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# Total Synthesis of Cyclosporin O by Convergent Approach Employing Fmoc-Amino Acid Chlorides Mediated by Zinc Dust

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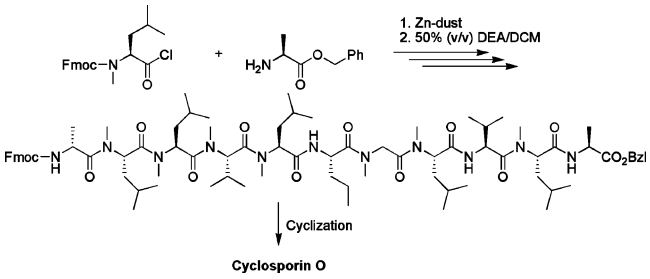


An epimerization free and efficient total synthesis of immunosuppressant cyclosporin O (CsO) by step-by-step assembly of amino acids in solution phase is reported. The couplings were performed by employing Fmoc-amino acid chlorides and were mediated by zinc dust under neutral conditions. The yield and purity of the coupling of sterically hindered *N*-methylamino acids to *N*-methylamino acids at positions 8, 9, 10, and 11 were enhanced by repeating the coupling thrice at these particular junctures. All the 10 intermediate peptides pertaining to CsO and the final CsO were isolated and completely characterized through IR, $^1$H NMR, mass spectrometry, and HPLC techniques.

*N*-Methylated and *N*-benzylated amino acids are widely distributed in several biologically active naturally occurring peptides.[1−3] These peptides have been intensively studied because of the structural perturbations induced in their backbone through cis−trans isomerization of the peptide bond and by reduced number of intramolecular hydrogen bonds owing to removal of a proton donating N−H group.[4−6] One of the most prominent examples is cyclosporin (Cs). Cs compounds are cyclic undecapeptides containing seven *N*-methyl amino acids.[7] Though the biosynthesis of Cs[8−12] is routinely carried out by the enzyme "cyclosporin synthetase", its chemical synthesis is much more difficult.[13]

The difficulties encountered in the incorporation of *N*-alkyl amino acids are attributed to the severe steric hindrance and the cis-conformation of peptide bond that has been formed owing to the bulkier *N*-alkyl group, the tendency to undergo cyclization to form stable diketopiperazines,[6,14] high degree of racemization,[15] and the sensitivity of N-alkylated peptides toward acid.[16] Often the yields of the required peptide are quite low, and it is very difficult to isolate the peptide even by preparative HPLC. The target peptide is contaminated with wrong peptide sequences that are formed by truncation of the sequence because of unattainable acylation or with the peptides that are formed by mismatch sequences that arise from incomplete peptide coupling.[17] The first synthesis of Cs was reported by Wenger.[18,19] Other attempts made for the synthesis of Cs have used diphenylphosphinic chloride (DppCl),[20,21] *N*,*N*-bis(2-oxo-3-oxazolidinyl)phosphinic chloride (BOP−Cl),[22,23] and *N*-[(1*H*-azabenzotriazol-1-yl)(dimethylamino)-methylene]-*N*-methylmethanaminium hexafluorophosphate-*N*-oxide (HATU).[24,25]7-Aza-benzotriazole-1-yl-oxy tris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP)[26] has been used for the solid-phase synthesis (SPPS) of [MeLeu$^1$]CsA and [*N*-methylleucine($\beta$-hydroxy)$^1$]CsA. It was found that azabenzotriazole-based reagents were effective for coupling sterically hindered N-methylated amino acids in the SPPS. During the cleavage of peptide from the resin considerable amount of degradation of peptide was observed and hence mild acid labile linkers like (4-hydroxymethyl-3-methoxyphenoxy)-acetic acid (HMPA), 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid

