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CYCLOHEXYL ETHER AS A NEW HYDROXY-PROTECTING GROUP FOR SERINE IN SOLID-PHASE PEPTIDE SYNTHESIS[1]

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Abstract A new hydroxy-protecting group for Ser, cyclohexyl, has been developed, and its application to the solid-phase peptide synthesis has been demonstrated successfully in combination with N^{α}-Boc protection and the Merrifield resin support.

In the solid-phase peptide synthesis (SPPS),[2] the hydroxy function of Ser is usually masked to prevent the unwanted *O*-acylation.[3] The most common protecting group for Ser in *tert*-butoxycarbonyl (Boc)-dependent SPPS is benzyl (Bzl), which can be readily deprotected by hydrogenation over Pd or acidolysis with anhydrous HF, 1 M trifluoromethanesulfonic acid (TFMSA)-thioanisole in trifluoroacetic acid (TFA),[4] etc. The Bzl group is, however, partially lost during the treatment with 50 % TFA in CH_2Cl_2 (3 % loss in 23 h treatment)[5]. The hydroxy function regenerated by losing the Bzl group at every N^{α}-deprotection step would be acylated at the subsequent coupling steps to give a variety of *O*-branched peptides. *O*-Acylserines may also undergo β-elimination with production of dehydroalanine peptides.[3] Particularly in SPPS of long peptides or proteins, accumulation of side products would be very serious, even though the hydroxy-protecting group is lost only in a small amount. The Bzl group is, therefore, not completely adequate for Boc-dependent SPPS of long peptides and proteins. More TFA-stable protecting groups have been thus required, and developed mainly by introducing electron-withdrawing substituents onto the benzyl moiety, such as chloro-,[6] bromo-,[5] and *N*,*N*-dimethylcarbamoyl-.[7] For instance, only 1.3 % of 4-bromobenzyl was reported to be lost on treatment with 50 % TFA in CH_2Cl_2 for 71 h.[5] Alternatively, *sec*-alkyl skeletons, such as cyclohexyl (Chx), have been known to be suitable as highly TFA-stable protecting groups. For example, an apparent rate constant of decomposition of Asp(*O*-Chx) in 50 % TFA in CH_2Cl_2 was less than a hundredth that of Asp(*O*-Bzl).[8] The Chx group on the hydroxy function of Tyr was also much more stable to TFA than the Bzl group.[9] The Chx protection of these amino acids can be smoothly removed with HF or 1 M TFMSA-thioanisole in TFA in a short period. These features have prompted us to apply the Chx group to the side-chain protection of Ser in peptide synthesis. This communication deals with the introduction of the Chx group to the hydroxy function of Ser, and the evaluation

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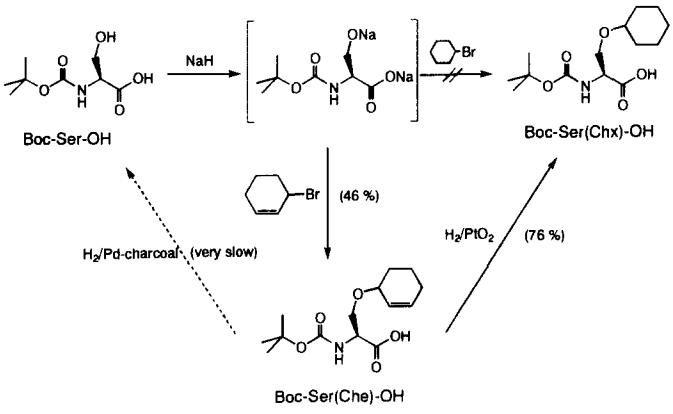
for the Boc-dependent SPPS.

