Cyclohexyl ether as a new hydroxy-protecting group for serine and threonine in peptide synthesis †,[1]

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A new hydroxy-protecting group for Ser and Thr, cyclohexyl (Chx), has been developed, and its application to peptide synthesis is described. The Chx group is introduced to the hydroxy functions of Boc-Ser-OH and Boc-Thr-OH in two steps; Boc-Ser-OH and Boc-Thr-OH are treated with NaH and then allowed to react with 3-bromocyclohexene to afford *N*-Boc-*O*-(cyclohex-2-enyl)-Ser and *N*-Boc-*O*-(cyclohex-2-enyl)-Thr in satisfactory yields, which are hydrogenated in the presence of PtO_2 to give Boc-Ser(Chx)-OH and Boc-Thr(Chx)-OH in good yields. The *O*-Chx group is stable to various acidic and basic conditions including TFA and 20% piperidine in DMF. It is not removed with catalytic hydrogenation over Pd–charcoal. The Chx group is, however, removed quantitatively with 1 mol dm^{-3} trifluoromethanesulfonic acid–thioanisole in TFA over a short period. These results indicate that the Chx group is suitable for the hydroxy-protection of Ser and Thr in peptide synthesis based on Boc-chemistry and can be used also in combination with either N^{α}-Fmoc or N^{α}-Z-protection. The apparent rate constant for removal of the Chx group with 50% TFA in CH_2Cl_2 (k_{Chx}) is found to be less than a twentieth of that of the Bzl group (k_{Bzl}), confirming the substantial stability of the Chx group under common Boc-deprotection conditions. Simulations of solid-phase peptide syntheses using k_{Chx} and k_{Bzl} indicate that the Chx group would be adequate for solid-phase synthesis of large peptides and proteins. Solid-phase synthesis of a peptide and conventional solution synthesis of a protected peptide segment using Chx protection are also demonstrated.

Introduction

Protection of side-chain functional groups of trifunctional amino acids has been a major consideration in peptide chemistry. A multitude of protecting groups and refined systems for their removal have been developed, and currently the tactics of side-chain protection are an almost routine procedure in the solid-phase synthesis of simple peptides.[2] However, synthesis of large peptides or proteins by solid-phase and conventional solution methods requires more stringent or absolute selectivity in removal of α-amino-protecting groups and side-chain protecting groups. Preparation of combinatorial peptide libraries[3] also seems to require the strict selectivity of protective-group cleavage, since the resulting libraries are usually screened for various functions including biological activity without purification and intensive characterization. Protecting groups with unique stability/removability profiles have opened new synthetic strategies for complex peptides, such as multiple antigenic peptides having two distinct epitopes,[4] and regioselectively sulfonated Tyr-containing peptides.[5] Thus the development of new protecting groups remains a major challenge in peptide chemistry.

The hydroxy functions of Ser and Thr are usually masked to prevent the unwanted *O*-acylation.[6] The most common protecting group for Ser and Thr in solid-phase peptide synthesis (SPPS) based on the Boc-chemistry is benzyl (Bzl), which can be readily deprotected by hydrogenation over Pd or acidolysis with anhydrous HF, 1 mol dm^{-3} trifluoromethanesulfonic acid (TFMSA)–thioanisole in TFA,[7] *etc.* The Bzl group is, however, partially lost during the treatment with 50% TFA in CH_2Cl_2.§ 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, which may also undergo β-elimination with production of dehydroalanine peptides.[6] Particularly in SPPS of large peptides or proteins, accumulation of side products would severely complicate the isolation of the desired product, even though the hydroxy-protecting group is lost only in a small amount. The Bzl group is, therefore, not entirely adequate for SPPS of long peptides and proteins. More TFA-stable hydroxy-protecting groups have been thus required for efficient peptide synthesis, and developed mainly by introducing electron-withdrawing substituents on the phenyl ring of the benzyl moiety, such as bromo[8] and *N*,*N*-dimethylcarbamoyl.[9]¶

Alternatively, *sec*-alkyl skeletons, such as cyclohexyl (Chx), have been known to be suitable as highly TFA-stable protecting groups.[10–13] For example, an apparent rate constant in decomposition of Asp(OChx) in 50% TFA in CH_2Cl_2 was reported to be less than a hundredth of that of Asp(OBzl).[11] A Chx group on the hydroxy function of Tyr was also much more stable to TFA than a Bzl group.[12] The Chx protection of these amino acids can be smoothly removed with anhydrous HF or 1 mol dm^{-3} 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 and Thr in peptide synthesis. This paper deals with the synthesis of Boc-Ser(Chx)-OH and Boc-Thr(Chx)-OH (Fig. 1), stability and removability of the *O*-Chx group under various conditions, and its application to SPPS and conventional peptide synthesis in solution.

† Amino acids used in this study are of L-configuration. Abbreviations used in this report for amino acids, peptides, and their derivatives are those recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature: *Biochem. J.*, 1984, **219**, 345; *Eur. J. Biochem.*, 1984, **138**, 9; *Pure Appl. Chem.*, 1984, **56**, 595.

