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# Efficient Solid-Phase Synthesis of Sulfated Tyrosine-Containing Peptides Using 2-Chlorotrityl Resin : Facile Synthesis of Gastrin/Cholecystokinin Peptides

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***Abstract:*** An efficient Fmoc-based solid-phase method for the synthesis of sulfated tyrosine-containing peptides is described. This synthetic approach involves two key features: (1) use of the 2-chlorotrityl resin as a solid support, and (2) a two-step cleavage/deprotection protocol. Various molecular forms of gastrin-II and cholecystokinin (CCK) were prepared by this approach without difficulty.


Synthetic tyrosine sulfate [Tyr($SO_3H$)]-containing peptides may serve as useful tools to gain insight into the sulfation in peptides and proteins. Many synthetic methods for Tyr($SO_3H$)-containing peptides have been reported including the solid-phase approach[1]; however, a general and convenient synthetic method for them has not yet been established. The major difficulty in the synthesis of Tyr($SO_3H$)-containing peptide lies in the intrinsic acid-lability of the Tyr($SO_3H$) residue. In our previous reports[1-g,h], we achieved the direct solid-phase synthesis of the relatively short peptides having one or more Tyr($SO_3H$) residue(s), in which Fmoc-Tyr($SO_3Na$)-OH was used as a building block for the introduction of the Tyr($SO_3H$) residue into the peptide-chain, and Fmoc-based chemistry[2] was employed throughout the synthesis. The acidic deprotection conditions that we found (90% aqueous TFA at 4 °C) proved to be effective to minimize the deterioration of the sulfate moiety of Tyr($SO_3H$)[1-g]. However, detachment of the peptide from the polymer support remained at about 40 % under these cleavage/deprotection conditions despite the use of an acid-labile PAL-resin[3]. In order to overcome this low recovery of peptides from the resin, and to eventually improve the overall yield of the desired sulfated peptides, we focused our attention on the 2-chlorotrityl resin (Clt-resin)[4] as the solid support. A Clt-resin is highly sensitive to acid, and detachment of the peptide from this resin can be achieved under extremely weak acidic conditions. Application of this resin to the solid-phase synthesis of the *non-sulfated form* of gastrin/CCK-related peptides, peptides having many acid-sensitive amino acid residues, has been reported[4-c]. In this communication, we report an efficient and convenient solid-phase method for the synthesis of Tyr($SO_3H$)-containing peptides and its application to the longer sulfated peptides. Our synthetic approach involves two key features in order to complete the synthesis in an efficient manner: (1) utilization of the Clt-resin, and (2) a two-step cleavage/deprotection protocol (Fig. 1).

For the synthesis of gastrin/CCK peptides, the C-terminus dipeptide, Fmoc-Asp-Phe-$NH_2$, was linked to a Clt-resin through the side-chain β-carboxyl group of Asp, then each Fmoc-amino acid including Fmoc-Tyr($SO_3Na$)-OH was introduced to the peptide-chain by the PyBOP[5]-mediated coupling protocol [Fmoc-amino acid (3 eq)–PyBOP reagent (3 eq)–NMM (9 eq), 20 °C, 90 min]. The fully assembled peptide-resin was subjected to the two-step cleavage/deprotection reaction; first with a mixture of AcOH-trifluoroethanol-$CH_2Cl_2$

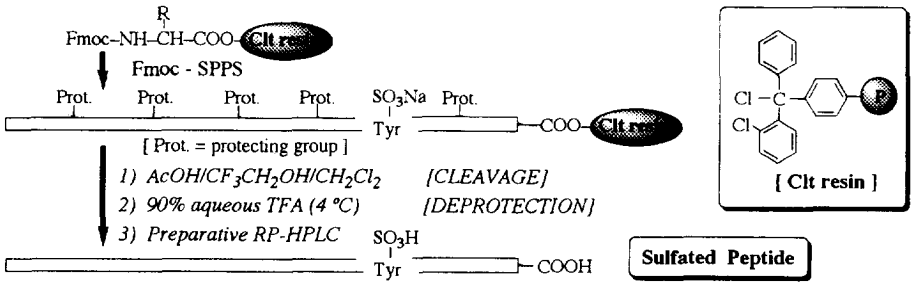


*Fig. 1. Synthetic Outline of Sulfated Tyrosine-Containing Peptide Using a Clt-Resin.*

(1:1:3 v/v, 25 °C, 45 min), followed with 90% aqueous TFA (4 °C, 5 h). The former weak acid treatment could nearly quantitatively detach the protected peptide from the Clt-resin (>95%) and the loss of the sulfate was negligible during this treatment. The latter acid treatment at *low temperature* minimized deterioration of the sulfate on Tyr (*ca.* 10% judged by RP-HPLC) despite the prolonged treatment. After purification with RP-HPLC, highly homogeneous human little gastrin-II (17 amino acid residues) was obtained in 38% yield and CCK-12 (12 amino acid residues) in 25% yield from the cleavage/deprotection step, respectively. The existence of the sulfate moiety in each peptide was confirmed by FT-IR spectra, and LSIMS[6]. The yield of CCK-12 obtained by this method was significantly improved compared to the previous synthesis using the PAL-resin[7].

