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Structure–activity relationship of marinostatin, a serine protease inhibitor isolated from a marine organism

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A 12-residue MST isolated from a marine organism is a potent serine protease inhibitor that has a double cyclic structure composed of two ester linkages formed between the β-hydroxyl and β-carboxyl groups, Thr[3]-Asp[9] and Ser[8]-Asp[11]. MST was synthesized by a regioselective esterification procedure employing two sets of orthogonally removable side-chain protecting groups for the Asp and Ser/Thr residues. In the MST molecule, there were no significant changes observed in yield by changing the order of esterification. SAR study of MST revealed that the minimum required structure for expressing the inhibitory activity is the sequence (1–9) in a monocyclic structure where Pro[7] located in the ring plays a crucial role in keeping the structural rigidity. By applying the structural motif of MST, we rationally designed protease inhibitory specificities that differ from those of the natural product. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: ester linkage; MST; NMR analysis; peptide synthesis; SAR study; serine protease inhibitor; solution structure

Introduction

MST isolated from extracellular products of the marine organism, *Pseudoaltermonus sagamiensis*, is a serine protease inhibitor consisting of 12 amino acids with two internal ester linkages that are formed between the β-hydroxyl and β-carboxyl groups, Thr[3]-Asp[9] and Ser[8]-Asp[11] (Figure 1) [1–3]. MST strongly inhibits serine proteases such as subtilisin, chymotrypsin, and elastase at an enzyme–inhibitor ratio of 1 : 1, but not trypsin [2]. To confirm the reported primary structure of MST including ester linkages, it was synthesized by a regioselective esterification procedure employing two sets of orthogonally removable protecting groups at the side-chains of Asp and Ser/Thr. Spectral and analytical data of the synthetic MST were in good agreement with those in the literature [4]. The inhibitory potency of the synthetic MST against subtilisin (*K*i, 0.59 ± 0.05 nM) was found to be comparable with a reported value for native MST (*K*i, 1.5 nM) [3]. Serine protease inhibitor loops generally show similar canonical conformations with a hydrophobic core arising from the disulfide linkages across the families, which is responsible for the direct binding to the catalytic sites of proteases [5]. In the case of MST, however, it is considered that its ester linkages may play a crucial role in expressing the canonical inhibitory activity and that its inhibitory potential is attributable to the hydrogen bond linking between the backbone NH proton of Arg[5] and the carbonyl oxygen atom of the ester linkage with Thr[3]-Asp[9], which protects the scissile bond of Met[4]-Arg[5] [6].

The relatively small and unique structure of MST prompted us to explore the characteristics including such a strong inhibitory potency. In the present study, we examined the effect of the order

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Abbreviations used: *2-PySH, 2-mercaptopyridine; 4-MeBzl, 4-methylbenzyl; 6-Cl-HOBt, 6-chloro-1-hydroxybenzotriazole; All, allyl; AMC, 7-amino-4-methylcoumarin; BrZ, 2-bromobenzyloxycarbonyl; CARL, subutilisin Carlsberg; cHx, cyclohexyl; Cl_2Bzl, 2,4-dichlorobenzyl; Dap, 2,3-diaminopropionic acid; DQF-COSY, double quantum filtered-correlated spectroscopy; EDC·HCl, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; HCTU, 1-[bis(dimethylamino)methylene]-5-chloro-1H-benzotriazolium 3-oxide hexafluorophosphate; HFIP, hexafluoroisopropanol; HOHAHA, homo-nuclear hartmann-hahn experiment; HOOBt, 3,4-dihydro-3-hydroxy-4-oxo-benzotriazine; MNBA, 2-methyl-6-nitrobenzoic anhydride; MST, marinostatin; NMP, 1-methyl-2-pyrrolidinone; OMTKY3, turkey ovomucoid third domain; Pen, 3-pentyl; TASF, tris(dimethylamino)sulfonium difluoro-trimethylsilicate; TBS, t-butyldimethylsilyl; Tos, tosyl; Trt(2-Cl), 2-chlorotrityl.*

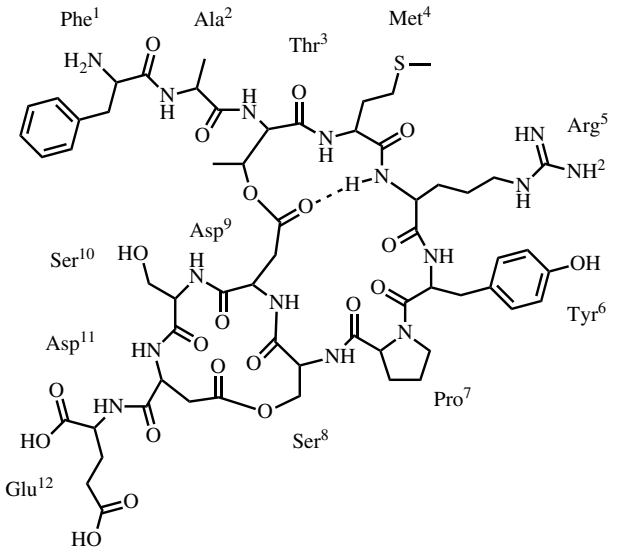

Figure 1. Structure of MST (**1**).

of esterification on the efficiency to form the intramolecular ester linkage by changing its combination of the side-chain protecting groups for Asp and Ser/Thr. Next, we synthesized a series of MST analogs and measured their inhibitory activities to elucidate the structure–activity relationship (SAR) by employing the same synthetic strategy as that for MST. Furthermore, we rationally designed protease inhibitory specificities that are different from those of the natural product by applying the structural motif of MST.

Materials and Methods

Boc, Fmoc, and Z-amino acids were obtained from the Peptide Institute, Inc. (Osaka, Japan) or Watanabe Chemical Industries (Hiroshima, Japan). All other chemicals and solvent of special grade were obtained from Nacalai Tesque (Kyoto, Japan) and were used without further purification. Syntheses were carried out on a 433A peptide synthesizer (Applied Biosystems, Foster City, CA, USA). Amino acid analysis was carried out on a Hitachi L-8800 (Ibaraki, Japan) after hydrolysis of the peptide with 6 N HCl at 110 °C for 22 h. HPLC was performed on an HP G1600A (Palo Alto, VA, USA) and a Shimadzu liquid chromatograph Model LC-8A or LC-20AT (Kyoto, Japan). Molecular weights were measured with an ESI-MS (HP1100 LC/MSD) and a MALDI-TOF MS (Voyager-DE STR, Applied Biosystems, Framingham, MA, USA).