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§ 3% (Ser) and 5% (Thr) loss after 23 h treatment.[8]

¶ 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.[8]

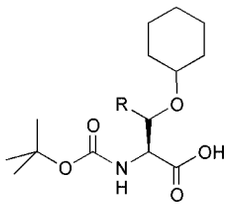
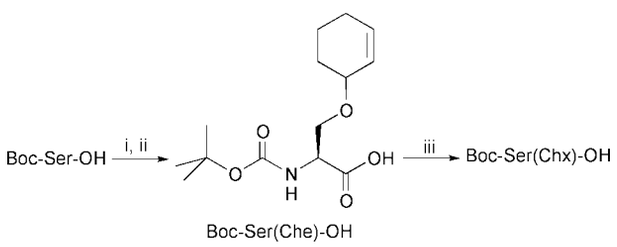


Fig. 1 Boc-Ser(Chx)-OH (R = H) and Boc-Thr(Chx)-OH (R = Me).

Results and discussion

Synthesis of Boc-Ser(Chx)-OH and Boc-Thr(Chx)-OH

Although Boc-Ser(Bzl)-OH can be readily prepared from Boc-Ser-OH and benzyl bromide by the Williamson synthesis,[14] a similar reaction using cyclohexyl bromide instead of benzyl bromide failed to give Boc-Ser(Chx)-OH. This failure would be a result of the insufficient reactivity of the *sec*-alkyl halide in nucleophilic substitution. Thus we tried the two-step synthesis of Boc-Ser(Chx)-OH, as shown in Scheme 1, involving the



Scheme 1 *Reagents*: (i) NaH; (ii) 3-bromocyclohexene; (iii) H_2, PtO_2.

introduction of the cyclohex-2-enyl (Che) moiety and its hydrogenation to Chx, since the Che ether can be deduced to be synthesized readily by the nucleophilic substitution reaction of 3-bromocyclohexene (Che-Br), which has a highly reactive allyl-halide-like structure, with the sodium alkoxide derived from Boc-Ser-OH. As expected, Boc-Ser(Che)-OH could be obtained in a satisfactory yield. Hydrogenation of Boc-Ser(Che)-OH over Pd-charcoal failed to give Boc-Ser(Chx)-OH.‖ Boc-Ser(Che)-OH could, however, be hydrogenated over PtO_2 to afford Boc-Ser(Chx)-OH in good yield. 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.[14]

Boc-Thr(Chx)-OH could be synthesized in a similar manner, and the overall yield of Boc-Thr(Chx)-OH from Boc-Thr-OH was higher than that of Boc-Thr(Bzl)-OH.[14]

Stability/removability profile of the *O*-Chx group

The acid stability of the *O*-Chx group was tested using Fmoc-Ser(Chx)-OH, which was synthesized from Boc-Ser(Chx)-OH in the usual manner. Fmoc-Ser(Chx)-OH was treated with TFA, 4 mol dm^{-3} HCl in AcOEt, 1 mol dm^{-3} trimethylsilyl bromide (TMSBr)–thioanisole in TFA,[15] and 1 mol dm^{-3} TFMSA–thioanisole in TFA, and then portions of the test solutions were subjected to HPLC to determine the amounts of survived Fmoc-Ser(Chx)-OH. No appreciable removal of the Chx group was observed in the treatment with TFA and 4 mol dm^{-3} HCl in AcOEt as included in Table 1. Furthermore, the Chx group was stable to 1 mol dm^{-3} TMSBr–thioanisole in TFA, by which the Bzl and Z groups were removed quantitatively. These results clearly indicate the substantial acid stability of the Chx group. The Chx group was, however, removed quantitatively with 1 mol dm^{-3} TFMSA–thioanisole in TFA at rt within 30 min.

‖ Boc-Ser(Che)-OH was recovered with a small amount of Boc-Ser-OH, which was regenerated possibly by undesirable hydrogenolytic cleavage of the Che ether bond.

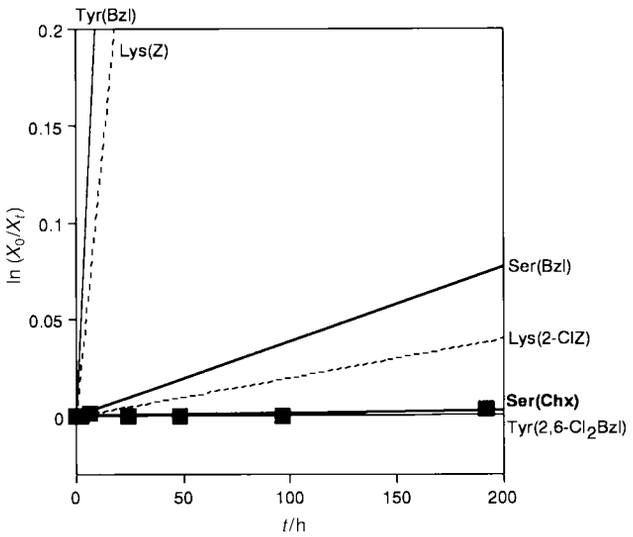


Fig. 2 Best-fit lines for apparent first-order-deprotection of Ser(Chx) in comparison with Ser(Bzl), Lys(Z), Lys(2-ClZ), Tyr(Bzl) and Tyr(Cl_2Bzl). 2-ClZ, 2-chlorobenzyloxycarbonyl; Cl_2Bzl, 2,6-dichlorobenzyl.

