of CsA by solid phase synthetic methods has not yet been achieved, in part because conventional coupling procedures with sterically hindered or N-methyl amino acids often result in incomplete couplings under SPPS conditions, leading to deletion sequences. A variety of reagents have been evaluated in attempts to overcome this synthetic challenge.[12] Encouraging results for coupling hindered, N-methylated amino acid residues in solution have been reported for BOP-Cl[11-13] and PyBroP.[14] More recently, Carpino et al. have shown that Fmoc-Aib-F gave quantitative yields for the solid phase synthesis of a model hexapeptide that contained four adjacent sterically hindered Aib-units.[15] We report here our results to evaluate these and other new coupling reagents for their utility in preparing peptide sequences related to CsA by SPPS.

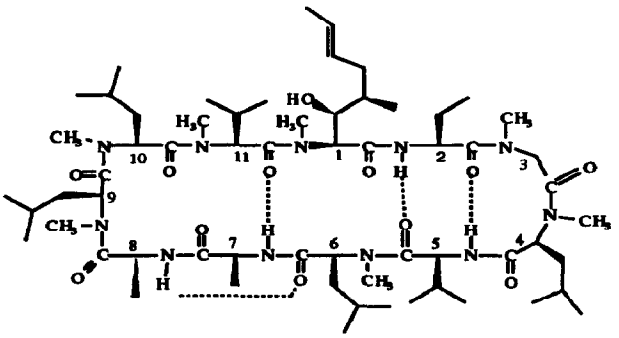

Figure 1. Cyclosporin A

Three tripeptides were chosen to model difficult couplings in the synthesis of CsA: H-Val-MeLeu-Ala-NH_2 (**1**), H-MeLeu-MeLeu-Ala-NH_2 (**2**), and H-MeLeu-MeVal-Ala-NH_2 (**3**). Tripeptide **1** models the CsA 5-6 amide bond, **2** models the 9-10 amide bond, and **3** models the 10-11 amide bond. Incorporation of the amino terminal residue in each model tripeptide was carried out by use of a double

coupling protocol (2 x 3 h), employing 3 eq of Fmoc-AA-OH (0.2 M in DMF), 3 eq of coupling reagent (0.2 M in DMF), and either 3 or 6 eq of base (DIEA or NMM). The extent of reaction was monitored by U.V. analysis of the Fmoc deprotection step (30% piperidine in DMF, 2 min + 8 min).[16] Characterization of all peptides upon cleavage from the resin (TFA:H_2O (95:5), 4 h) was carried out by RP-HPLC[17] and HR-FABMS analysis. The intermediate dipeptides were prepared using Fmoc-PAL-Nle-MBHA-polystyrene-resin[18], linking Fmoc-Ala-OH to the resin with DIPCDI, followed by Fmoc-MeLeu-OH or Fmoc-MeVal-OH using BOP/NMM.[19]

Preliminary evaluation of the following reagents was carried out with the model systems (Table 1): PyBroP, BOP, Fmoc-Val-F, HBTU, BOP-Cl, DIPCDI/HOAt, and HATU. PyBroP gave lower yields in this system than anticipated for the coupling of N-methylated amino acids[14,20], and the yields were not improved by addition of DMAP. BOP-Cl gave low yields of coupled peptide as well. However, large enhancements of coupling yields were obtained for all three peptides with PyBroP and BOP-Cl when 6 eq of base were used. Quantitative couplings were achieved for model tripeptide **1** when an excess of base was maintained throughout the reaction. Couplings with Fmoc-Val-F[21] , DIPCDI/HOAt, and HATU were quantitative when the pH was maintained during the reaction at a pH ~ 9.[22] However, increasing the base gave inconsistent results with model tripeptides **2** and **3**, in which both amino acids are N-methylated and sterically hindered.

Table 1: Synthesis of model tripeptides **1-3**

Entry	Coupling reagents[a,b]	Base	Yield(%)[c,d]		
			1	**2**	**3**
1	PyBroP	DIEA (3 eq)	11	6	29
2	PyBroP/DMAP	DIEA (3 eq)	---	7	4
3	PyBroP	NMM (3 eq)	---	16	---
4	PyBroP	DIEA (6 eq)	88	65	40
5	BOP	DIEA (3 eq)	50	70	25
6	BOP	DIEA (6 eq)	70	80	27
7	HBTU	DIEA (3 eq)	---	87	34
8	HBTU	DIEA (6 eq)	---	57	22
9	BOP-Cl	DIEA (3 eq)	---	10	5
10	BOP-Cl	DIEA (6 eq)	---	82	42
11	Fmoc-Val-F	DIEA (3 eq)	80	---	---
12	Fmoc-Val-F	DIEA (6 eq)	>99	---	---
13	DIPCDI/HOAt	DIEA (3 eq)	>99	>99	>99
14	DIPCDI/HOAt	DIEA (6 eq)	>99	>99	>99
15	HATU	DIEA (3 eq)	>99	>99	>99
16	HATU	DIEA (6 eq)	>99	>99	>99

(a) In each coupling 3 eq of Fmoc-Xxx-OH and 3 eq of coupling reagent, or 3 eq of Fmoc-Val-F were used.