Synthesis of MST (1)

Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(tBu)-Asp(OAll)-Ser(Bzl)-Asp(OtBu)-OH

The protected MST(1–11) was synthesized with an ABI 433A peptide synthesizer using Fmoc strategy on an Asp(O^{t}Bu)-Trt(2-Cl) resin (0.32 g, 0.25 mmol) [7]. The following side-chain-protected Fmoc amino acids were employed: Arg(Tos), Asp(OAll), Asp(O^{t}Bu), Ser(^{t}Bu), Ser(Bzl), Thr(TBS), and Tyr(Pen) [8]. Z-Phe was used for *N*-terminal incorporation. Deprotection of N^{α}-Fmoc groups was performed using 20% morpholine (3 min ×5) to reduce the aspartimide formation [9]. The peptide chain was elongated using FastMoc protocols of coupling with Fmoc amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/8 equiv.) in NMP (single coupling, 20 min). The protected peptide resin (0.86 g, 0.25 mmol) was treated with HFIP/$CHCl_3$ (1/4, v/v, 20 ml) for 1 h. The resin was filtered and the filtrate was concentrated *in vacuo* to give an oily residue, which was precipitated with ether; yield 0.51 g (quant.); ESI-MS: 1002.6 ($[M+2H]^{2+}$), theoretical value: 1002.5.

Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(tBu)-Asp(OAll)-Ser(Bzl)-Asp(OtBu)-Glu(OcHx)-OBzl

To a solution of Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(*t*Bu)-Asp(OAll)-Ser(Bzl)-Asp(O*t*Bu)-OH (0.50 g, 0.25 mmol), Glu(OcHx)-OBzl·HCl (93 mg, 0.26 mmol), and HOOBt (43 mg, 0.26 mmol) in DMF (5 ml) at 0 °C was added EDC (46 µl, 0.26 mmol). The reaction mixture was stirred for 1.5 h at room temperature and poured into an iced water to give a residue that was extracted with $CHCl_3$. The organic layer was washed successively with 5% $NaHCO_3$, water, 5% citric acid, water, and brine, and dried over anhydrous $MgSO_4$. Precipitation from $CHCl_3$ and ether yielded 0.51 g (90%). ESI-MS: 1153.6 ($[M+2H]^{2+}$), theoretical value: 1153.1.

Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(tBu)-Asp-Ser(Bzl)-Asp(OtBu)-Glu(OcHx)-OBzl

To a solution of Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(*t*Bu)-Asp(OAll)-Ser(Bzl)-Asp(O*t*Bu)-Glu(OcHx)-OBzl (0.50 g, 0.22 mmol) in DMF (5 ml) under an argon atmosphere were added $Pd(PPh_3)_4$ (50 mg, 43 µmol) and morpholine (0.19 ml, 2.2 mmol) at room temperature. The reaction mixture was stirred for 1 h, quenched with water at 0 °C, and then extracted with $CHCl_3$. The organic layer was washed successively with 5% citric acid, water and brine, and dried over anhydrous $MgSO_4$. The filtrate was concentrated *in vacuo*. Purification of the resulting residue by flash column chromatography [silica gel 30 g, $CHCl_3$/methanol 100/0–10/1 (v/v)] gave 0.34 g (68%). ESI-MS: 1133.6 ($[M+2H]^{2+}$), theoretical value: 1133.1.

Z-Phe-Ala-Thr-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(tBu)-Asp-Ser(Bzl)-Asp(OtBu)-Glu(OcHx)-OBzl

To a solution of Z-Phe-Ala-Thr(TBS)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(*t*Bu)-Asp-Ser(Bzl)-Asp(O*t*Bu)-Glu(OcHx)-OBzl (0.33 g, 0.15 mmol) in DMF (3 ml) was added TASF (0.20 g, 0.73 mmol) at room temperature. After being stirred for 50 min, the product was precipitated with water at 0 °C, and collected by filtration. The product was purified by reprecipitation from $CHCl_3$/methanol (3/1, v/v) and ether, and dried *in vacuo*: yield 0.29 g (93%); ESI-MS: 1076.2 ($[M+2H]^{2+}$), theoretical value: 1076.0.

Z-Phe-Ala-Thr-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(tBu)-Asp-Ser(Bzl)-Asp(OtBu)-Glu(OcHx)-OBzl (with the ester linkage of Thr^3-Asp^9)

To a stirred solution of MNBA (29 mg, 84 µmol) and DMAP (4.3 mg, 35 µmol) in anhydrous DCM (13 ml) were added a solution of Z-Phe-Ala-Thr-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser(*t*Bu)-Asp-Ser(Bzl)-Asp(O*t*Bu)-Glu(OcHx)-OBzl (0.15 g, 70 µmol) in anhydrous DCM (5.6 ml) and a solution of TEA (21 µl, 0.15 mmol) in anhydrous DCM (5.6 ml) simultaneously using mechanical driven syringes for 3.5 h. After being stirred for 20 h at room temperature, the reaction mixture was quenched with H_2O at 0 °C and then extracted with chloroform. The organic layer was washed successively with 5% $NaHCO_3$, water, 5% citric acid, water, and brine, and then dried

over anhydrous $MgSO_4$. The filtrate was concentrated *in vacuo*. The product was precipitated with ether, and the precipitates were collected by filtration and dried *in vacuo*: yield 0.12 g (81%); ESI-MS: 1067.1 ($[M+2H]^{2+}$), theoretical value: 1067.0.