The stability of the Chx group under various basic conditions was examined using Boc-Ser(Chx)-OH by similar procedures to those for acid-stability tests. As shown in Table 1, the Chx group was not removed with 10% Et_3N in DMF, 10% aq. $NaHCO_3$, 2 mol dm^{-3} aq. NaOH, and 20% piperidine in DMF. These results indicate that the Chx group is satisfactorily stable under various basic conditions including those for saponification of esters and for β-elimination of fluoren-9-ylmethyl-based protecting groups.

Boc-Ser(Chx)-OH was exposed to catalytic hydrogenolytic conditions, by which the Bzl and 4-bromobenzyl groups were removed quantitatively. Removal of the Chx group was not detectable.

From these results, the Chx group appears suitable for the hydroxy-protection for Ser and Thr in peptide synthesis based on the Boc-chemistry. In addition, the *O*-Chx group can be used in combination with N^{α}-Fmoc and N^{α}-Z protections, if desired. Furthermore, the cyclohexyl ether has a unique stability/removability profile different from other major hydroxy-protecting groups, such as Bu^t, Bzl, and fluoren-9-ylmethyl, and thus they are removable individually without significant loss of the Chx group.

Kinetics of Chx loss in 50% TFA in CH_2Cl_2

To compare the acid stability of Chx and Bzl in detail, we examined the kinetics of Chx loss under common Boc-deprotecting conditions.

Fmoc-Ser(Chx)-OH was dissolved in 50% TFA in CH_2Cl_2, and the amount of survived Fmoc-Ser(Chx)-OH was determined with HPLC after 2, 6, 24, 48, 96, and 192 h. Only 3.9% loss was observed after 192 h, revealing the excellent stability of the Chx group. The apparent first-order rate constant for removal of the Chx group with 50% TFA in CH_2Cl_2 (k_{Chx}) was determined from the slope of the best-fit line through data points plotted on a grid of $\ln(X_0/X_t)$ [where X_0 is the initial concentration of Fmoc-Ser(Chx)-OH and X_t is the concentration of Fmoc-Ser(Chx)-OH at a given time t] *vs.* time (Fig. 2) based on the rate law, $k_{Chx} \cdot t = \ln(X_0/X_t)$. The k_{Chx} was determined as 0.46×10^{-8} s^{-1}. Previously, the apparent rate constant of Bzl loss under similar conditions was determined as 10.7×10^{-8} s^{-1}.[16] These values allowed calculation of the amounts of Chx and Bzl to be removed in a single standard N^{α}-Boc-deprotection (rt, 20 min) as 0.00055 and 0.013%, respectively. This indicates that the Chx group withstands Boc-deprotection conditions considerably better than Bzl does.

Table 1 Stability and removability of the Chx group

Conditions	% R-Ser(Chx)-OH survived[a]					
	0.5	1	2	4	8	24 h
TFA, rt		100		100	100	100
4 mol dm^{-3} HCl in AcOEt, rt		100		100	100	100
1 mol dm^{-3} TMSBr–thioanisole in TFA, ice-bath temperature	100		98			
1 mol dm^{-3} TFMSA–thioanisole in TFA, rt	0					
10% aq. $NaHCO_3$, rt						100
10% Et_3N in DMF, rt			100	100		100
20% piperidine in DMF, rt		100	100	100		100
2 mol dm^{-3} aq. NaOH, rt		100		100		100
H_2/Pd–C in MeOH–water			100		100	

[a] In acid treatments, R = Fmoc. In other, R = Boc.

Table 2 Calculated yields (%) of peptides that maintain complete protection of Ser residues for SPPS of three peptides from the human immunoglobulin Eu

	Variable region of light chain (108 amino acid residues)	Complete light chain (214 amino acid residues)	Complete heavy chain (440 amino acid residues)
Number of Ser residues ⟶	16	32	53
Cycles[a] ⟶	774	3389	11 223
Protection of 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 $cycle^{-1}$. [c] Ref. 16.

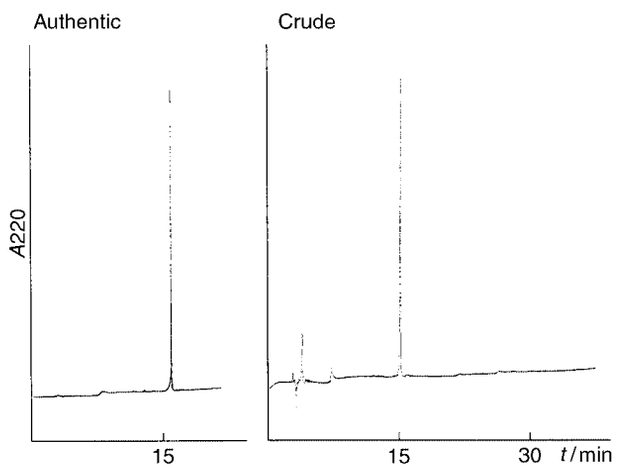


Fig. 3 Analytical HPLC profile of crude Ac-Tyr-Ile-Gly-Ser-Arg-β-Ala-OH (conditions are given in the Experimental section).