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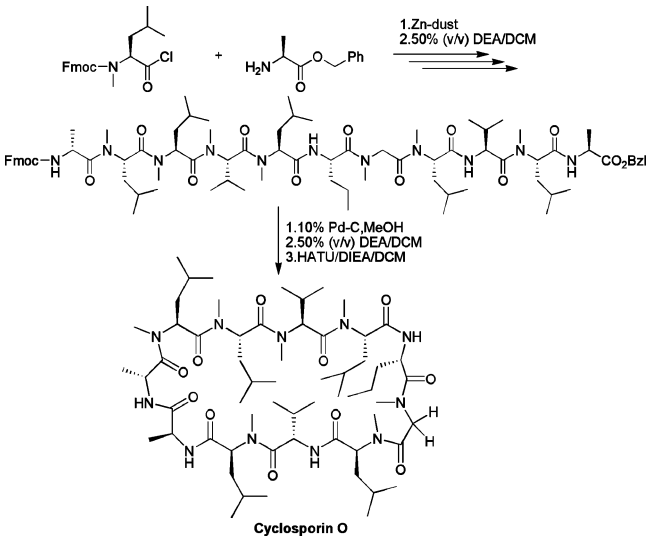
(HMPB), and photolinkers were used as handles.[27] A recent report on the synthesis of peptides containing *N*-methyl amino acids is the N-methylation of $N^{\alpha}$-acylated linear peptides and $N^{\alpha}$-aryl sulfonyl peptides on solid support by using $CH_3I/Ag_2O$ in DMF[28] and dimethylsulfate and DBU as base,[29] respectively.

Cyclosporin O (CsO), one of the important members of the Cs family that occurs naturally contains MeLeu and norvaline (Nva) at positions 1 and 2 in place of (4*R*)-[4(*E*)-2-butenyl]-4,*N*-dimethyl-L-threonine (MeBmt) and (2*s*)-2-aminobutanoic acid (Abu), respectively. Cs compounds bearing Nva such as CsG are reported to be less nephrotoxic than the parent CsA.[30] Hence, development of an efficient method for the synthesis of CsO becomes imminent. The synthesis of CsO both in solution as well as by solid-phase method was performed by a combination of reagents, 2-bromo-3-ethyl-4-methyl thiazolium tetrafluoroborate (BEMT), 2-bromo-1-ethyl pyridinium tetrafluoroborate (BEP), and 5-(1*H*-benzotriazol-1-yloxy)-3,4-dihydro-1-methyl 2*H*-pyrrolium hexachloroantimonate (BDMP) in the presence of 1-hydroxy-7-azabenzotriazole (HOAt).[31−33] Another successful synthesis of CsO was accomplished in solution phase via 4 + 7 fragment coupling and the cyclization of final undecapeptide between L-Ala and D-Ala using 1-(1-pyrrolidinyl-1*H* −1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene)pyrrolidinium hexafluorophosphate *N*-oxide (HAPyU). The SPPS of CsO using bis(trichloromethyl)carbonate (BTC)/collidine and acid labile trityl chloride polystyrene resin was reported.[34,35] The couplings were performed by in situ generation of acid chloride in the presence of collidine and were found to be complete in 3 h. This was an extension of the Falb et al.[36] method for the formation of *N*-alkyl amides on Rink amide resin. In another report the CsO fragment, Boc-MeLeu-MeLeu-MeVal-MeLeu-OMe was synthesized by treating its unmethylated precursor with excess MeI and $Ag_2O$. The fragment was further utilized in the synthesis of CsO.[37] To the best of our knowledge, most of the earlier reports on the synthesis of Cs were either on solid phase or by fragment coupling in solution phase. In this report we describe the utility of acid chloride/zinc dust for the synthesis of CsO by step-by-step assembly of amino acids using Fmoc chemistry.

Acid chlorides, one of the powerful modes of activation of carboxylic acids, preparation is simple as well as less expensive. Their utility in the synthesis of peptides possessing dialkyl amino acids is known.[38] When acid chlorides are employed as acylating agents the coupling requires a base for the abstraction of liberated HCl. Instead, zinc dust[39−45] can be used for this purpose under non-Schotten−Baumann conditions in homogeneous medium employing DCM or $CHCl_3$ resulting in the complete elimination of base-catalyzed side reactions, namely, the formation of oxazolone and racemization. The coupling proceeds under neutral conditions and thus can be carried out over an extended period of time. This advantage offered by amino acid chlorides becomes much more conspicuous for the coupling of *N*-methyl amino acids. Following the reports of difficulties encountered in the synthesis of Cs, we choose to exploit the utility of zinc dust for the coupling of hindered *N*-methyl amino acids. The overall strategy involved in the synthesis of CsO is illustrated in Scheme 1.