Z-Phe-Ala-Thr-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser-Asp-Ser(Bzl)-Asp-Glu(OcHx)-OBzl (with the ester linkages of Thr^3-Asp^9 and Ser^8-Asp^{11})

Z-Phe-Ala-Thr(*t*Bu)-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser-Asp(O*t*Bu)-Ser(Bzl)-Asp-Glu(OcHx)-OBzl with the ester linkage of Thr^3-Asp^9 (0.29 g, 0.14 mmol) was dissolved in TFA (100 ml) at 0 °C, and then allowed to react for 1 h at room temperature. After removal of excess TFA *in vacuo*, the product was precipitated with diethylether, and collected by filtration, and dried to yield 0.27 g (97%); ESI-MS: 1011.4 ($[M+2H]^{2+}$), theoretical value: 1010.9. A solution of this peptide (50 mg, 25 µmol) and TEA (3.4 µl, 25 µmol) in anhydrous DCM/DMF (5/1, v/v, 2.4 ml) was added to a solution of MNBA (10 mg, 30 µmol), DMAP (1.5 mg, 12 µmol), and TEA (4.1 µl, 25 µmol) in anhydrous DCM/DMF (5/1, v/v, 3.6 ml) with a mechanical driven syringe for 3.5 h. After being stirred for 19 h at room temperature, the reaction mixture was quenched with water at 0 °C, and then extracted with chloroform. The organic layer was washed successively with 5% $NaHCO_3$, water, 5% citric acid, water, and brine, and then dried over anhydrous $MgSO_4$. The filtrate was concentrated *in vacuo*. The product was precipitated with ether and the precipitates were collected by filtration, and dried to yield 40 mg (81%). ESI-MS: 1002.5 ($[M+2H]^{2+}$), theoretical value: 1001.9.

MST (1)

Z-Phe-Ala-Thr-Met-Arg(Tos)-Tyr(Pen)-Pro-Ser-Asp-Ser(Bzl)-Asp-Glu(OcHx)-OBzl with the ester linkages of Thr^3-Asp^9 and Ser^8-Asp^{11} (70 mg, 35 µmol) was treated with HF (2 ml) in the presence of *p*-cresol (0.50 ml, 4.8 mmol) and 2-PySH (20 mg, 0.18 mmol) at −2 to −5 °C for 1 h to give a crude product, which was purified by RP-HPLC using a YMC-Pak ODS column (30 × 250 mm) at a flow rate of 20 ml/min. The RP-HPLC run was eluted with a linear gradient of MeCN in 0.1% TFA (10–30%, 80 min) to obtain 21 mg (44%). MALDI-TOF MS: 1383.38 ($[M+H]^+$), theoretical value: 1383.46 ($[M+H]^+$); amino acid analysis: Asp 1.98 (2), Thr 0.98 (1), Ser 1.99 (2), Glu 1.00 (1), Ala 1.04 (1), Met 0.94 (1), Tyr 1.00 (1), Phe 0.99 (1), Arg 0.97 (1), Pro 1.00 (1); ^{1}H-NMR [H_2O/D_2O (v/v, 9/1)]: Phe^1 δ 4.24 (Hα), 3.24, 3.09 (Hβ), 7.26 (2, 6H), 7.35 (3, 5H), 7.32 (4H). Ala^2 8.64 (NH), 4.47 (Hα), 1.35 (Hβ). Thr^3 8.72 (NH), 4.58 (Hα), 5.52 (Hβ), 1.35 (Hγ). Met^4 8.95 (NH), 4.66 (Hα), 2.32, 1.81 (Hβ), 2.58, 2.44 (Hγ). Arg^5 7.72 (NH), 4.10 (Hα), 1.79, 1.70 (Hβ) 1.70, 1.53 (Hγ), 3.26, 3.18 (Hδ), 7.36 (NH), 6.94, 6.48 (NH_2). Tyr^6 8.52 (NH), 4.52 (Hα), 2.96, 2.86 (Hβ), 7.11 (2, 6H), 6.83 (3, 5H). Pro^7 3.49 (Hα), 1.76, 1.63 (Hβ), 1.65, 1.56 (Hγ), 3.62, 3.34 (Hδ). Ser^8 7.19 (NH), 4.66 (Hα), 4.92, 4.25 (Hβ). Asp^9 9.29 (NH), 4.47 (Hα), 2.97, 2.97 (Hβ). Ser^{10} 8.59 (NH), 4.55 (Hα), 3.84, 3.79 (Hβ). Asp^{11} 7.83 (NH), 4.78 (Hα), 2.72, 2.63 (Hβ). Glu^{12} 8.36 (NH), 4.23 (Hα), 2.14, 1.93 (Hβ), 2.41, 2.41 (Hγ).

Synthesis of MST Analogs

[Ser^3]-MST(1–12) (2)

Z-Phe-Ala-Ser(*t*Bu)-Met-Arg(Tos)-Tyr(Pen)-Ser(TBS)-Asp(O*t*Bu)-Ser(Bzl)-Asp-Glu(OcHx)-OBzl was detached from the peptide resin which was assembled onto a Fmoc-Asp[OTrt(2-Cl) resin]-Glu(OcHx)-OBzl (0.39 g, 0.25 mmol) as described above for MST (**1**) to yield 0.41 g (70%). The ester formation of Ser^8-Asp^{11}, followed by that of Ser^3-Asp^9, was performed as reported previously [4] to obtain 39 mg (60%). ESI-MS: 1368.5 ($[M+H]^+$), theoretical value: 1368.6; amino acid analysis: Asp 1.98 (2), Ser 2.62 (3), Glu 1.00 (1), Ala 1.02 (1), Met 0.89 (1), Tyr 0.96 (1), Phe 0.97 (1), Arg 0.97 (1), Pro 1.00 (1).

[Ser^8-Asp^{11}]-MST(1–12) (3)

This peptide was prepared as described above for [Ser^3]-MST(1–12) (**2**) to yield 39 mg (60%). ESI-MS: 1400.5 ($[M+H]^+$), theoretical value: 1400.6; amino acid analysis: Asp 2.02 (2), Thr 0.98 (1), Ser 1.82 (2), Glu 1.02 (1), Ala 1.01 (1), Met 0.94 (1), Tyr 1.00 (1), Phe 1.01 (1), Arg 1.00 (1), Pro 1.03 (1).