Simulation of SPPS of large peptides

The substantial stability of Chx would be advantageous for improving the purity of the resulting peptides, particulary in SPPS of large peptides. For exemplification, SPPS of large peptides was simulated by Merrifield's protocol,[16] where three large peptides from human immunoglobulin Eu[17] are used as models. The yields of three large peptides that lost no protection of Ser residues were calculated using k_{Chx} and k_{Bzl}, and the results are summarized in Table 2. Calculated yields were higher with Chx than with Bzl, particularly for larger peptides. From these simulations the *O*-Chx protection is expected to be more favorable for SPPS of large peptides than is *O*-Bzl protection.

Application of Boc-Ser(Chx)-OH to SPPS

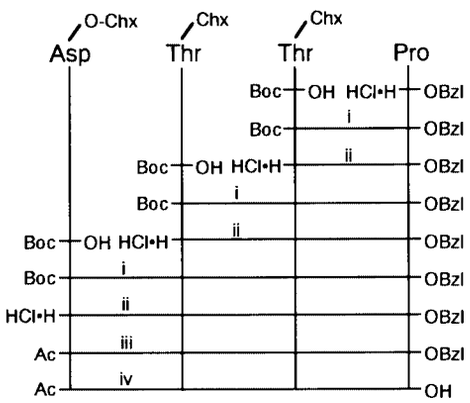
To demonstrate the applicability of the Chx group to practical Boc-SPPS, a simple hexapeptide, Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH (βAla: β-alanine), which is an analogue of laminin-related antimetastatic peptide YIGSR,[18] was synthesized. The desired sequence was constructed on the Merrifield resin[19] bearing Boc-βAla by the successive couplings of Boc-Arg(Tos)-OH[20] (Tos: *p*-tolylsulfonyl), Boc-Ser(Chx)-OH, Boc-Gly-OH, Boc-Ile-OH, and Boc-Tyr(Cl_2Bzl)-OH[16] (Cl_2Bzl: 2,6-dichlorobenzyl) with benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate[21] (BOP) in the presence of HOBT. N^{α}-Boc groups were removed by a standard protocol, 20 min treatment with 50% TFA in CH_2Cl_2. The protected peptide resin was treated with 1 mol dm^{-3} TFMSA–thioanisole in TFA containing *m*-cresol at rt for 60 min. There were no difficulties during the synthesis, and the HPLC profile of the crude product showed a single major peak at a retention time identical with that of an authentic sample prepared by the solution method,[22] as shown in Fig. 3. This demonstrates the conformity of the Chx group to a common Boc-SPPS protocol.

Application of Boc-Thr(Chx)-OH to the conventional peptide synthesis in solution

The segment-condensation strategy, where protected peptide segments are coupled to afford a larger protected peptide, have been successfully employed in conventional solution peptide synthesis.[23] A protected peptide segment having the free carboxy function is required in the segment-condensation strategy. To prepare such a protected peptide segment, the carboxy-terminal protecting group of a fully protected peptide intermediate should be removable without loss of protecting groups for the α-amino and side-chain functionalities. Since the

Chx group is stable under catalytic hydrogenolytic conditions as stated above, benzyl-based protecting groups would be selectively removable from the protected peptides having Chx side-chain protection.

To demonstrate this, Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OH, a key intermediate for the synthesis of analogs of an anti-human immunodeficiency virus type-1 peptide,[24] was synthesized using Boc-Thr(Chx)-OH. As summarized in Scheme 2, the fully protected peptide, Boc-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl, was synthesized in a stepwise manner. Although the amino acid sequence, Asp(OChx)-Thr(Chx)-Thr(Chx), implies steric hindrance (since the β-branching Thr residues are consecutive and the Chx group is somewhat bulkier than the Bzl group) satisfactory yields could be acheived at every coupling step without excess of the carboxylic component and coupling reagent. After removal of the amino-terminal Boc group, the resulting amine was acetylated with acetic anhydride to give Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl. The carboxy-terminal Bzl group was then removed by hydrogenation over Pd–charcoal to give Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OH in an almost quantitative yield. This is demonstrative of the usefulness of a Boc/*O*-Chx/Bzl protection scheme in the synthesis of protected peptide segments.



Scheme 2 *Reagents*: (i) BOP, Et_3N; (ii) 4 mol dm^{-3} HCl in AcOEt; (iii) acetic anhydride, Et_3N; (iv) H_2, Pd–charcoal.

Conclusions

The Chx group could be easily introduced onto the hydroxy function of Ser and Thr in two steps from Boc-Ser-OH and Boc-Thr-OH *via O*-Che derivatives. The Chx group was stable under various acidic and basic conditions, including deprotection conditions for Boc, Z, and Fmoc, and thus adequate for use in combination with any of these N^{α}-protecting groups. It could be removed with 1 mol dm^{-3} TFMSA–thioanisole in TFA in a short period. The stability of the *O*-Chx group under common Boc-deprotecting conditions was >20-fold higher than that of the *O*-Bzl group. Simulation of SPPS of immunoglobulin-related large peptides revealed that the Chx group would be more favorable for use in SPPS of large peptides than would Bzl. Two peptides containing Ser or Thr were successfully synthesized by solid-phase and conventional solution methods using Chx as side-chain protection. The results obtained here clearly demonstrate the usefulness of the Chx group in peptide synthesis. The Chx group is the first *sec*-alkyl-based protecting group for the alcoholic hydroxy function. This new hydroxy-protecting group would be useful not only for peptide synthesis, but also for the synthesis of various compounds having multiple functional groups, where selective removal of specific protecting groups is required, because of its unique stability/removability profile, different from that of any other hydroxy-protecting group available now.