Next, in order to extend this methodology for the longer and more complicated Tyr($SO_3H$)-containing peptides, big gastrin-II (G-34 (II), 34 amino acid residues) and CCK-33 (33 amino acid residues) were prepared. In order to carry out the synthesis with a minimum of protecting groups, Asn and Gln residues were introduced to the peptide-chain with the side-chain unprotected. For this purpose, Fmoc-Asn/Gln-OPfp was used as an acylation reagent in the presence of HOOBt[8]. As for the protecting group for His and Arg, we chose Fmoc-His(Boc)[9] and Fmoc-Arg(Pbf)[10], respectively. These protecting groups were the most sensitive to our deprotection conditions (90% aq.TFA, 4 °C) among the various protecting groups available for these amino acids. After completion of the peptide-chain assembly, the protected peptide-resin corresponding to G-34 (II) was subjected to a two-step cleavage/deprotection. In this case, complete deprotection of the protecting groups required an 8 h treatment with 90% aqueous TFA at 4 °C. Purification of the crude peptide on RP-HPLC afforded big gastrin-II in 18% yield from the cleavage/deprotection and the final product exhibited pseudo-molecular ions, $[M+H]^+$ and $[M-H]^-$, in the LSIMS consistent with the molecular weight calculated for the sulfated peptide[11].

As for CCK-33, complete deprotection was achieved with 90% aq.TFA in 7 h at 4 °C plus an additional 1 h treatment at 25 °C because of the difficulty in complete deprotection of the three Pbf groups on the Arg residues. Purification of the crude CCK-33 (Fig. 2-a) on HPLC afforded CCK-33 (Fig. 2-b) in 10% yield from the cleavage/deprotection step[12]. The observed molecular mass in the LSIMS coincided with the molecular weight calculated for the parent ion of the sulfated peptide (Fig. 2-c). Penke *et al.*[1-e] and, more recently, Han *et al.*[1-i] reported the solid-phase synthesis of CCK-related peptides, in which the global cleavage/deprotection was achieved with 50% TFA/$CH_2Cl_2$ in the presence of sulfur-containing scavengers at room temperature. We pointed out in our previous report[1-g] that the reaction temperature was a decisive determinant for the decomposition of Tyr($SO_3H$) during the acid treatment. Also, water involved in the deprotection system has enough scavenger effects without imparting damage to the Tyr($SO_3H$) residue, while sulfur-containing scavengers, such as thioanisole and ethanedithiol, accelerate decomposition of Tyr($SO_3H$) to Tyr. In these

respects, our two-step cleavage/deprotection strategy seems to be favorable for this kind of peptide containing an extremely acid-labile amino acid residue. The slightly low yield of CCK-33 may be due in some part to the three oxidation-sensitive Met residues in this sequence.

Thus, despite the highly acid-labile nature of the Tyr($SO_3H$) residue, relatively large sulfated peptides, big gastrin-II and CCK-33, were obtained without difficulty. It is worth noting that the difference in acid-sensitivity of the Tyr($SO_3H$) residue in gastrin-peptides and in CCK-peptides was observed during the deprotection reactions; Tyr($SO_3H$) in the gastrin-peptides was more sensitive to the acidic conditions compared to that in the CCK-peptides. It seems very likely that this difference may be partly due to the ionic interactions between the oppositely charged side chains of the Tyr($SO_3H$) and Arg residues in close proximity in the CCK-peptides as Moroder *et al.* had commented[1-a].

In conclusion, we have developed an efficient and convenient solid-phase method for the synthesis of sulfated peptides using the Clt-resin and a two-step cleavage/deprotection strategy. This method should be generally applicable to the preparation of sulfated peptides having 30~40 amino acid residues. To our knowledge, the synthesis of big gastrin-II has not been successfully achieved so far. Also, precise hormonal activities of big molecular forms of CCK-peptides, CCK-39 or CCK-58, have not been fully investigated due to their limited availability. The synthetic method developed here might be helpful for the physiological studies of this class of important peptide hormones.