**SCHEME 1. Synthesis of CsO by Step-by-Step Assembly of Amino Acids**




The synthesis of all Fmoc-*N*Me-amino acid chlorides except Fmoc-MeVal-Cl was accomplished by the $SOCl_2$/DCM method. The use of regular protocol for the synthesis of Fmoc-MeVal-Cl was not satisfactory. Alternatively, an in situ generated acid chloride was used. A suspension of Fmoc-MeVal-OH (1 mmol)

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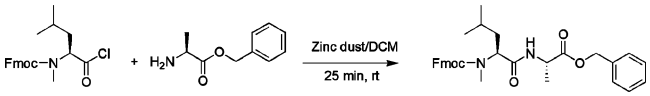
**SCHEME 2. Synthesis of Fmoc-MeLeu$^6$-Ala$^7$-OBzl 1**




in DCM was added to dicyclohexylamine (DCHA,1 mmol), and the mixture was stirred for 3 min. To the resulting clear solution, pyridine (1.2 mmol) and thionyl chloride (1.2 mmol) were added, and the mixture was stirred for an additional minute. The hydrochloride salt of DCHA/pyridine formed was filtered off, and the clear filtrate was taken for the coupling. To verify this method for possible racemization during the coupling, it was subjected to a racemization test. Synthesis and HPLC analysis of racemization prone Carpino's diastereomeric dipeptides Fmoc-L-Phg-Phe-OMe and Fmoc-D-Phg-Phe-OMe were chosen for this purpose. Fmoc-L-Phg-Cl and Fmoc-D-Phg-Cl, prepared by DCHA/pyridine/$SOCl_2$, were coupled in separate experiments with $H_2N$-Phe-OMe in the presence of zinc dust. The HPLC analysis of the LL and DL pairs revealed that the method was free from racemization. This was in accordance with the earlier report on the in situ generation of acid chloride by Matsuda et al.[46]

Toward the first step, Fmoc-MeLeu$^6$-Ala$^7$-OBzl **1** was synthesized using Fmoc-MeLeu-Cl in presence of zinc dust (Scheme 2). A solution of $H_2N$-Ala-OBzl (1 mmol) in DCM (3 mL) was added to a mixture of Fmoc-MeLeu-Cl (1.2 mmol) and freshly activated zinc dust (1.5 mmol) in DCM (3 mL) and stirred at room temperature until the completion of the reaction. The progress of the reaction was monitored by the TLC and IR. The formation of **1** was complete in 25 min, and workup of the reaction mixture resulted in pure peptide in 93% yield. Tos·$H_2N$-Ala-OBzl was synthesized following the microwave irradiation method,[47] and the tosyl salt was neutralized with aqueous $Na_2CO_3$ prior to coupling. The resulting amino-free amino acid benzyl ester was extracted into DCM and used directly for coupling.

For the deprotection of Fmoc group, the use of *tris*(2-aminoethyl)amine (TAEA) and diethyl amine (DEA) was explored. TAEA is well-known for deblocking Fmoc group in solution phase.[48]Upon completion of deprotection, excess of TAEA and dibenzofulvene (DBF) adduct are removed using aqueous work up. However in our studies, when TAEA was employed for deprotection of Fmoc group, the similar work up conditions resulted in the loss of amino-free peptide ester accounting for low yield of tripeptide. On the contrary, DEA being a low boiling solvent (bp 55 °C), can be completely removed by simple evaporation. The deprotection was complete in 30 min and the DBF adduct could be removed through column chromatography after coupling.

After the removal of the Fmoc group using DEA in DCM (1:1), the amino-free dipeptide benzyl ester was reacted with Fmoc-Val-Cl in presence of zinc dust to obtain the tripeptide Fmoc-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **2** in 90% yield. The amino-free dipeptide benzyl ester, $H_2N$-MeLeu$^6$-Ala$^7$-OBzl was used directly without isolation. Fmoc-Val-Cl was synthesized employing $SOCl_2$, and the optimum formation of **2** was observed after stirring the reaction mixture for 40 min. The formation of **2** was dependent on the coupling conditions. The coupling of same acid chloride in presence of diisopropylethylamine (DIEA) resulted in less yield of the tripeptide. HPLC purity of the pure tripeptide was about 98%. The summary of the study is given in the Table 1.

**TABLE 1. A Comparison Study of the Coupling of Fmoc-Val-Cl/OH to H-MeLeu-Ala-OBzl**

| coupling method | solvent | base (equiv) | yield (%)[a] |
|---|---|---|---|
| acid chloride/zinc dust | DCM | | 90 |
| acid chloride/base | DCM | DIEA (2) | 68 |
| BOP-Cl | DCM | DIEA (2) | 60 |
| PyBrOP | DCM | DIEA (2) | 48 |
| HBTU | THF | DIEA (2) | 53 |

[a] Isolated yield after column purification.