Experimental

R_f-Values in TLC (Kieselgel 60 F_{254}, Merck) refer to $CHCl_3$–MeOH–AcOH (90:8:2), and spots were detected with UV light and/or staining with 0.1% ninhydrin in acetone. Optical rotations were measured with a JASCO DIP-370 polarimeter, and $[\alpha]_D$-values are in units of 10^{-1} deg cm^2 g^{-1}. HPLC was conducted with a WATERS 600 system, and A and B in the mobile-phase system refer to 0.05% aq. TFA and 0.05% TFA in MeCN, respectively. ^1H NMR spectra were recorded on a JEOL Lambda 400 MHz spectrometer for samples in $CDCl_3$ solution. *J*-Values are given in Hz. $SiMe_4$ was used as the internal standard.

DMF was distilled from ninhydrin before use. Boc-Thr-OH and Boc-Asp(*O*-Chx)-OH were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan). Che-Br was purchased from Aldrich Chemical Company (USA), and used without purification. Fluoren-9-ylmethyl *N*-succinimidyl carbonate (Fmoc-OSu) was purchased from Novabiochem (USA). Merrifield resin (0.33 mequiv. Cl g^{-1}, 1% divinylbenzene-cross-linked polystyrene, 100–200 mesh) was purchased from Peptide Institute, Inc. (Osaka, Japan). HOBT used was its hydrate. Other reagents were of reagent grade and used without purification.

Boc-Ser(Che)-OH·CHA **

To a solution of Boc-Ser-OH (6.2 g, 30 mmol) in DMF (150 cm^3) was added NaH (60–72% oil dispersion; 2.4 g, ≈60 mmol) portionwise under N_2 stream at 0 °C. After the evolution of H_2 gas had ceased, 3-bromocyclohexene (5.8 g, 36 mmol) was added to the above solution. The reaction mixture was stirred at room temperature (rt) overnight. After removal of the solvent under reduced pressure below 40 °C, the residue was dissolved in water (80 cm^3) under cooling with ice, and the solution was washed with diethyl ether (20 cm^3 × 3). The aqueous phase was acidified to pH 4 with 10% aq. citric acid, and extracted with $CHCl_3$ (200 cm^3). The extract was washed with water, dried over Na_2SO_4, and then evaporated to give a yellowish oil (3.9 g, 46%), which was chromatographically pure and could be used in the next hydrogenation reaction without purification; δ_H 5.88–5.85 (1 H, m), 5.74–5.69 (1 H, m), 5.42 (1 H, d, *J* 6.6), 4.44–4.43 (1 H, m), 4.01–3.95 (1 H, m), 3.92–3.90 (1 H, m), 3.77–3.69 (1 H, m), 2.07–1.91 (2 H, m), 1.85–1.61 (2 H, m), 1.56–1.52 (2 H, m), 1.46 (9 H, s).

To an ice-cooled solution of a small part of the product (0.80 g) in diethyl ether (50 cm^3) was added CHA (0.99 g) to afford the *title salt* as a precipitate, which was collected by filtration and washed with diethyl ether successively; yield 0.98 g; mp 170–175 °C (decomp.); $[\alpha]_D^{25}$ +37.2 (*c* 1.0 in DMF) (Found: C, 61.2; H, 9.40; N, 7.34. $C_{20}H_{36}N_2O_5·0.4H_2O$ requires C, 61.3; H, 9.47; N, 7.15%).

Boc-Ser(Chx)-OH·CHA

Boc-Ser(Che)-OH [prepared from Boc-Ser(Che)-OH·CHA (1.0 g, 2.6 mmol) in the usual manner] was dissolved in a mixture of MeOH (50 cm^3) and water (10 cm^3), and hydrogenated in the presence of PtO_2 (0.1 g) for 90 min. After removal of the solvent, the residue in $CHCl_3$ (5 cm^3) was applied to a silica gel column (Wako Gel C-200, 10 g), which was equilibrated and eluted with $CHCl_3$. After removal of the solvent from the effluent (54–378 cm^3), the oily residue was dissolved with diethyl ether. CHA (0.26 g, 2.6 mmol) was added to the solution to afford the *title salt* as a precipitate, which was collected by filtration and washed with diethyl ether; yield 0.67 g (90%); mp 195–198 °C (decomp.); $[\alpha]_D^{25}$ +27.2 (*c* 1.0 in MeOH) (Found: C, 60.8; H, 10.0; N, 7.24. $C_{20}H_{38}N_2O_5·0.5H_2O$ requires C, 60.7; H, 9.94; N, 7.08%); δ_H (free acid form) 5.40 (1 H, d, *J* 7.8), 4.43–

** CHA = cyclohexylamine.

4.40 (1 H, m), 3.96–3.93 (1 H, m), 3.66 (1 H, dd, *J* 4.5 and 9.4), 3.32–3.27 (1 H, m), 1.87–1.83 (2 H, m), 1.73–1.68 (2 H, m), 1.52–1.49 (2 H, m), 1.46 (9 H, s), 1.31–1.20 (4 H, m).