Linear MST(1–12) (4)

The protected peptide was synthesized with an ABI 433A peptide synthesizer on a Boc-Glu(OcHx)-PAM resin (0.59 g, 0.5 mmol) using *in situ* neutralization protocols of coupling with Boc-amino acid/HCTU/6-Cl-HOBt/DIEA (4/4/4/6 eq) in NMP (single coupling, acetylation after each coupling step). The following side-chain-protected Boc-amino acids were employed: Asp(OcHx), Arg(Tos), Ser(Bzl), Thr(Bzl), and Tyr(BrZ). The peptide resin (0.96 g, 0.25 mmol) was treated with HF (12 ml) in the presence of *p*-cresol (3.0 ml, 28 mmol) and 2-PySH (0.14 g, 1.3 mmol) at −2 to −5 °C for 1 h to give a crude product, which was purified by RP-HPLC as described above to obtain 0.21 g (60%). ESI-MS: 1418.2 ($[M+H]^+$), theoretical value: 1418.5; amino acid analysis: Asp 1.99 (2), Thr 0.97 (1), Ser 1.79 (2), Glu 1.00 (1), Ala 0.99 (1), Met 0.98 (1), Tyr 0.99 (1), Phe 0.99 (1), Arg 0.99 (1), Pro 1.04 (1).

[Thr^3-Asp^9]-MST(1–12) (5)

This peptide was prepared as described above for MST (**1**) to yield 36 mg (45%). ESI-MS: 1400.5 ($[M+H]^+$), theoretical value: 1400.6; amino acid analysis: Asp 2.00 (2), Thr 0.96 (1), Ser 1.80 (2), Glu 1.01 (1), Ala 1.00 (1), Met 0.94 (1), Tyr 0.98 (1), Phe 1.00 (1), Arg 1.00 (1), Pro 1.02 (1).

[Thr^3-Asp^9]-MST(1–9) (6)

Protected peptide was synthesized with an ABI 433A peptide synthesizer using Fmoc chemistry on an Fmoc-Asp(Wang resin)-OBzl (0.40 g, 0.25 mmol). The following side-chain-protected Fmoc amino acids were employed: Arg(Tos), Ser(Bzl), Thr(*t*Bu), and Tyr(Cl_2Bzl). Z-Phe was used for *N*-terminal incorporation. The chain assembly was performed by employing the same conditions as those for MST (**1**) except for the Fmoc deprotection which was carried out using 20% piperidine/NMP (2.5 min ×4). This peptide was prepared as described above for [Ser^3]-MST(1–12) (**2**) to yield 26 mg (52%). ESI-MS: 1069.4 ($[M+H]^+$), theoretical value: 1069.5; amino acid analysis: Asp 1.00 (2), Thr 0.97 (1), Ser 0.93 (1), Ala 1.01 (1), Met 0.90 (1), Tyr 0.99 (1), Phe 0.99 (1), Arg 1.00 (1), Pro 1.00 (1).

[Thr^3-Asp^9]-MST(3–9) (7)

This peptide was prepared as described above for [Thr^3-Asp^9]-MST(1–9) (**6**) to yield 26 mg (52%). ESI-MS: 851.4 ($[M+H]^+$), theoretical value: 851.4; amino acid analysis: Asp 1.00 (1), Thr 0.95 (1), Ser 0.88 (1), Met 0.92 (1), Tyr 0.97 (1), Arg 0.99 (1), Pro 1.00 (1).

[Cys^3-Cys^9, Cys^8-Cys^{11}]-MST(1–12) (8)

This peptide was prepared as reported previously [3]. ESI-MS: 1408.4 ([M+H]$^+$), theoretical value: 1408.5; amino acid analysis: Ser 0.92 (1), Glu 1.00 (1), Ala 1.01 (1), 1/2(Cys)$_2$ 2.70 (4), Met 0.94 (1), Tyr 0.98 (1), Phe 0.98 (1), Arg 0.98 (1), Pro 0.99 (1).

[Dap^3-Asp^9, Dap^8-Asp^{11}]-MST(1–12) (9)

Protected peptide was synthesized with an ABI 433A peptide synthesizer using Fmoc chemistry on an Fmoc-Asp[OTrt(2-Cl) resin]-Glu(cHx)-OBzl (0.56 g, 0.25 mmol). The following side-chain-protected Fmoc amino acids were employed: Dap(Boc), Dap(Alloc), Arg(Tos), Asp(O^{t}Bu), Ser(Bzl), and Tyr(Cl$_2$Bzl). Z-Phe was used for *N*-terminal incorporation. The chain assembly was performed by employing the same conditions as those for MST (**1**) to obtain 0.80 g. After removal of the Alloc group on Dap^8 by treatment with Pd(PPh$_3$)$_4$/Me$_2$NH·BH$_3$ (0.24 g, 4.1 mmol) in DCM, the protected peptide was detached from the Trt(2-Cl) resin by treatment with CHCl$_3$/HFIP (20 ml, v/v = 4/1) to yield 0.32 g (68%). To a solution of the protected peptide (0.55 g, 0.24 mmol) in DMF (83 ml) was added EDC·HCl (93 mg, 0.49 mmol) and HOBt (66 mg, 0.49 mmol). After being stirred at room temperature for 19 h, the reaction mixture was concentrated *in vacuo* to give the residue, which was precipitated from CHCl$_3$/methanol and ether to yield 0.51 g (93%). After treating this peptide (0.26 g, 0.12 mmol) with TFA (5 ml) for 1 h, the second amide linkage with Dap^3-Asp^9 was formed by adding EDC (25 µl, 0.14 mmol) and HOBt (19 mg, 0.14 mmol) in DMF (37 ml). The reaction mixture was stirred at room temperature for 22 h, and then concentrated *in vacuo* to yield the residue, which was precipitated from CHCl$_3$/methanol and ether to yield 0.24 g (97%). The HF treatment of the peptide (0.15 g, 72 µmol) was followed by purification on RP-HPLC as described above to give 48 mg (48%). ESI-MS: 1366.5 ([M+H]$^+$), theoretical value: 1366.6; amino acid analysis: Asp 1.98 (2), Ser 0.87 (1), Glu 1.00 (1), Ala 1.00 (1), Met 0.89 (1), Tyr 0.95 (1), Phe 0.97 (1), Arg 0.97 (1), Pro 0.96 (1).

[Ala^7]-MST(1–12) (10)

This peptide was prepared as described above for [Ser^3]-MST(1–12) (**2**) to yield 21 mg (27%). ESI-MS: 1356.5 ([M+H]$^+$), theoretical value: 1356.6; amino acid analysis: Asp 2.05 (2), Thr 0.99 (1), Ser 1.90 (2), Glu 1.04 (1), Ala 2.05 (2), Met 0.97 (1), Tyr 0.96 (1), Phe 1.01 (1), Arg 1.00 (1).