Subsequent deprotection and coupling of **2** with Fmoc-MeLeu-Cl resulted in the tetrapeptide Fmoc-MeLeu$^4$-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **3** in 90% yield. The pentapeptide Fmoc-Sar$^3$-MeLeu$^4$-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **4** was obtained by the condensation of Fmoc-Sar-Cl with the amino-free tetrapeptide which was obtained from **3**. The yield of the pentapeptide was 84%. The yield of **4** was optimum when 2 equiv of Fmoc-Sar-Cl were used for the coupling. However, further increase in molar ratio to 2.5 equiv did not show any increase in the yield. The hexapeptide Fmoc-Nva$^2$-Sar$^3$-MeLeu$^4$-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **5** and the heptapeptide Fmoc-MeLeu$^1$-Nva$^2$-Sar$^3$-MeLeu$^4$-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **6** were obtained starting from their corresponding precursor amino-free peptide benzyl esters in 91 and 90% yields, respectively.

The incorporation of Fmoc-MeVal at the 11-position and Fmoc-MeLeu at 9- and 10-positions was crucial in the stepwise assembly. CsO being the longest known sequence composed exclusively of highly hindered N-methylated amino acids, the assembly of this subunit MeLeu$^9$-MeLeu$^{10}$-MeVal$^{11}$-MeLeu$^1$ was the most difficult job to accomplish during its assembly. Nevertheless, with the acid chloride/zinc dust method, the yields and the purity of the peptides were good. During the synthesis of the octapeptide Fmoc-MeVal$^{11}$-MeLeu$^1$-Nva$^2$-Sar$^3$-MeLeu$^4$-Val$^5$-MeLeu$^6$-Ala$^7$-OBzl **7**, the formation of a peptide bond between Fmoc-MeVal-Cl and amino-free heptapeptide was sluggish, and the yield was only 45% after first coupling. The LC−MS spectrum of the crude **7** showed the presence of the free N-deprotected heptapeptide as major component. The unreacted amino-free heptapeptide present in the reaction mixture could not be separated either by extraction with 10% citric acid or by purification through column chromatography owing to undifferential $R_f$ values. This resulted in the formation of mismatch sequences when the peptide chain was subjected to further extension.

The presence of such undesired peptides resulted in contamination of the target peptide, and hence the separation of such peptides became much more cumbersome because of their undifferentiated $R_f$ values. Consequently, to avoid such complications and improve the yield and purity, the coupling of MeVal to the heptapeptide **6** was repeated two more times. The repetition of the coupling twice, each time employing Fmoc-MeVal-Cl (1.5 equiv) and zinc dust (1.6 equiv), and with a duration of 2.5 h, led to the octapeptide **7** in 79% yield. The analytical HPLC of the pure octapeptide was found to be 95%. Thus, with the use of triple coupling and longer duration of reaction time, the yield of the octapeptide **7** enhanced drastically

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**TABLE 2. Summary of Coupling Conditions Employed during CsO Synthesis**

| acid chloride equiv used | no. of couplings | coupling duration | peptide obtained | yield (%) |
|---|---|---|---|---|
| 1.1 | one | 0.41 h | di | 93 |
| 1.1 | one | 0.66 h | tri | 90 |
| 1.1 | one | 0.41 h | tetra | 90 |
| 2.0 | one | 2.0 h | penta | 84 |
| 1.1 | one | 1.5 h | hexa | 91 |
| 1.1 | one | 1.5 h | hepta | 90 |
| 1.5 | three | 2.5 h | octa | 79 |
| 1.5 | three | 2.5 h | nona | 73 |
| 1.5 | three | 2.5 h | deca | 76 |
| 1.5 | three | 2.5 h | undeca | 82 |

from 45% to 79%. A similar approach was adopted further for the incorporation of the remaining three residues. The nonapeptide Fmoc-MeLeu$^{10}$-MeVal$^{11}$-MeLeu$^{1}$-Nva$^{2}$-Sar$^{3}$-MeLeu$^{4}$-Val$^{5}$-MeLeu$^{6}$-Ala$^{7}$-OBzl **8**, the decapeptide Fmoc-MeLeu$^{9}$-MeLeu$^{10}$-MeVal$^{11}$-MeLeu$^{1}$-Nva$^{2}$-Sar$^{3}$-MeLeu$^{4}$-Val$^{5}$-MeLeu$^{6}$-Ala$^{7}$-OBzl **9** and the undecapeptide Fmoc-D-Ala-MeLeu$^{9}$-MeLeu$^{10}$-MeVal$^{11}$-MeLeu$^{1}$-Nva$^{2}$-Sar$^{3}$-MeLeu$^{4}$-Val$^{5}$-MeLeu$^{6}$-Ala$^{7}$-OBzl **10** were obtained in 73%, 76%, and 82% yields, respectively (Table 2).

