RACEMIZATION-FREE SYNTHESIS OF C-TERMINAL CYSTEINE-PEPTIDE USING 2-CHLOROTRITYL RESIN

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We investigated the effects of bases, resins, and S-protecting groups on the extent of racemization at the C-terminal cysteine during Fmoc-based(Fmoc=fluoren-9-yl-methoxy-carbonyl) solid phase peptide synthesis. The use of 2-chlorotrityl resin was most effective in suppressing the racemization caused by the base treatment for Fmoc-cleavage. Somatostatin was successfully synthesized with practically no racemization using 2-chlorotrityl resin by Fmoc-chemistry.

KEYWORDS solid phase peptide synthesis; racemization; C-terminal cysteine; base treatment; 2-chlorotrityl resin; somatostatin

Racemization during the esterification of the C-terminal amino acid or during the chain elongation is one of the most critical problems in Fmoc-based solid phase peptide synthesis(SPPS). In the anchoring of C-terminal amino acid to the starting resin, it has been reported that the use of the catalyst, 4-dimethylaminopyridine, promotes racemization especially at Cys and His residues.[1,2] To suppress this racemization during the anchoring, several alternative techniques have been reported.[2-4] Concerning the racemization during chain elongation, Atherton *et al.* have reported that the use of piperidine for Fmoc-cleavage causes significant racemization at a C-terminal cysteine residue.[5] We also observed a side product in the Fmoc-based SPPS of human C-type natriuretic peptide 22(hCNP22),[6] which was derived from the racemization at the C-terminal Cys residue during the chain elongation.[7] The racemization was detected for C-terminal Cys residue having free acid but not for cysteine-amide. These findings led us to investigate the effect of various bases, resins, and S-protecting groups on the extent of the racemization at the C-terminal Cys, and to seek a racemization-free method of Fmoc-based SPPS. Now, we report that the C-terminal Cys-peptide can be successfully synthesized without racemization by Fmoc-based solid phase method using 2-chlorotrityl(Clt) resin.

In order to examine the effect of base, we first exposed Boc-Cys(Acm)-Wang resin(Acm=acetamidomethyl) to four different kinds of bases, i.e., 5% and 20% piperidine(PIP)/dimethylformamide(DMF), 5% diisopropylethylamine(DIEA)/DMF, and 5% Et_3N/DMF(Fig. 1A). For estimation of the racemization, resin-bound Cys(Acm) was cleaved by the treatment with trifluoroacetic acid(TFA)-thioanisole(9:1) for 60min at 25°C. The D-isomer content of the product was examined using a SUMICHIRAL OA-5000 column. With increasing concentration of piperidine for Fmoc cleavage, a higher degree of racemization was observed. The use of Et_3N or DIEA did not affect the optical purity of Cys(Acm). Next, we examined the effect of other S-protecting groups on the extent of the racemization(Fig. 1B). After the treatment of the resin-bound dipeptide, Val-Cys(R)-Wang resin(R=trimethylacetamidomethyl(Tacm),[8] MeBzl, or tBu), with 20%PIP/DMF, each dipeptide was cleaved from the resin with TFA-thioanisole(9:1) as described above. The L-D-isomer content of the product was analyzed using reverse phase(RP)-HPLC.[9] More than 10% formation of the L-D-isomers was detected for all of the S-protecting groups, although there were some differences in the isomer contents among the products derived from these protecting groups. Then the effect