Boc-Thr(Che)-OH·CHA

The *title compound* was synthesized from Boc-Thr-OH (2.0 g, 8.7 mmol), NaH (60–72% oil dispersion; 0.6 g, *ca.* 17 mmol), and 3-bromocyclohexene (1.7 g, 8.7 mmol) in essentially the same manner as that for Boc-Ser(Che)-OH·CHA, yield 1.7 g (62%); mp 167–169 °C (decomp.); $[a]_D^{25}$ +3.5 (*c* 1.0 in DMF) (Found: C, 54.6; H, 6.49; N, 10.9. $C_{21}H_{38}N_2O_5·1.6H_2O$ requires C, 54.4; H, 6.88; N, 11.3%); δ_H (free acid form) 5.89–5.86 (1 H, m), 5.73–5.65 (1 H, m), 5.38 (1 H, d, *J* 6.8), 4.35–4.32 (1 H, m), 4.22–4.20 (1 H, m), 4.03–3.97 (1 H, m), 2.06–1.55 (6 H, m), 1.46 (9 H, s), 1.22 (3 H, d, *J* 6.4).

Boc-Thr(Chx)-OH

The *title compound* was obtained from Boc-Thr(Che)-OH·CHA (1.7 g, 4.3 mmol) in essentially the same manner as that for Boc-Ser(Chx)-OH as a colorless amorphous powder (1.3 g, 79%); $[a]_D^{25}$ +2.5 (*c* 1.0 in DMF) (Found: C, 56.1; H, 6.36; N, 11.0. $C_{15}H_{27}NO_5·2H_2O$ requires C, 55.9; H, 6.68; N, 11.4%); δ_H 5.33 (1 H, d, *J* 7.8), 4.29 (1 H, dd, *J* 3.1 and 7.9), 4.17–4.14 (1 H, m), 3.42–3.38 (1 H, m), 1.81–1.78 (2 H, m), 1.68–1.65 (2 H, m), 1.49–1.46 (2 H, m), 1.43 (9 H, s), 1.30–1.20 (4 H, m), 1.15 (3 H, d, *J* 6.4).

Fmoc-Ser(Chx)-OH

To a solution of Fmoc-OSu (203 mg, 0.60 mmol) in 1,2-dimethoxyethane (50 cm^3) was added a solution of H-Ser(Chx)-OH·HCl [prepared from Boc-Ser(Chx)-OH·CHA (259 mg, 0.67 mmol) and 4 mol dm^{-3} HCl in AcOEt (2.0 cm^3) in the usual manner] in 5% aq. $NaHCO_3$ (20 cm^3) portionwise. The resulting mixture was stirred at rt overnight. After removal of the insoluble substance, the solution was concentrated to a third of the initial volume with a rotary evaporator. The condensed solution was acidified with 6 mol dm^{-3} aq. HCl under cooling with ice, and then extracted with AcOEt. The extract was washed with water, dried over Na_2SO_4, and evaporated to dryness. *n*-Hexane was added to the oily residue to afford crystals of the *title acid*, which were collected by filtration and recrystallized from $CHCl_3$–*n*-hexane, yield 220 mg (89.5%), mp 155–157 °C; $[a]_D^{25}$ –27.0 (*c* 0.5, DMF) (Found: C, 70.55; H, 6.81; N, 3.56. $C_{24}H_{27}NO_5$ requires C, 70.4; H, 6.65; N, 3.42%).

Stability and removability of the Chx group

TFA and 4 mol dm^{-3} HCl in AcOEt. Fmoc-Ser(Chx)-OH (20 μmol) and Fmoc-Ala-OH (20 μmol) were dissolved in the test reagent (1.0 cm^3). Parts of the solution were collected at times given in Table 1, and subjected to HPLC. The percentage survival of the Chx group was determined as follows: % survival = $100 \times (S_t \times A_0/A_t)/S_0$, where S_0 is the peak area of Fmoc-Ser(Chx)-OH on analysis of the initial solution; S_t is the peak area of Fmoc-Ser(Chx)-OH on analysis of the test solution stored for *t* h; A_0 is the peak area of Fmoc-Ala-OH on analysis of the initial solution; and A_t is the peak area of Fmoc-Ala-OH on analysis of the test solution stored for *t* h.

1 mol dm^{-3} TMSBr–thioanisole in TFA. Fmoc-Ser(Chx)-OH (50 μmol) and Fmoc-Ala-OH (50 μmol) were dissolved in TFA (5.0 cm^3). A part of the solution (0.6 cm^3) was stored as a standard. To the remaining part of the solution cooled with an ice-bath were added thioanisole (290 mm^3) and TMSBr (310 mm^3). The mixture was stirred at ice-bath temperature. After 30 and 120 min, parts of the solution were subjected to HPLC. % Survival of the Chx group was determined as described above.

1 mol dm^{-3} TFMSA–thioanisole in TFA. Fmoc-Ser(Chx)-OH (50 μmol) and Fmoc-Ala-OH (50 μmol) were treated with the title reagent (5.0 cm^3) in a similar manner to the above except for the reaction time (30 min) and temperature (rt).

10% Et_3N in DMF, 10% aq. $NaHCO_3$, 2 mol dm^{-3} aq. NaOH, and 20% piperidine in DMF. Boc-Ser(Chx)-OH (20 μmol) and Boc-Ala-OH (20 μmol) were dissolved in the test reagent (1 cm^3), and % survival was determined in a similar manner to the above.

