Solid-Phase Synthesis of Cyclosporin Peptides

Yvonne M. Angell, Timothy L. Thomas, George R. Flentke, and Daniel H. Rich*

School of Pharmacy and Department of Chemistry University of Wisconsin—Madison, 425 North Charter Street Madison, Wisconsin 53706

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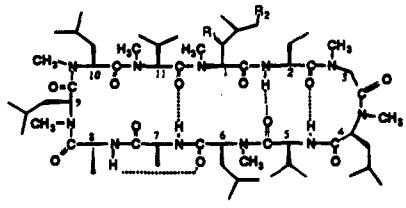
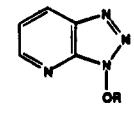
Cyclosporin A (CsA, Sandimmune, **1**), currently the drug of choice for preventing rejection of transplanted human organs,[1] is a cyclic undecapeptide, cyclo(-MeBmt[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-(D)-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]-), that contains seven *N*-methyl amino acids and the novel amino acid (4*R*)-4-[(2′*E*)-butenyl]-4,*N*-dimethyl-(L)-threonine (abbreviated as MeBmt)[2] in the 1-position.[1] Although a number of synthetic routes are used for the solution-phase synthesis of CsA,[3-5] a solid-phase synthetic approach[6] has not been achieved. The difficulty lies in assembling the linear undecapeptide precursor under SPPS conditions where conventional coupling procedures with sterically hindered or *N*-methyl amino acids often result in incomplete couplings.[7] Recently, we showed[8] that the CsA 2–7 sequence (H-Abu-Sar-MeLeu-Val-MeLeu-Ala-OH) could be synthesized on a solid phase by use of the coupling reagent HATU (**4**), a uronium-based reagent that contains the novel additive, 1-hydroxyazabenzotriazole (**3**), HOAt, invented by Carpino.[9] Here we describe the first synthesis of a cyclosporin derivative by solid-phase methods.



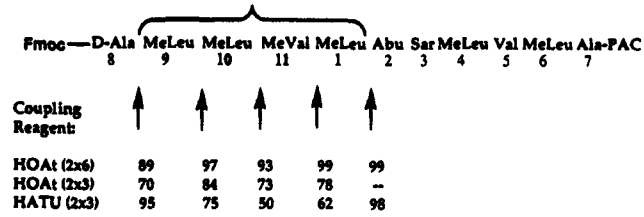
1, R_1 = OH; R_2 = $CH_2CHCHCH_3$
2, R_1 = R_2 = H.
5, R_1 = OH; R_2 = H



3, R = H
4, R = C(=NMe_2)NMe_2

Conversion of the CsA 2–7 sequence into the linear undecapeptide precursor for CsA derivatives requires high-yield addition of three sterically hindered, *N*-methyl amino acids (MeVal; MeLeu; MeLeu) onto the sterically hindered MeBmt. [MeLeu[1]]CsA, **2** in which MeLeu replaces MeBmt in the 1-position, was chosen as the synthetic target for structure–function reasons (*vide infra*). Both HOAt and HATU were tested for their effectiveness in coupling hindered amino acids to the hexapeptide. The fluorenylmethoxycarbonyl (Fmoc) group was used to protect the amino acids.[10] Both Fmoc-Ala–PAC–polystyrene (*p*eptide *ac*id linker) and PAC–PEG–polystyrene resins were evaluated and gave comparable yields.[11] Each coupling was performed twice with 3 equiv of Fmoc-amino acid/coupling reagent/DIEA. Since quantitative couplings were rarely achieved, unreacted peptide chains were capped routinely by reaction with acetic anhydride-pyridine (1:2). *N,N*-Dimethylformamide (DMF) was used to swell and wash the resin. Fmoc deblocking was carried out with piperidine-DMF (3:7) and monitored for completion by checking the UV absorbance at 301 nm (ϵ = 7000 M^{-1} cm^{-1}) of the fulvene–piperidine adduct obtained from deblocking.[12]

Scheme 1. Strategy for Solid-Phase Synthesis of Linear Undecapeptide[a]



[a] See text for experimental details. Percentage yields represent coupling completeness for the individual step as monitored by the UV absorbance of the fulvene–piperidine adduct.[12]

The couplings of Fmoc-MeLeu at the 9- and 10-positions and Fmoc-MeVal at the 11-position are particularly difficult. Nevertheless, with either HATU or HOAt/DIPCDI, the linear undecapeptide could be obtained in reasonable yields when double couplings and longer reaction times were used. The yields obtained from these couplings are shown in Scheme 1. HOAt/DIPCDI gave the highest yields when two 6-h couplings were used.