The Fmoc-protected linear undecapeptide **10** was subjected to catalytic hydrogenation using 10% Pd on carbon to deprotect benzyl ester. In a typical procedure a 50 mg of the undecapeptide **10** was dissolved in 2 mL of methanol. To this, a 10 mg of 10% palladium on carbon was added and hydrogen gas was bubbled through. Complete deprotection of the benzyl ester was observed after 3 h. The undecapeptide was worked up and purified through column. This resulted in Fmoc-protected undecapeptide acid in 92% yield (Figure 1). This was deprotected, and the resulting free undecapeptide was cyclized using *O*-(azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′,-tetramethyluronium hexafluorophosphate (HATU)/DIEA at room temperature to obtain CsO as a white solid in 85% yield.

In summary, the synthesis of CsO was carried out efficiently by step-by-step linear condensation approach employing Fmoc-amino acid chlorides in the presence of zinc dust under neutral conditions. The amino-free peptide benzyl esters were not isolated and were directly employed for coupling with the next amino acid. The assembly of four consecutive *N*-methyl amino acids required triple coupling and extended coupling duration to enhance the yield and purity of the respective peptides. All the 10 intermediate Fmoc-protected peptide fragments starting from the dipeptide **1** to the linear undecapeptide **10** were isolated as well as characterized by $^{1}$H NMR, mass spectroscopy, and HPLC. The isolated yields of the peptides, after column chromatography, were above 90%. However, for the coupling of consecutive *N*-methyl amino acids, the yields were in the range of 73−79%. Catalytic hydrogenation of the linear undecapeptide with Pd−C/MeOH resulted in Fmoc-protected undecapeptide acid in 92% yield. The cyclization of free undecapeptide with HATU in DCM resulted in CsO in 85% yield. Thus, starting from dipeptide **1**, the final CsO was obtained in an overall of 15−18% yield.

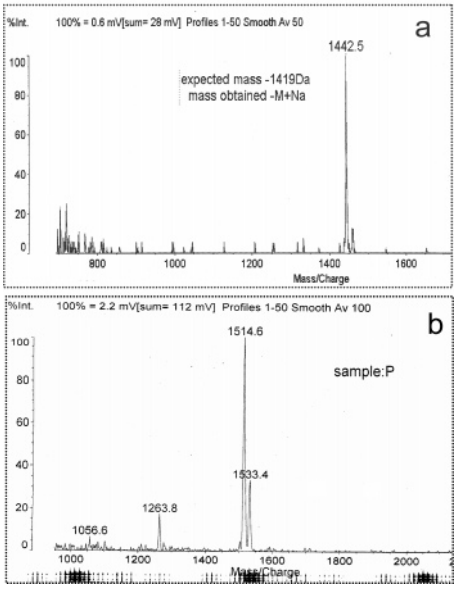


**FIGURE 1.** MALDI-TOF spectra of Fmoc-protected peptide fragments (a) decapeptide **9** and (b) undecapeptide **10**.

## Experimental Section

**General Procedure for the Coupling of Acid Chloride Mediated by Zinc Dust.** To a solution of amino-free amino acid benzyl ester/free peptide ester (1 mmol) in DCM (3 mL) was added Fmoc−amino acid chloride (1.2 mmol) and zinc dust (0.105 g, 1.5 mmol). After completion of the reaction, the reaction mixture was filtered, and the filtrate was diluted with $CHCl_3$ (10 mL) and washed with 10% citric acid solution (5 mL × 2), 10% aqueous $Na_2CO_3$ (5 mL × 2), and water (5 mL × 2). The organic layer was dried over anhydrous $Na_2SO_4$ and evaporated under reduced pressure. The residue was purified through silica gel column chromatography (100−200 mesh; eluant, varying concentrations of EtOAc/*n*-hexane).

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**Supporting Information Available:** $^{1}$H NMR, HPLC, and mass spectra of peptides **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8**, **9**, **10** and CsO; mass spectra of Fmoc-undecapeptide acid (**11**). This material is available free of charge via the Internet at http://pubs.acs.org.

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