Methods to Circumvent a Difficult Coupling in the Solid-Phase Synthesis of Cyclosporine Analogues

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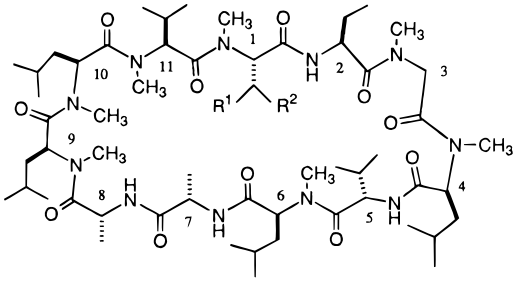
Cyclosporine (Cs (**1**), [cyclo(-MeBmt[1]-Abu[2]-Sar[3]-MeLeu[4]-Val[5]-MeLeu[6]-Ala[7]-D-Ala[8]-MeLeu[9]-MeLeu[10]-MeVal[11]-) where MeBmt = (4*R*)-4-[(2′*E*)-butenyl]-4,*N*-dimethyl-L-threonine][1]), the important immunosuppressant (Sandimmune) used for preventing rejection of transplanted organs, has been synthesized efficiently in solution,[2] but not by the solid-phase synthesis (SPS) methods pioneered by Merrifield.[3] The difficulty in synthesis appears to be due to the seven, sterically hindered *N*-methyl amino acids, which resist coupling, especially between *N*-methyl amino acids comprising the 11−1 bond. Recently we reported the synthesis of [MeLeu[1]]Cs (**2**) in which the linear undecapeptide precursor was assembled stepwise on a solid support, cleaved from the resin, and cyclized in solution,[4] an approach that succeeded because the novel additive hydroxyazabenzotriazole (HOAt)[5] afforded quantitative coupling of *N*-methyl amino acids on the resin. Subsequently, Wenger and Ko reported a different method for synthesizing a different precursor by SPS.[6] Here we report the successful syntheses of [MeSer[1]]Cs (**3**), [MeThr[1]]-Cs (**5**), and [MeLeu(3-OH)[1]]Cs (**7**), analogues of Cs with a β-hydroxy amino acid in the 1-position, by three procedures that overcome the difficult 11−1 coupling.

Early on we found that substantial degradation of the N-alkylated undecapeptides occurred during deprotection of Cs-derived peptides with high concentrations of TFA,[7] so the mild acid labile linkers HMPA,[8] HMPB,[9] and HAL[10] and the photolabile linker[11] were used in place of the PAC linker (see the Supporting Information for structures). Cs 2−7 hexapeptide was synthesized on PEG−polystyrene resin (or Tentagel resin) as previously described[5] except that the Fmoc protecting group was removed with DBU/piperidine/NMP (1:1:48) and unreacted amines were capped with *N*-acetylimidazole. Acylation of the hexapeptide−resin by



1: Cs , R^1 = OH; R^2 = $CH(CH_3)CH_2CH{=}CHCH_3$
2: [MeLeu[1]]Cs, R^1 = H; R^2 = isopropyl
3: [MeSer[1]]Cs, R^1 = OH; R^2 = H
4: [MeSer(OBn)[1]]Cs : R^1 = OBn; R^2 = H
5: [MeThr[1]]Cs, R^1 = OH; R^2 = CH_3
6: [MeThr(OBn)[1]]Cs : R^1 = OBn; R^2 = CH_3
7: [MeLeu(3-OH)[1]]Cs, R^1 = OH; R^2 = isopropyl

Fmoc-MeSer(OBn)-OH and Fmoc-MeThr(OBn)-OH proceeded in nearly quantitative yields, but acylation of the 1-position residue of the heptapeptide resins with Fmoc-MeVal-OH proved to be difficult (Scheme S1 for peptide sequences). Quantitative yields were not achieved despite a double-coupling protocol with DIPCDI/HOAt. Wenger and Ko also found coupling between the MeVal[11] and a β-OH amino acid in the 1-position particularly difficult and were unable to obtain the desired product despite repeated treatments with Fmoc-MeVal anhydride.[6]

Detailed studies of the reaction of Fmoc-MeVal-OH with MeSer(OBn)−heptapeptide resin and MeThr(OBn)−heptapeptide resin were performed to identify optimal reaction conditions. Difficult couplings are sequence dependent and have been ascribed to interchain interactions and/or poor solvation that lead to limited accessibility to the amino group in the growing peptide chains.[12] This problem has been overcome by use of the polar aprotic solvent NMP,[13] elevated temperatures,[14] mixed solvents containing TFE[15] and HFIP,[16] and chaotropic agent washes.[17] Although different combinations of the solvents DMSO, THF, toluene, and NMP did not improve the yield, acylation of the MeSer(OBn)−heptapeptide resin with Fmoc-MeVal-OH gave the octapeptide nearly quantitatively when we switched the solvent to NMP and raised the reaction temperature to 60 °C. However, these conditions gave only a 40% yield of octapeptide when applied to the MeThr(OBn)−heptapeptide resin. Neither solvent additives (such as TFE and HFIP or the chaotropic agent KSCN) nor use of the amino acid fluoride Fmoc-MeVal-F,[18,19] with or without added base, improved the yields. A quadruple acylating protocol eventually gave a 75% yield of Fmoc-MeVal-MeThr(OBn)−heptapeptide resin. Despite capping with *N*-acetylimidazole, substantial amounts of the MeVal-deletion cyclic *deca*peptide were obtained, but this undesired byproduct could be eliminated by manually

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(1) See the Supporting Information for abbreviations.