H_2/Pd. Boc-Ser(Chx)-OH (20 μmol) and Boc-Ala-OH (20 μmol) were dissolved in MeOH–water (4:1 v/v; 1 cm^3). To the solution was added a small amount of Pd–charcoal. The suspension was stirred under H_2 atomosphere at rt. Parts of the solution were collected at 2 and 8 h, passed through cotton filters individually, and subjected to HPLC. % Survival was determined in a similar manner to the above.

Determination of k_{Chx}

Fmoc-Ser(Chx)-OH (50 μmol) and Fmoc-Ala-OH (50 μmol) were dissolved in 50% TFA in CH_2Cl_2 (5.0 cm^3), and the solution was stored at 25 °C in a glass tube with a tight cap and Teflon sealing. At 0, 2, 6, 24, 48, 96, and 192 h after mixing, parts of the solution were subjected to HPLC [column, WATERS μBondasphere $5C_{18}$ (3.9 × 150 mm); solvent, A:B 80:20 to 20:80 in 60 min (1.0 cm^3 min^{-1})], and peak areas of Fmoc-Ser(Chx)-OH (t_R 39.23 min) and Fmoc-Ala-OH (t_R 25.45 min) were determined. The X_0/X_t-value was corrected using the internal standard; $X_0/X_t = (S_0·A_t)/(S_t·A_0)$. The best-fit line through data points plotted on a grid of ln (X_0/X_t) *vs.* *t* is shown in Fig. 2. The rate constant, k_{Chx}, was determined as 0.46×10^{-8} s^{-1} from its slope.

Simulation of SPPS of large peptides related to human immunoglobulin Eu

The yields of three large peptides that maintain complete protection of Ser residues were calculated as described in a previous report.[16]

Boc-βAla-O-Merrifield resin

Boc-βAla-OH (0.31 g, 1.65 mmol) was converted to its Cs salt in the usual manner.[25] Boc-Ala-OCs was added to a suspension of Merrifield resin (0.33 mequiv. g^{-1}; 5 g) in DMF (50 cm^3). The mixture was stirred gently at rt for 4 h. The resin was collected by filtration, washed successively with DMF, CH_2Cl_2, MeOH, and *n*-hexane, and dried over $CaCl_2$ *in vacuo*, yield 5.24 g [0.30 mmol βAla g^{-1} (determined by the weight increase)].

Ac-Tyr-Ile-Gly-Ser-Arg-βAla-OH

Starting from Boc-βAla-O-Merrifield resin (500 mg, 0.15 mmol), SPPS was carried out by the following protocol: 1) CH_2Cl_2 (5 cm^3), 1 min × 5; 2) 50% TFA in CH_2Cl_2 (5 cm^3), 2 min × 1, 20 min × 1; 3) CH_2Cl_2 (5 cm^3), 1 min × 5, then DMF (5 cm^3), 1 min × 5; 4) Boc-amino acid (0.45 mmol), BOP (0.45 mmol), HOBT (0.45 mmol), $EtNPr^i_2$ (0.45 mmol), DMF (5 cm^3), 30 min; 5) DMF (5 cm^3), 1 min × 5; repeat from step 1). After removal of the terminal Boc group, acetic anhydride (0.45 mmol) and $EtNPr^i_2$ (0.45 mmol) were added to the resin in DMF (5 cm^3), and the mixture was agitated for 60 min. The resulting peptide resin was washed as follows: DMF (5 cm^3), 1 min × 5; CH_2Cl_2 (5 cm^3), 1 min × 5; MeOH, 1 min × 5; and then *n*-hexane (5 cm^3), 1 min × 5, and dried over $CaCl_2$ *in vacuo*, yield 587 mg.

To an ice-cooled suspension of the peptide resin obtained in TFA (3.8 cm^3) containing thioanisole (0.59 cm^3, 5.0 mmol) and *m*-cresol (0.17 cm^3) were added TFMSA (0.44 cm^3, 5.0 mmol). The mixture was gently stirred at ice-bath temperature for 30 min, and then at rt for 60 min. Diethyl ether (50 cm^3) and water (20 cm^3) were added to the reaction mixture. The water

layer was washed with diethyl ether (50 cm^3 × 5), neutralized with $NaHCO_3$, and applied to a Sephadex G-10 column (2.8 × 48 cm), which was equilibrated and eluted with 3% aq. AcOH. Individual fractions (11 cm^3) were collected, and the desired fractions (#10–15) were combined and lyophilized to give a white fluffy powder (64 mg, 60% from the starting resin); t_R = 15.38 min [90.0%, column YMC-R-ODS (4.6 × 250 mm), A : B 95 : 5 to 20 : 80 in 45 min (1.0 cm^3 min^{-1}), the major peak was confirmed to co-elute with an authentic sample[22] prepared by the conventional solution method].