[Arg^4, Met^5]-MST(1–12) (11)

This peptide was prepared as described above for [Ser^3]-MST(1–12) (**2**) to yield 17 mg (33%). ESI-MS: 1382.5 ([M+H]$^+$), theoretical value: 1382.6; amino acid analysis: Asp 1.97 (2), Thr 0.95 (1), Ser 1.78 (2), Glu 1.00 (1), Ala 0.99 (1), Met 0.89 (1), Tyr 0.95 (1), Phe 0.98 (1), Arg 0.98 (1), Pro 1.00 (1).

Protease Inhibitory Activities

The inhibitory activity of synthetic peptides against subtilisin or trypsin was measured according to a literature procedure [3]. The substrate (Suc-Ala-Ala-Pro-Phe-MCA for subtilisin, Bz-Arg-MCA for trypsin) obtained from the Peptide Institute, Inc. (Osaka, Japan) was dissolved in DMSO at a concentration of 25 µM. CARL (Calbiochem, Germany, 110 nM) or bovine trypsin (Sigma-Aldrich, St. Louis, MO, USA, 110 nM) was incubated with an appropriate amount of peptides in 2.0 ml of 25 mM phosphate buffer (pH 7.0) containing 1.0 mM $CaCl_2$ for 1 min at 30 °C. The reaction was started by the addition of 0.1 ml of the corresponding substrate solution. The release of AMC was monitored by measuring fluorescence intensity at 440 nm with excitation at 350 nm. The residual enzyme activity was plotted against the concentration of the peptides to calculate the *K*i value [7].

NMR Spectroscopy

NMR samples were prepared by dissolving the synthetic peptides in 200 µl of 100% D_2O or 90% H_2O/10% D_2O solution to give final concentrations of approximately 4 mM (6 mg/ml). All NMR spectra were acquired at 10 °C on a Bruker DMX750 spectrometer equipped with pulse-field gradients. Complete sequence-specific ^{1}H resonance assignments were obtained according to the standard strategy of the spin system identification followed by sequential assignments [8] using 2D DQF-COSY spectrum, 2D HOHAHA spectrum with a mixing time of 70 ms, and 2D NOESY spectrum with a mixing time of 150 ms. ^{13}C resonances were assigned using 2D ^{1}H-^{13}C HSQC spectrum together with the results of ^{1}H resonance assignments. ^{3}J coupling constants for nonoverlapping signals were measured using 1D ^{1}H spectra with high S/N and digital resolution, where possible, and from separations between antiphase absorptive and dispersive peak extrema observed in the DQF-COSY spectra. All NMR spectra were processed using XWINNMR (Bruker Instruments, Rheinstetten, Germany) and the NMRPipe software [9], and were analyzed using the PIPP software [10]. ^{1}H and ^{13}C chemical shifts were referenced indirectly to TSP [sodium 3-(trimethylsilyl)propionate].

Structure Calculations

NOE-derived interproton distance restraints were classified into three ranges: 1.8–2.7 Å, 1.8–3.5 Å, 1.8–5.0 Å, corresponding to strong, medium, and weak NOEs. An additional 1.5 Å was added to the upper bound for NOEs involving methyl protons and methylene protons that were not assigned stereospecifically [11]. Torsion angle restraints on ϕ and ψ were derived from $^3J_{HNH\alpha}$ coupling constants and short-range NOEs. The side-chain χ_1 and χ_2 angle restraints were derived from short-range NOEs combined with $^3J_{H\alpha H\beta}$ and $^3J_{H\beta H\gamma}$ coupling constants, respectively. Structures of MST analogs were calculated using the hybrid distance geometry-dynamic simulated annealing method as contained in X-PLOR 3.1 [12]. For structure calculations of MST (**1**), 167 interproton distance restraints (comprising 73 *intra*- and 94 *inter*-residue restraints) obtained from 2D NOESY spectra were employed along with two distance restraints for one hydrogen bond between the NH proton of Arg^5 and the carbonyl oxygen of the ester linkage with Thr^3-Asp^9. In addition, 19 dihedral angle restraints (10 ϕ, 2 ψ, 6 χ_1 and 1 χ_2) were included in the structure calculation. A peptide bond between Tyr^6 and Pro^7 was set to a *cis* configuration, i.e. $\omega \approx 0°$, based on observations of an extremely strong sequential H^α–H^β NOE between these two residues and a large difference between Pro^{7}C$^\beta$ and C$^\gamma$ chemical shifts. For structure calculations of MST[P7A] (**10**), 137 interproton distance restraints (65 *intra*- and 72 *inter*-residue restraints) and 24 dihedral angle restraints (10 ϕ 6 ψ, 7 χ_1 and 1 χ_2) were used. For both cases, a final set of 20 lowest energy structures were selected from 100 calculations. None of them had NOE or dihedral angle violations of >0.5 Å or 5°, respectively.

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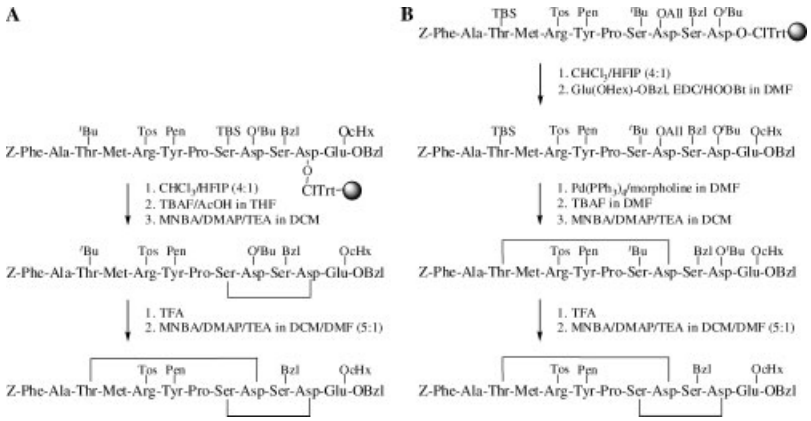

Figure 2. Regioselective formation of intramolecular ester linkages for MST. Route A, the previous work; route B, this work. Analogs **2**, **3**, **9**, **10,** and **11**, and analogs **5** and **8** were synthesized according to route A and B, respectively. The substitution of Ser for Thr[3] was done for analog **2**. In the case of **8** and **9**, Cys[3,8]-Cys[9,11] and Dap[3,8]-Asp[9,11], respectively, were substituted for Thr[3]/Ser[8]-Asp[9,11].