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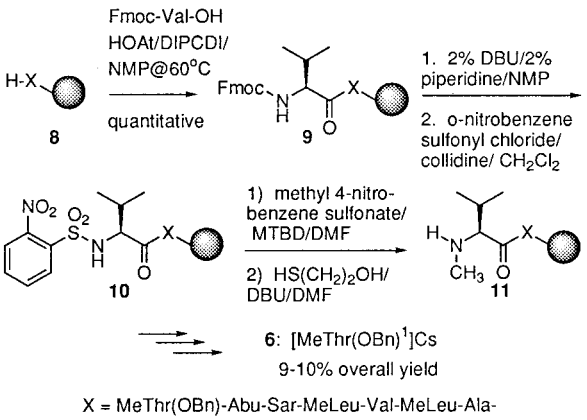
Scheme 1



capping with pyridine/acetic anhydride (2:1) following the difficult coupling; this procedure greatly increased the ease of purification. Using these optimized conditions, the linear undecapeptide precursors for [MeSer(OBn)[1]]Cs (**4**) and [MeThr(OBn)[1]]Cs (**6**) were synthesized smoothly using NMP at 60 °C for the acylation of the last four amino acid residues beginning with MeVal[11].

To complete the synthesis of the cyclosporin analogues, the undecapeptide precursors were Fmoc-deprotected, cleaved from the resin, and cyclized in solution in a manner analogous to that used to synthesize [MeLeu[1]]Cs (**2**). [MeSer(OBn)[1]]Cs (**4**) and [MeThr(OBn)[1]]Cs (**6**) were obtained in excellent yields (5−16% overall). Cleavage of the benzyl ethers using Pearlman's catalyst ($Pd(OH)_2/C$) proceeded smoothly to afford the target analogues [MeSer[1]]Cs (**3**) and [MeThr[1]]Cs (**5**), which were characterized by the usual methods.[20]

Site-selective N-methylation on-resin (Scheme 1) circumvented the problem of poor coupling between Fmoc-MeVal-OH and the MeThr(OBn)−heptapeptide resin (**8**). Use of Fmoc-Val-OH instead of Fmoc-MeVal-OH in the 11-position gave a quantitative yield of Fmoc-Val-MeThr(OBn)−heptapeptide resin **9**. The methylated H-MeVal-MeThr(OBn)−heptapeptide resin **11** was synthesized by the site-selective N-methylation reaction invented by Scanlan and Miller.[21] The linear undecapeptide was synthesized using the protocol outlined above for [MeThr(OBn)[1]]Cs (**6**). Cleavage[22] from the resin followed by cyclization using $(PrPO_2)_3$/DMAP/CH_2Cl_2 gave the desired [MeThr(OBn)[1]]Cs (**6**) in 9% overall yield after flash chromatography. Even though the yield of the analogue did not improve significantly, no deletion peptides were observed.

The difficult formation of the 11−1 bond is affected by the peptide sequence. Although Fmoc-MeVal-OH acylation of alternate sequences prepared by SPS from Sar[3] and Abu[2] linked to the resin[23] did not give improved coupling yields (see Supporting Information for alternate linear undecapeptide sequences), synthesis of the undecapeptide precursor[24] proceeded smoothly in NMP at 60 °C when Fmoc-MeVal-OH was attached directly to HMPB-PEG-PS.[25] After cleavage, cyclization,[26] and purification, [MeThr(OBn)[1]]-Cs (**6**) was obtained in 9% overall yield, but approximately one-third of this product was the 11-position diastereomer, formed by epimerization at the 11-position during the activation step. This approach was also used to synthesize the more hindered [MeLeu(3-OH)[1]]Cs (**7**). After assembly of the linear precursor, cleavage, and cyclization, the title compound, [MeLeu(3-OH)[1]]Cs (**7**), was obtained in 10% overall yield as a 2:1 mixture of MeVal[11] diastereomers.

The total synthesis of [MeSer[1]]Cs (**3**), [MeThr[1]]Cs (**5**), and [MeLeu(3-OH)[1]]Cs (**7**) by a combination of solid-phase and solution methods has accelerated the production of Cs analogues from months using only solution-phase techniques to a matter of days, methods that will facilitate screening of Cs analogues for new activities.[27] Successful synthesis was achieved by the use of DIPCDI/HOAt for the couplings, NMP at 60 °C for acylations of the 1-position and following residues, and either pyridine/acetic anhydride for capping of any unreacted amines or site-selective N-methylation of H-Val-MeThr(OBn)−heptapeptide resin, procedures that eliminate the formation of the cyclic decapeptide, an undesired side product that is difficult to separate from the desired product. We have used these methods to synthesize cyclosporin analogues by stepwise assembly of side-chain attached Cs precursor peptides followed by on-resin cyclization, an entirely solid-phase approach that enables Cs analogues to be synthesized in a combinatorial fashion. We are currently optimizing these procedures and will report them in due course.

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Supporting Information Available: Abbreviations, structures of linkers and alternate undecapeptide sequences, Scheme S1 showing difficult couplings, and experimental procedures and compound characterization data for compounds **3**−**7** (20 pages).

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(20) Analogues were characterized by ^1H NMR, FAB-MS, RP-HPLC, and TLC. Please see Supporting Information for analytical data for the Cs analogues.

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