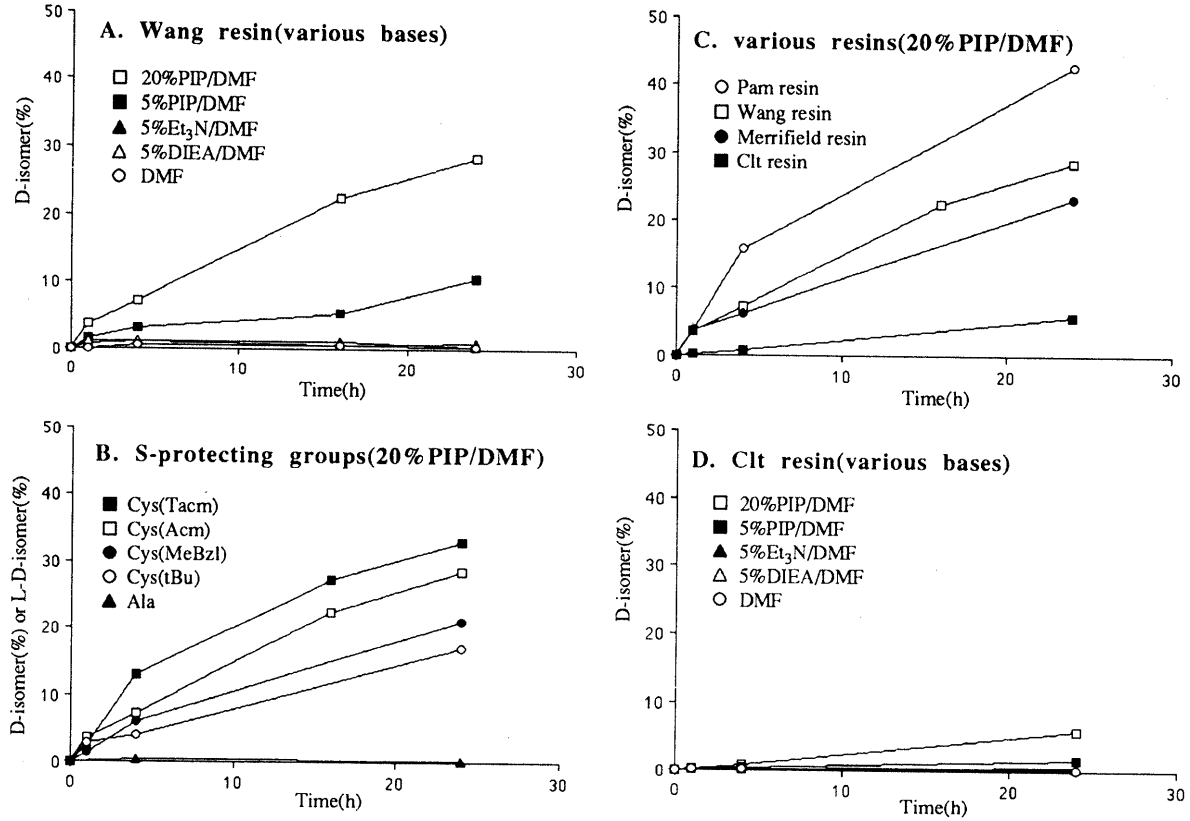
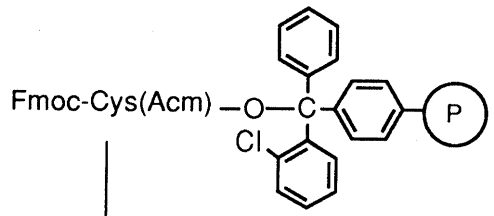


Fig. 1. Time Course Curves of D-Isomer or L-D-Isomer Formation in Samples of Cys Derivative Attached on Resin under Various Basic Conditions

of resin was examined using Wang-, Merrifield-, Pam-, and Clt-resin.[10] Boc-Cys(Acm) bound to each resin was exposed to various bases, and the optical purity of the Cys(Acm) liberated from each resin with an acid(TFA or HF) was analyzed on the SUMICHIRAL OA-5000 column. The products prepared from Pam-, Wang-, and Merrifield-resin contained significant amounts of isomers in that order. However, only a little racemization (<5%) was detected even after 24h treatment with 20% PIP/DMF when Clt resin was used(Fig. 1C and D). These results suggest that the use of Clt resin circumvents the racemization at the C-terminal cysteine caused by the base treatment, probably due to its high steric hindrance.