Table 1. Effect of the order of esterification reaction mediated by the MNBA/DMAP method on the ratio of products

	Ratio of products (monomer : dimer[a] : Asi-peptide)	
	First esterification	Second esterification
Route A	94 : 3 : 3[b]	92 : 6 : 2[c]
Route B	93 : 5 : 2[c]	90 : 2 : 8[b]

[a] Dimer refers to cyclic and linear dimerized peptides.
[b] Asi[11]-peptide.
[c] Asi[9]-peptide.

Results and Discussion

Effect of Changing the Order of Esterification on the Synthesis of MST

MST was synthesized by a regioselective esterification procedure employing two sets of orthogonally removable side-chain protecting groups for the Asp and Ser/Thr residues as previously reported [4]. This strategy can allow esterification to take place sequentially. In this synthesis, MST was constructed by forming the first ester linkage between Ser[8] and Asp[11] after removal of TBS and Trt(2-Cl) groups from the respective side-chain functions, followed by the second one between Thr[3] and Asp[9], after removal of their ^{t}Bu groups (Route A in Figure 2). In the present study, to examine whether the order of esterification can affect the efficiency to form the intramolecular ester linkage, we also synthesized the alternative starting linear-peptide, in which the side-chain functional groups of Ser[8] and Asp[11] were protected with ^{t}Bu groups, and those of Thr[3] and Asp[9] with TBS and allyl (All) groups, respectively (Route B in Figure 2). This combination of the protecting groups for Asp and Ser/Thr allowed for changing the order of esterification. The other side-chain protecting groups and the *N*/*C*-terminal ones must be compatible with both Boc and Fmoc chemistry and must be readily removed by the final HF treatment. Considering this, the 3-pentyl (Pen) group was chosen to protect the phenolic hydroxyl group of Tyr[6], as it is a base-resistant protecting group and cleavable by HF without the formation of any significant amounts of the alkyltyrosine rearrangement product [13]. The linear-peptide was assembled in solution from the *N*-terminal segment (1–11) and the *C*-terminal Glu(OHex)-OBzl. The former was elongated on an Fmoc-Asp(O^{t}Bu)-Trt(2-Cl) resin using Fmoc chemistry and detached from the resin by treatment with $CHCl_3$/HFIP (v/v, 4/1) to obtain in the form of the fully protected segment with a free α-carboxyl group [14]. During the chain assembly using repeated Fmoc deprotection performed by 20% piperidine/NMP (2.5 min $\times$4), the Asp(OAll)[9]-Ser(^{t}Bu)[10] sequence quantitatively converted to the aspartimide one, Asi-Ser(tBu) [15]. The Asi formation could be significantly reduced ($\sim$18%) by substituting 20% morpholine/NMP for 20% piperidine/NMP, although prolonged deprotection steps (3 min $\times$5) were required for complete removal of the Fmoc group.

To evaluate the efficacy to produce the intramolecular ester linkages by changing the order of ester formation precisely in the MST molecule, the same reaction conditions, as those used for the previous study, were employed. That is, a *pseudo*-high dilution procedure, involving a slow addition of the linear-peptide into the reaction mixture, with the aid of MNBA/DMAP [16], which proved to be essential not only for accelerating intramolecular esterification but also for preventing intermolecular esterification and the Asi formation at Asp[9] or Asp[11]. The formation of the first ester linkage with Thr[3]-Asp[9] after successive removal of the All group and the TBS group, followed by that of the second linkage with Ser[8]-Asp[11] after removal of the ^{t}Bu groups, was sequentially carried out to produce the protected MST. In the MST molecule, however, no significant changes in terms of the efficiency yielding individual ester linkages were observed by changing the order of esterification as shown in Table 1. The protected MST thus obtained was treated by HF in the presence of *p*-cresol and 2-PySH [17] at -2 to -5 °C for 1 h to give a crude product, which was purified by RP-HPLC and characterized by amino acid analysis, MALDI-TOF MS and NMR.

Structure–Activity Relationship of MST

It is noted that the residues of OMTKY3 in the OMTKY3–CARL complex and the corresponding residues of the solution structure of MST were well superimposed as previously reported [6]. In addition, the Ramachandran angles of the MST residues coincide with those of OMTKY3 binding loop [18]. Thus, it is expected that MST binds to an active pocket of CARL in the same manner as OMTKY3. To achieve a presumption of the interaction mechanism between MST and proteases, we developed a MST–CARL docking model using the crystal structure of OMTKY3–CARL complex as a template. The distances for each contact are summarized in

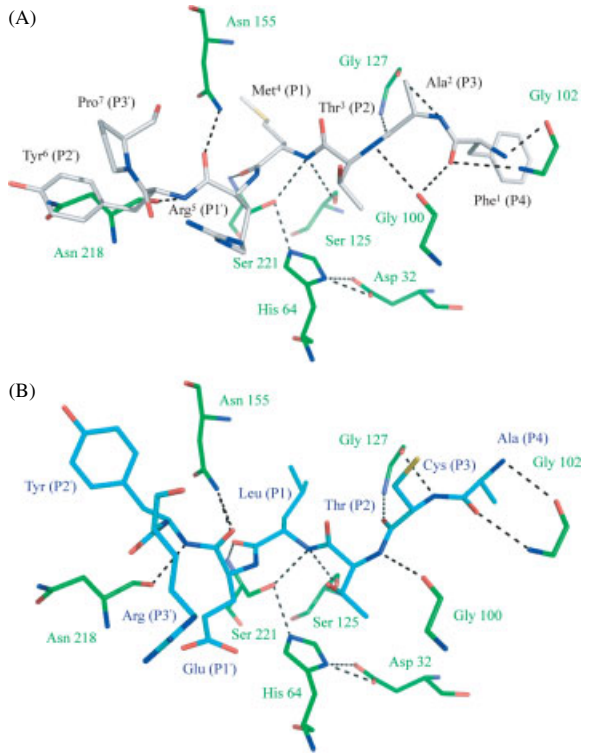

Figure 3. (A) Active site representation of the MST–CARL docking model. The docking model was constructed manually, by superimposing the Cα atoms from P3′ to P4 residues of MST to those of OMTKY3 bounded to CARL (PDB code, IYU6). The six residues of MST (carbon, white; nitrogen, blue; oxygen, red) are shown as stick model. The residues of CARL (carbon, green; nitrogen, blue; oxygen, red) interacting with MST and those of catalytic triad are also shown. (B) Active site representation of the OMTKY3–CARL complex crystal structure. The six residues of OMTKY3 (carbon, cyan; nitrogen, blue; oxygen, red) are shown as stick model. These images were made with PyMol.