In contrast to the Bzl protection,[10] the reaction of Boc-Ser-OH with NaH and cyclohexyl bromide failed to introduce the Chx group onto the hydroxy function. Thus we tried two-step synthesis using an allyl-type derivative, 3-bromocyclohexene, instead of cyclohexyl bromide, as shown in Scheme 1. Boc-Ser(cyclohexen-3-yl)-OH [Boc-Ser(Che)-OH] was obtained in a moderate yield. Hydrogenation of the resulting Boc-Ser(Che)-OH over Pd-charcoal did not give Boc-Ser(Chx)-OH; Boc-Ser(Che)-OH was recovered with a small amount of Boc-Ser-OH. This indicates that an undesirable reaction, hydrogenolytic cleavage of the Che ether, has occurred at an exceedingly slow rate. However, Boc-Ser(Che)-OH could be hydrogenated over PtO_2 to afford Boc-Ser(Chx)-OH in a satisfactory yield with a trace amount of Boc-Ser-OH, which could be removed readily by silica-gel column chromatography. Boc-Ser(Chx)-OH was a colorless oil, and could be converted to a stable crystalline form by means of cyclohexylamine.[11] Although this route requires an additional hydrogenation step, the overall yield of Boc-Ser(Chx)-OH from Boc-Ser-OH was comparable to that of Boc-Ser(Bzl)-OH. Fmoc-Ser(Chx)-OH could be obtained in a good yield from H-Ser(Chx)-OH•HCl, prepared from Boc-Ser(Chx)-OH and 4 N HCl in AcOEt, in the usual manner.



Scheme 1

Stability of the Chx group under the Boc-deprotecting condition was examined by treating Fmoc-Ser(Chx)-OH with 50 % TFA in CH_2Cl_2 at room temperature. HPLC analysis indicated that no appreciable amount of Chx was removed in 96 h, and the removal was only 3.9 % after 192 h. From the result, the apparent rate constant (k_{app}) for removal of the Chx group was determined as 0.46×10^{-8} sec^{-1}. Previously, the k_{app} for the cleavage of the Bzl ether under similar conditions was reported as 10.7×10^{-8} sec^{-1}.[12] These k_{app} values allowed calculation of amounts of Chx and Bzl removed in a single standard N^{α}-Boc deprotection (20 min) to be 0.00055 and 0.013 %, respectively. The Chx ether could be, therefore, concluded to be much more stable in TFA treatment than the Bzl ether. The substantial stability of Chx would be advantageous for improving the purity of the resulting peptides. Furthermore, this influence will be more pronounced in SPPS of larger peptides. For

exemplification, SPPS of large peptides was simulated by the Merrifield protocol.[12] The yields of three large peptides that maintain complete protection of Ser residues were calculated by using k_{app} values for Chx and Bzl, and are shown in Table 1. Calculated yields were higher with Chx than Bzl for hydroxy-protection of Ser, particularly for larger peptides. These simulations suggest that the Chx group would make possible efficient SPPS of large peptides. In addition, the Chx group was also stable to 20 % piperidine in DMF, an N^{α}-Fmoc-deprotecting reagent, at room temperature for up to 24 h. The Chx group was not susceptible also to catalytic hydrogenation over Pd, whereas the 4-bromobenzyl group was removed by a similar treatment. The Chx group could be quantitatively removed by treating with 1 M TFMSA-thioanisole in TFA at room temperature for 30 min. These results indicate that this newly developed protecting group is fit to use in Boc-chemistry, and can be used also in combination with N^{α}-Fmoc- and N^{α}-benzyloxycarbonyl (Z) protections.

Table 1 Calculated yields of peptides that maintain complete protection of Ser residues for SPPS of three peptides from the human immunoglobulin Eu[13]

peptide	variable region of light chain (108 amino acids)	complete light chain (214 amino acids)	complete heavy chain (440 amino acids)
number of Ser	16	32	53
cycles[a]	774	3,389	11,223
protection for Ser		calculated yield (%)[b]	
Chx	99.6	98.1	94.0
Bzl	90.5[c]	64.7[c]	23.7[c]

[a] Sum of the TFA exposures of the individual Ser residues, where the TFA exposure of the residue at position P is P-1 cycles.

[b] Mole % of the peptides having all Ser residues still protected upon complete assembly of the resin-bound peptide; based on deprotection by 50 % TFA in CH_2Cl_2 for 20 min/cycle.

[c] ref. 12.