SPPS by Fmoc strategy

H-Ala-Gly-Cys(Acm)-Lys(Boc)-Asn-Phe-Phe-Trp-Lys(Boc)-Thr(tBu)-Phe-Thr(tBu)-Ser(tBu)-Cys(Acm)-**Clt resin**

1) 1M HBF_4-thioanisole/TFA (4°C, 30min)
2) preparative FPLC

[Cys(Acm)^{3,14}]-somatostatin

3) $AgBF_4$-anisole/TFA (4°C, 60min)
4) Air oxidation at pH7.8(peptide concentration, 0.11 mM)
5) Preparative FPLC

purified somatostatin(yield: 26%)

Fig. 2. Synthesis of Somatostatin Using Clt Resin

In order to demonstrate the usefulness of the Clt resin, we synthesized somatostatin using Fmoc-Cys(Acm)-Clt resin as a starting resin(Fig. 2). The attachment of C-terminal Cys(Acm) residue to the Clt resin was carried out according to the published procedure without racemization.[10c] The peptide backbone was constructed by the combination of diisopropylcarbodiimide condensation and piperidine deprotection.[11] The fully protected peptide resin was treated with 1M HBF_4-

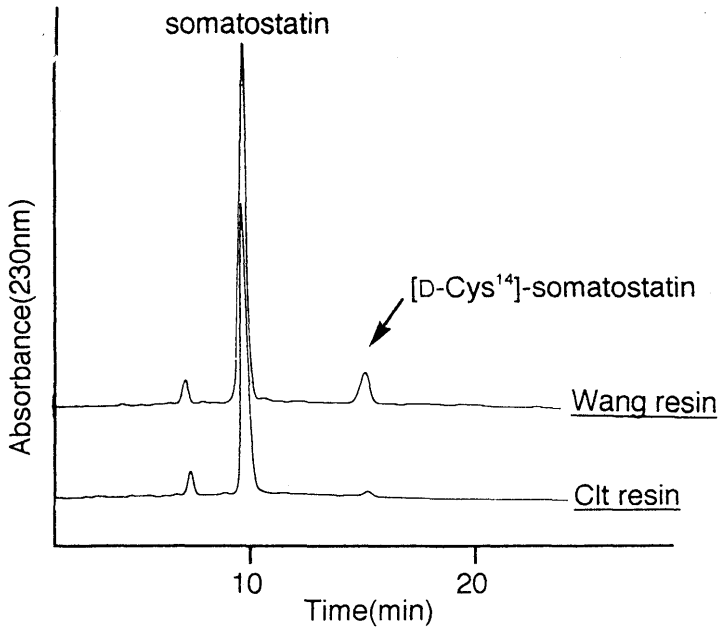


Fig. 3. HPLC Elution Patterns of Crude Somatostatin after Air-Oxidation

conditions: column, YMC AM302(4.6 X150mm); eluent, 27%MeCN/0.1%TFA; flow rate, 1.0ml/min; O.D. 230nm.

thioanisole/TFA[12] in the presence of m-cresol and ethanedithiol to cleave the peptide from the resin and, at the same time, to remove all the protecting groups except for two S-Acm groups. The S-protected product was purified by fast protein liquid chromatography(FPLC) and then treated with $AgBF_4$-anisole/TFA[13] to remove the remaining two S-Acm groups. To construct the intramolecular disulfide bridge, the reduced somatostatin was subjected to air-oxidation at pH 7.8 under high dilute condition. The progress of the reaction was monitored by RP-HPLC. The crude air-oxidized compound was purified by preparative FPLC to give a homogeneous peptide in 26% yield(calculated from the starting Fmoc-Cys(Acm)-Clt resin). For comparison, SPPS of somatostatin using the same procedure as above but starting from the conventional Wang-resin was conducted. As shown in Fig. 3, the crude product after air-oxidation contained 10% [D-Cys[14]]-somatostatin.

In conclusion, we found that Clt resin is suitable especially for the synthesis of C-terminal Cys-peptides in Fmoc-based SPPS. Using the Clt resin, the peptides containing C-terminal cysteine can be obtained with practically no racemization in excellent yield.

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