Table 2. Distances within 4 Å between CARL and OMTKY3/MST

Contact	CARL–OMTKY3[a] (Å)	CARL–MST (model, Å)
P1 (N)–Ser 125 (CO)	3.30	3.73
P1 (N)–Ser 221 (O)	2.96	3.33
P1 (CO)–Asn 155 (N)	2.71	–
P1 (CO)–Ser 221 (N)	3.21	2.84
P2 (N)–Gly 100 (CO)	3.64	2.88
P2′ (N)–Asn218 (CO)	2.83	1.94
P3 (N)–Gly127 (CO)	3.10	3.32
P3 (CO)–Gly127 (N)	2.95	2.81
P4 (N)–Gly 102 (CO)	3.07	2.66
P4 (CO)–Gly 102 (N)	2.89	3.36

[a] Data from literature [18].

Table 2. This indicated that the six residues MST(1–6) are involved in direct contacts with CARL (Figure 3). To elucidate the structural requirements of MST including the ester linkages for expressing the protease inhibitory potential, we designed its analogs (**2**–**10**) for SAR study (Table 3), and their structures are illustrated in Figure 4. These analogs were based on substituting Ser for Thr^3 (**2**), opening the ester linkage(s) between Thr^3 and Asp^9 and/or

Table 3. MST analogs and their *K*i values against CARL

Peptide	*K*i (nM)[a]	Conformation of AA^7
MST (**1**)	0.59 ± 0.05	*cis*
2	0.87 ± 0.20	*cis*
3	>50 000	–
4	>50 000	–
5	14.0 ± 1.6	*cis* : *trans* = 85: 15
6	17.9 ± 1.8	*cis* : *trans* = 85: 15
7	>50 000	*cis* : *trans* = 85: 15
8	92.7 ± 3.8	*cis* : *trans* = 70: 30
9	23.4 ± 0.8	*cis* : *trans* = 70: 30
10	84.3 ± 1.2	*trans*

[a] Mean of 4–6 determinations ± SD.

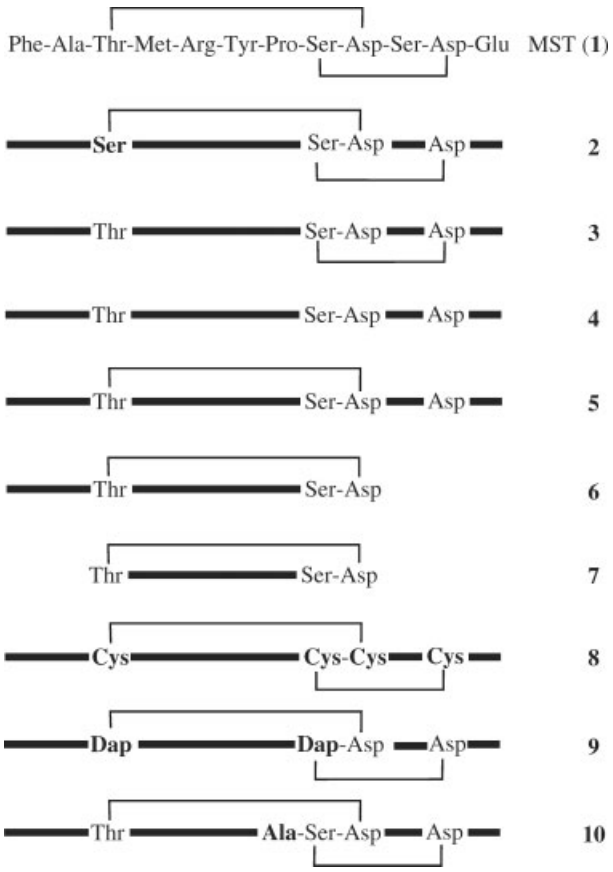

Figure 4. Structures of MST (**1**) and its analogs **2**–**10**.

Ser^8 and Asp^{11} (**3, 4**, and **5**), truncating the peptide chain (**6** and **7**), substituting the disulfide (**8**) or amide bonds (**9**) for the ester linkages, and substituting Ala for Pro^7 (**10**). These analogs were synthesized as in the case of MST by applying the regioselective ester, amide or disulfide formation with two sets of orthogonally removable side-chain protecting groups for Ser/Thr and Asp, Dap and Asp, or Cys and Cys, respectively. The peptides were evaluated for the potency inhibiting protease activity of CARL (Table 3). The inhibitory potency was not affected when the MST structure was modified by replacement of Thr^3 with Ser (**2**) to remove the β-methyl group. Opening of the ester linkage between Thr^3 and

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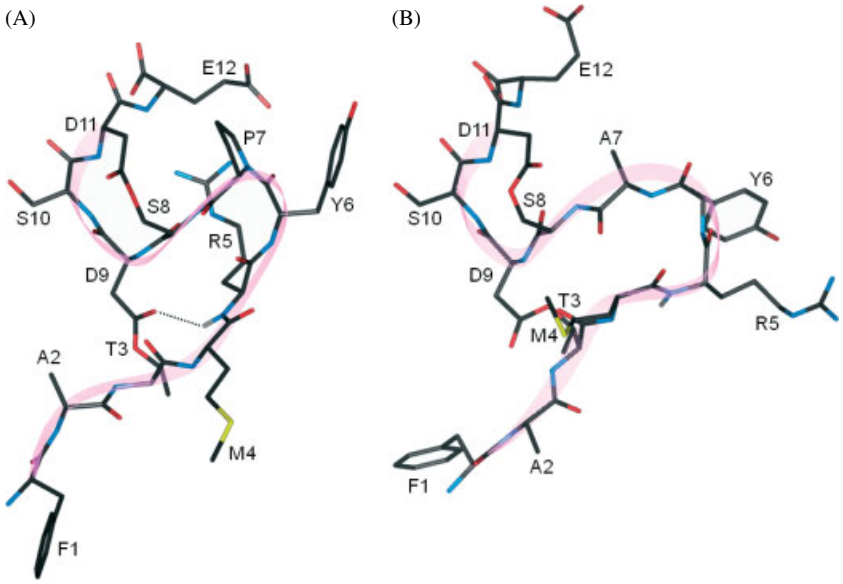

Figure 5. NMR solution structures of (A) MST (**1**) and (B) [Ala^7]-MST (**10**). A dotted line in (A) represents a hydrogen bond formed between the NH proton of Arg^5 and the carbonyl oxygen of the ester linkage with Thr^3-Asp^9.