The linear undecapeptide was cleaved from the resin (TFA/H_2O (95:5); 3–4 h) and cyclized in solution according to our usual strategy[3,5] [0.3 mM in CH_2Cl_2; $(PrPO_2)_3$ (propyl phosphonic anhydride) and DMAP ((dimethylamino)pyridine)]. The cyclized peptide was obtained in 10–15% yield, purified by column chromatography and RP-HPLC,[13] and characterized by NMR and FABMS (1146.57 calcd, 1146.7 found). The NMR

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(2) Abbreviations used: CsA, cyclosporin A; DIEA, diisopropylethylamine; DIPCDI, diisopropylcarbodiimide; DMAP, 4-(dimethylamino)-pyridine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOAt, 1-hydroxy-7-azabenzotriazole; RP-HPLC, reverse phase high-performance liquid chromatography; HRFAB-MS, high-resolution fast atom bombardment mass spectroscopy; MBHA, 4-methylbenzhydrylamine resin; MeBmt, (4*R*-)-4-[(2′*E*)-butenyl]-4,*N*-dimethyl-(L)-threonine; PAC, *p*etide *ac*id linker; PEG–PS, polyethylene glycol-cross-linked polystyrene graft resin support; SPPS, solid-phase peptide synthesis.

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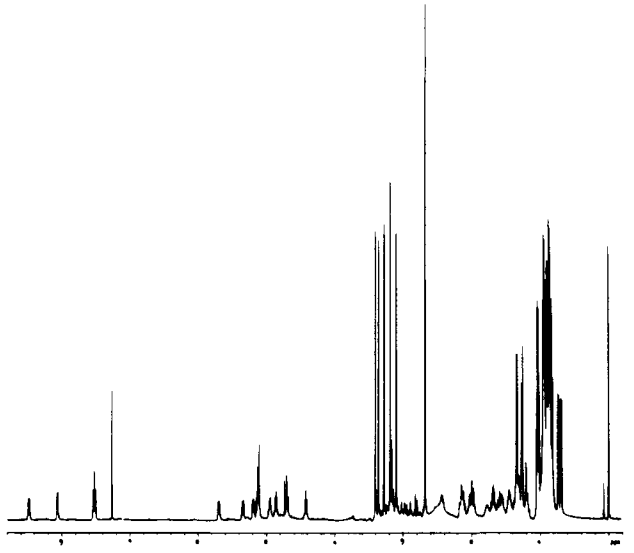

Figure 1. 500 MHz NMR of [MeLeu^1]CsA in $CDCl_3$. Note 5 N-methyls at 3.40, 3.35, 3.27, 3.19 and 3.09 ppm, and 2 N-methyls shifted upfield at 2.68 ppm; also 4 NH's at 7.51, 7.52, 8.05, and 8.47 ppm. The corresponding signals in CsA are: 7 N-methyls at 2.70, 2.71, 3.11, 3.12, 3.25, 3.40, and 3.51, and 4 NH's at 7.17, 7.48, 7.68, and 7.96.

spectrum (Figure 1) is closely related to that of CsA in chloroform, but additional minor peaks are evident. Saturation transfer NMR experiments (data not shown) established that these additional peaks were due to minor conformers and not epimers and thus confirm the configurational sequence of the product. Similar amounts of minor conformers are detectable in the spectra of CsA analogs which retain the correct configurational sequence of amino acids, but CsA diastereomers give substantially different NMR spectra in chloroform.

The MeBmt side chain in CsA is critical for immunosuppression mediated by inhibition of calcineurin,[4,14] but some 1-position analogs remain highly effective PPIase inhibitors,[16] e.g., [*N*-methylleucine(β-hydroxy)]^{1}CsA, **5**. This and other nonimmunosuppressive CsA analogs inhibit HIV replication in newly infected lymphocytes[16,17] and probably exert this activity by inhibiting binding of the p55*gag* protein to cyclophilin.[18,19] We applied solid-phase synthesis to prepare [MeLeu^1]CsA in order to determine the importance of the hydroxyl group in position-1 for binding to PPIase. [MeLeu^1]CsA, **2**, was found to inhibit the PPIase activity of cyclophilin A [K_i = (1.83 ± 0.16) × 10^{-6}][15] about 180-fold weaker than the β-hydroxy derivative **5** (K_i = (11 ± 3) × 10^{-9}).[16] This increased dissociation constant is remarkable since X-ray structures of CsA–cyclophilin complexes[20] show that the β-hydroxyl group in MeBmt is intramolecularly hydrogen bonded to the MeLeu-4 carbonyl group and does not participate in direct hydrogen bonding interactions with the protein.

This first solid-phase synthesis of a CsA analog was made possible because the azabenzotriazole-based coupling reagents, HOAt and HATU,[9] are particularly effective for coupling sterically hindered, N-methylated amino acids.[8] For this synthesis, HOAt/DIPCDI proved to be a more effective reagent than HATU, but both reagents should prove useful for synthesizing other peptides and amide bonds with hindered or N-methylated substituents. The solid-phase synthesis of additional cyclosporin analogs is in progress.

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Supporting Information Available: HPLC and NMR data for [MeLeu^1]CsA (6 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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(13) Analytical HPLC is carried out on a Vydac C-18 column (4.6 × 250 mm): linear gradient over 50 min of CH_3CN-MeOH (1:1)/0.036% TFA and H_2O/0.045% TFA from 0:100 to 100:0, flow rate 1.2 mL/min, detection at 214 nm; single peak at t_R = 45 min.

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