(b) Fmoc-MeLeu-F was not employed since we were unable to synthesize the fluoride derivative of Fmoc-MeLeu-OH utilizing Carpino's procedure (see Ref. 21). Optimal reaction conditions for the synthesis of Fmoc-N-methyl-amino acid-fluorides are still needed.

(c) Yields indicate efficiency for coupling of the amino terminal residue to the dipeptide- resin (Error = ±0.04 A) ($A = c \times \varepsilon \times l$).

(d) UV absorbance of the fulvene-piperidine adduct was monitored at 301 nm ($\varepsilon = 7000\ M^{-1}\ cm^{-1}$).

The best yields were obtained for all three model tripeptides when 3 eq of HATU, the novel 1-hydroxyazabenzotriazole-containing uronium salt developed by Carpino[23], was used with 3 or 6 eq of DIEA. The increased efficiency of HATU and DIPCDI/HOAt relative to the other reagents tested could be governed by the formation of the HOAt active ester intermediate, which is postulated to have enhanced reactivity compared to that of the HOBt analogue due to the effect of the neighboring nitrogen.[23]

The use of HATU has been applied to the synthesis of the CsA 2-7 sequence (H-Abu-Sar-MeLeu-Val-MeLeu-Ala-OH). The peptide was synthesized by Fmoc-SPPS procedures utilizing HATU/DIEA. The Fmoc was removed, hexapeptide cleaved with TFA/H_2O (95:5), concentrated, washed with ether, and dried. The crude peptide was analyzed by RP-HPLC (Figure 2) and HR-FABMS.[17] Fewer than 4% of D-MeLeu diastereomers are formed in the synthesis. The crude peptide was purified by preparative RP-HPLC, isolated in 80% yield, and compared by HPLC and NMR with authentic samples prepared by solution phase methods. Our synthesis of the CsA 2-7 sequence complements the work of Carpino et al., who prepared H-D-Ala-MeLeu-MeLeu-MeVal-Phe-Val-OH, by SPPS by using HATU as the coupling reagent.[24] All amide bonds present in CsA now have been successfully formed on solid phase by use of HATU and HOAt reagents.

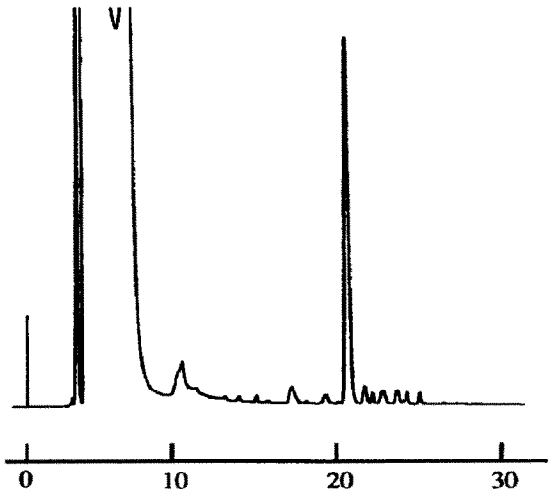

Figure 2. HPLC of crude CsA 2-7

Elution was carried out on a Vydac C-18 analytical column (4.6 x 250 mm), using a 40 min gradient of 0-80% CH_3CN (containing 0.036% TFA) in 0.045% TFA/H_2O at a flow rate of 1.2 mL/min. The CsA 2-7 hexapeptide has a retention time of 21 minutes (peaks eluting with retention times of 3-9 minutes are due to solvent elution). These conditions separated diastereomeric linear undecapeptide precursors of CsA analog, [N(Me)(β-OH)Leu]^1-CsA, diastereomeric hexapeptides comprising the CsA 2-7 sequence with D-MeLeu in the 4 and 6 positions, and diastereomers of the model tripeptides used in these coupling studies.

The azabenzotriazole-based coupling reagents gave results superior to other reagents tested in these systems. While conditions used for the coupling reactions are more stringent than those typical of peptide bond forming reactions, the results of this study should have broad applicability to the syntheses of various peptides and amides with hindered or N-methylated substituents. Application of HATU and DIPCDI/HOAt to the solid-phase synthesis of CsA and its analogues is in progress.

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13. Abbreviations: Aib: α-amino isobutyric acid; BOP: 1-benzotriazolyloxytris(dimethylamino)-phosphonium hexafluorophosphate; BOP-Cl: bis(2-oxo-3-oxazolidinyl)phosphinic chloride; DIEA: diisopropylethylamine; DIPCDI: diisopropylcarbodiimide; DMAP: 4-dimethylaminopyridine; DMF: dimethylformamide; Fmoc: 9-fluorenylmethoxycarbonyl; HOAt: 1-hydroxy-7-azabenzotriazole; HATU: O-(7-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HBTU: O-(benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; MBHA: 4-methylbenzhydrylamine resin; PAL: 5-(4-aminomethyl-3,5,-dimethoxyphenoxy)-valeric acid handle for peptide amides; PyBroP: bromo tris(pyrrolidino)phosphonium hexafluorophosphate; SPPS: solid phase peptide synthesis.
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