Asp^9 resulted in complete loss of the potency (**3**), while the analogs (**5**) and (**6**) having this ester linkage retained significant inhibitory activity even with removal of the other one with Ser^8-Asp^{11} and the *C*-terminal Ser^{10}-Asp^{11}-Glu^{12}. However, removal of the *N*-terminal Phe^1-Ala^2 resulted in complete loss of the potency (**7**). It is suggested that the subtilisin–substrate association consists mainly of hydrogen bonds producing an antiparallel β-sheet between the main chains, and hydrophobic interactions between the side-chains at the P1 and P4 positions and the corresponding binding pockets at the S1 and S4 subsites, respectively [19]. Therefore, the *N*-terminal Phe^1-Ala^2 (i.e. P4–P3) should be essential for addressing the MST molecule to proteases. Thus, the minimum size required for expressing the inhibitory activity is the sequence (1–9) with a monocyclic structure, i.e. the analog (**6**). Pro^7 located in this ring structure was found to take a *cis*-conformation from the NMR analysis [4,6]. The *cis*-isomer would be the conformation bound to proteases although the *cis*-Pro^7 does not interact directly with the protease surface. This may contribute to a rigid type VI β-turn structure composed of Tyr^6-Pro^7, which promotes the internal hydrogen bond between the NH proton of Arg^5 and the carbonyl oxygen atom of the ester linkage with Thr^3-Asp^9 to fix the scissile bond of Met^4-Arg^5. When substituting Ala for Pro^7, the analog (**10**) in which Ala should take a *trans*-conformation displayed 2 orders of magnitude less potency than the original level. The analog (**10**) assumes almost the same structure for the smaller ring as the wild-type, but a significantly different structure for the larger ring in which Arg^5-Tyr^6 segment forms a type II β-turn (Figure 5). Distortion of the ring structure in (**10**) determined by NMR should be caused by the *trans*-conformation at position 7, leading to loss of internal hydrogen bond even though two ester linkages are retained. The distances between the NH proton of Arg^5 and the carbonyl oxygen atom of the ester linkage with Thr^3-Asp^9 are 2.2 and 6.8 Å for **1** and **10**, respectively. On the other hand, substitution of the amide bonds or disulfide bridges for the ester linkages altered the population of the backbone conformation at Pro^7 (**8** and **9**, *cis*/*trans* = 70/30). Besides this, the latter analog, in particular, resulted in reduction of the potency to 2 orders of magnitude less than the original level due to no chance to form the internal hydrogen bond as in the case of (**10**). The *cis*-Pro^7 conformation involved in promoting the internal hydrogen bond could be responsible for enhancing the inhibitory potency. Furthermore, monocyclic analogs (**5** and **6**) having the ester linkage with Thr^3-Asp^9 which is one of the requirements for expressing the inhibitory potential possessed the ratio between *cis*- and *trans*-conformation at Pro^7 of 85 to 15. Thus, the other ester linkage with Ser^8-Asp^{11} is likely to assist to stabilize the *cis*-conformation at Pro^7. From these observations, we concluded that the ester linkage with Thr^3-Asp^9, the *cis*-conformation at Pro^7, and the *N*-terminal Phe^1-Ala^2 may play a critical role in making it such a small-sized protease inhibitor for the canonical inhibitory activity. Recently, we determined the crystal structure of MST–CARL complex. This would be providing an interpretation to confirm the findings deduced from the SAR study. The details of the structure will be reported elsewhere.

Rational Design of Protease Inhibitory Specificities

By applying the structural motif of MST, we tried to rationally design protease inhibitory specificities that differ from those of the natural products. The canonical inhibitors of serine proteases are involved in the substrate-like interaction. The recognition of both substrate and inhibitor by most serine proteases is performed by accommodating the solvent-exposed primary binding residue P1. Therefore, the characteristic of the side-chain function on P1 is critical for the protease–inhibitor association and specificity. MST has a strong inhibitory potential against subtilisin, chymotrypsin, and elastase, but not trypsin. Amino acid preference at the P1 position for subtilisin is Met/Leu, while that for trypsin is Arg/Lys. As expected, [Arg^4, Met^5]-MST (**11**), in which P1 and P1′ residues of native MST (i.e. Met^4-Arg^5) were mutually replaced, resulted in complete loss of activity against subtilisin. And this gave a stronger inhibitory potency against trypsin than that of leupeptin, a well-known trypsin inhibitor (Table 4). Thus, the structural motif of MST is a useful tool for rational design of various protease inhibitors.

Table 4. *Ki* values of MST (**1**) and [Arg^4, Met^5]-MST (**11**) against trypsin and CARL

Peptide	*Ki* (nM)[a] Trypsin	CARL
MST (**1**)	>50 000	0.59 ± 0.05
[Arg^4, Met^5]-MST (**11**)	5.3 ± 0.8	378 ± 15
Leupeptin	42.3 ± 7.5	–

[a] Mean of 3–6 determinations ± SD.

Conclusions

MST was synthesized by a regioselective esterification procedure employing two sets of orthogonally removable side-chain protecting groups for the Asp and Ser/Thr residues to form two internal ester linkages sequentially. In the MST molecule, there were no significant changes in terms of the efficiency yielding individual ester linkages by changing the order of esterification as long as a *pseudo*-high dilution procedure with the aid of MNBA/DMAP was applied for the ester formation. We also synthesized a series of MST analogs to elucidate SAR by employing the same strategy as that for MST. This revealed that the ester linkage with Thr^3-Asp^9, the *cis*-conformation at Pro^7 and the *N*-terminal Phe^1-Ala^2 are essential for inhibitory activity. Furthermore, we demonstrated that the structural motif of MST is a useful tool for rational design of various inhibitors by changing the MST serine protease specificities from subtilisin into trypsin.

Acknowledgements

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