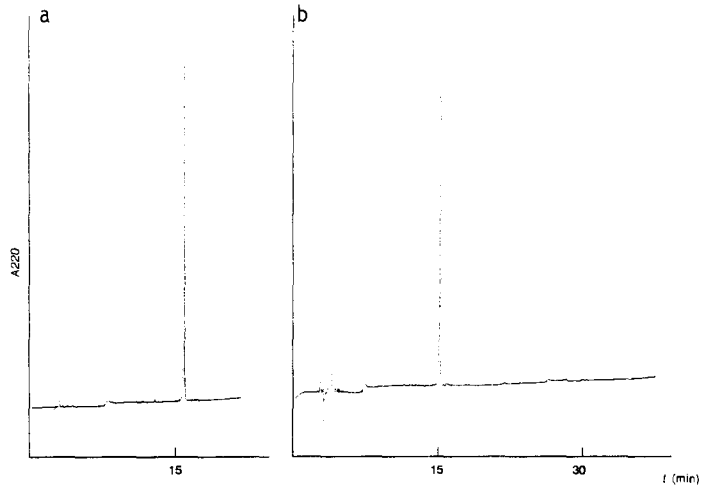


Fig. 1 Analytical HPLC profiles of (a) the authentic and (b) crude Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH. *column, YMC-Pack R-ODS (4.6 x 250 mm); solvents, a = 0.05 % aq. TFA, b = 0.05 % TFA in MeCN; gradient a/b 95/5 to 20/80 in 45 min; flow rate, 1.0 cm^3/min.*

To demonstrate applicability of the Chx group for practical Boc-SPPS, a hexapeptide, Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH (βAla; β-alanine) which is an analogue of an anti-metastatic peptide derived from the partial sequence of laminin,[14] was synthesized. The desired sequence was constructed by the successive Bop-HOBt couplings of Boc-Arg(Tos)-OH (Tos; *p*-tolylsulfonyl), Boc-Ser(Chx)-OH, Boc-Gly-OH, Boc-Ile-OH, and Boc-Tyr(Cl_2Bzl)-OH (Cl_2Bzl; 2,6-dichlorobenzyl) on the Merrifield resin bearing Boc-βAla (0.3 mmol/g). N^{α}-Boc groups were removed by treating with 50 % TFA in CH_2Cl_2 for 20 min. After removal of the terminal Boc group, the resulting amino function was acetylated with acetic anhydride. The protected peptide resin was treated with 1 M TFMSA-thioanisole in TFA containing *m*-cresol at room temperature for 60 min. The HPLC profile of the crude product showed a single major peak at a retention time identical with that of the authentic sample prepared by the solution method, as shown in Fig. 1. High purity of the crude product clearly demonstrates that no significant side reaction has occurred during the synthesis. This is indicative of the compatibility of the Chx group to a common Boc-SPPS protocol.

Consequently, the Chx group has proved to be quite useful as a hydroxy-protecting group for Ser in peptide synthesis. The Chx group could be easily introduced onto the hydroxy function of Ser in two steps from Boc-Ser-OH. The Chx ether was much more stable to TFA-treatment than the Bzl ether as expected, but could be removed with 1 M TFMSA-thioanisole in TFA in a short period. Boc-Ser(Chx)-OH was successfully employed for Boc-dependent SPPS of Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH without any serious side reactions. In addition, the Chx group was stable also under the conditions employed for the deprotection of Fmoc and Z. This suggests that the Chx group can be used also with N^{α}-Fmoc- and N^{α}-Z-protections. The Chx group would be thus useful for synthesis of various Ser-containing peptides.

References and Notes

1. *Abbreviations*: Ac, acetyl; Boc, *tert*-butoxycarbonyl; Bzl, benzyl; Che, cyclohexen-3-yl; Cl_2Bzl, 2,6-dichlorobenzyl; Chx, cyclohexyl; DMF, *N*,*N*-dimethylforamide; Fmoc, 9-fluorenylmethoxycarbonyl; SPPS, solid-phase peptide synthesis; TFMSA, trifluoromethanesulfonic acid; TFA, trifluoroacetic acid; Z, benzyloxycarbonyl; βAla, β-alanine; Tos, *p*-tolylsulfonyl.
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