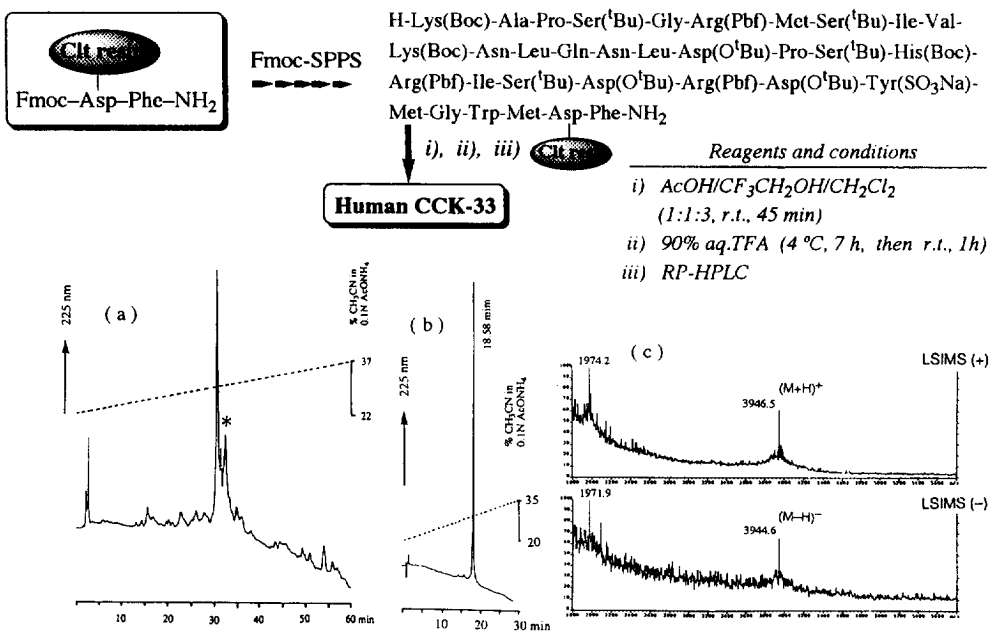


*Fig. 2. Synthesis of CCK-33. [(a) HPLC of the crude product after cleavage/deprotection..The asterisk shows the desulfated form; (b) HPLC of the purified peptide. HPLC was conducted on a Cosmosil 5C18-AR (3.9 x 150 mm) at a flow rate of 0.8 ml/min (a) and 1 ml/min (b); (c) LSIMS of the purified peptide].*

**Abbreviations**: Fmoc=fluoren-9-ylmethyloxycarbonyl, Boc=*tert*-butyloxycarbonyl, $^t$Bu=*tert*-butyl, Pbf=2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, PyBOP=benzotriazolyloxy-tris(pyrrolidino)phosphonium hexafluorophosphate, NMM=*N*-methylmorpholine, Pfp=pentafluorophenyl, HOOBt=3,4-dihydro-3-hydroxy-4-oxo-1,2,3-benzotriazine, TFA=trifluoroacetic acid.

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7. Cleavage and deprotection were concurrently achieved with 90% aq.TFA at 4 °C in the previous synthesis of CCK-12. Homogeneous CCK-12 was obtained in 7.4% yield based on the protected peptide-resin.
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11. *Human big gastrin-II*: [Pyr-Leu-Gly-Pro-Gln-Gly-Pro-Pro-His-Leu-Val-Ala-Asp-Pro-Ser-Lys-Lys-Gln-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr($SO_3H$)-Gly-Trp-Met-Asp-Phe-$NH_2$] Amino acid ratios of acid hydrolysate, 2 x Asp, 2.09; 1 x Ser, 1.05; 8 x Glu, 7.57; 5 x Pro, 4.97; 4 x Gly, 3.90; 2 x Ala, 2.00; 1 x Val, 0.91; 1 x Met, 0.72; 3 x Leu, 2.71; 1 x Tyr, 0.89; 1 x Phe, 1.00; 2 x Lys, 2.12; 1 x His, 0.94; 2 x Trp, ND. LSIMS, [M-H]$^-$ 3927.8 (calcd for $C_{176}H_{250}N_{43}O_{56}S_2$, average mass 3928.3).
12. *Human CCK-33* [Lys-Ala-Pro-Ser-Gly-Arg-Met-Ser-Ile-Val-Lys-Asn-Leu-Gln-Asn-Leu-Asp-Pro-Ser-His-Arg-Ile-Ser-Asp-Arg-Asp-Tyr(SO3H)-Met-Gly-Trp-Met-Asp-Phe-NH2]: Amino acid ratios of acid hydrolysate, 6 x Asp, 5.53; 4 x Ser, 3.18; 1 x Glu, 1.03; 2 x Pro, 2.12; 2 x Gly, 2.00; 1 x Ala, 0.89; 1 x Val, 0.66; 3 x Met, 2.53; 2 x Ile, 1.52; 2 x Leu , 1.80; 1 x Tyr, 0.93; 1 x Phe, 1.00; 2 x Lys, 1.91; 1 x His, 0.91; 3 x Arg, 2.72; 1 x Trp, ND. Recoveries of Val and Ile were slightly low because of the presence of the Ile-Val sequence. LSIMS, [M-H]$^-$ 3944.6 (calcd for $C_{167}H_{262}N_{51}O_{52}S_4$, average mass 3944.5).


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