H-Thr(Chx)-Pro-OBzl·HCl

To an ice-cooled solution of H-Pro-OBzl·HCl (3.4 g, 14 mmol) and Boc-Thr(Chx)-OH (3.7 g, 14 mmol) in DMF (200 cm^3) containing Et_3N (2.0 cm^3, 14 mmol) were added BOP (6.2 g, 14 mmol) and Et_3N (4.0 cm^3, 28 mmol). The mixture was stirred at rt overnight. After removal of the solvent, the residue was extracted with AcOEt (200 cm^3). The extract was washed successively with 5% aq. $NaHCO_3$, 10% aq. citric acid, and water, dried over Na_2SO_4, and evaporated to dryness. The oily residue was dissolved in 4 mol dm^{-3} HCl in AcOEt (14 cm^3). The solution was stirred at ice-bath temperature for 1 h and then at rt for 1 h, and then evaporated. Diethyl ether was added to the residue to afford crystals of the *title salt*, which were collected by filtration; yield 4.0 g (70%); mp 174–176 °C; $[\alpha]_D^{25}$ −57.7 (*c* 1.0, DMF) (Found: C, 51.6; H, 8.38; N, 5.40. $C_{22}H_{33}ClN_2O_4$·4.9H_2O requires C, 51.5; H, 8.38; N, 5.40%).

H-Thr(Chx)-Thr(Chx)-Pro-OBzl·HCl

To an ice-cooled solution of H-Thr(Chx)-Pro-OBzl·HCl (2.9 g, 7.1 mmol) and Boc-Thr(Chx)-OH (1.8 g, 7.1 mmol) in DMF (200 cm^3) containing Et_3N (1.0 cm^3, 7.1 mmol) were added BOP (3.1 g, 7.1 mmol) and Et_3N (2.0 cm^3, 14 mmol). The mixture was stirred at rt overnight. After removal of the solvent, the residue was extracted with AcOEt (200 cm^3). The extract was washed successively with 5% aq. $NaHCO_3$, 10% aq. citric acid, and water, dried over Na_2SO_4, and evaporated to dryness. The oily residue was dissolved in 4 mol dm^{-3} HCl in AcOEt (8.9 cm^3). The solution was stirred at ice-bath temperature for 1 h and then at rt for 1 h, and then evaporated. Diethyl ether was added to the residue to afford crystals of the *title salt*, which were collected by filtration; yield 3.9 g (90%); mp 115–119 °C; $[\alpha]_D^{25}$ −63.2 (*c* 1, DMF) (Found: C, 56.8; H, 8.55; N, 6.08. $C_{32}H_{50}ClN_3O_6$·4H_2O requires C, 56.5; H, 8.59; N, 6.18%).

Boc-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl

To an ice-cooled solution of H-Thr(Chx)-Thr(Chx)-Pro-OBzl·HCl (2.5 g, 4.1 mmol) and Boc-Asp(OChx)-OH (1.3 g, 4.1 mmol) in DMF (200 cm^3) containing Et_3N (0.60 cm^3, 4.1 mmol) were added BOP (1.8 g, 4.1 mmol) and Et_3N (1.2 cm^3, 8.2 mmol). The mixture was stirred at rt overnight. After removal of the solvent, the residue was extracted with AcOEt (200 cm^3). The extract was washed successively with 5% aq. $NaHCO_3$, 10% aq. citric acid, and water, dried over Na_2SO_4, and evaporated to give the *title compound* as an amorphous powder (3.2 g, 90%); $[\alpha]_D^{25}$ −42.1 (*c* 1.0, DMF) (Found: C, 60.3; H, 8.50; N, 6.06. $C_{47}H_{72}N_4O_{11}$·3.5H_2O requires C, 60.6; H, 8.54; N, 6.01%).

Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl

To an ice-cooled solution of H-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl·HCl [prepared from Boc-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl (2.0 g, 2.5 mmol) and 4 mol dm^{-3} HCl in AcOEt (4.6 cm^3) in the usual manner] in DMF (100 cm^3) containing Et_3N (0.40 cm^3, 2.5 mmol) was added acetic anhydride (0.3 cm^3, 2.7 mmol). The mixture was stirred at rt for 2 h. After removal of the solvent, the residue was extracted with AcOEt (200 cm^3). The extract was washed successively with 10% aq. citric acid and water, dried over Na_2SO_4, and evaporated to dryness. Diethyl ether was added to the residue to afford crystals of the *title product*, which were collected by filtration; yield 1.8 g (87%); mp 133–137 °C; $[\alpha]_D^{25}$ −19.9 (*c* 1.0, DMF) (Found: C, 59.7; H, 8.52; N, 6.48. $C_{43}H_{66}N_4O_{10}$·3.8H_2O requires C, 59.5; H, 8.55; N, 6.46%).

Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OH

Ac-Asp(OChx)-Thr(Chx)-Thr(Chx)-Pro-OBzl (1.2 g, 1.5 mmol) was dissolved in MeOH–water (10 : 1 v/v; 110 cm^3), and hydrogenated in the presence of Pd–charcoal (5%; 1.0 g) at rt for 4 h. After removal of the catalyst and solvent, the residue was extracted with AcOEt (100 cm^3), and the extract was dried over Na_2SO_4, and evaporated to dryness. Diethyl ether was added to the residue to afford crystals of the *title acid*, which were collected by filtration; yield 1.0 g (93%); mp 161–165 °C; $[\alpha]_D^{25}$ −19.8 (*c* 1.0, DMF) (Found: C, 48.7; H, 9.02; N, 6.70. $C_{34}H_{60}N_4O_{11}$·7.6H_2O requires C, 48.7; H, 9.05; N, 6.69%).

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