Tetrahedron Letters 50 (2009) 2377–2380

Contents lists available at ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Total synthesis of marinostatin, a serine protease inhibitor isolated from the marine bacterium *Pseudoallteromonas sagamiensis*

Misako Taichi [a,b], Toshimasa Yamazaki [c], Terutoshi Kimura [a], Yuji Nishiuchi [a,b,*]

[a] SAITO Research Center, Peptide Institute, Inc., Ibaraki, Osaka 567-0085, Japan
[b] Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan
[c] Protein Research Unit, National Institute of Agrobiological Science, Tsukuba, Ibaraki 305-8602, Japan

ARTICLE INFO

Article history:
Received 3 February 2009
Revised 26 February 2009
Accepted 27 February 2009
Available online 4 March 2009

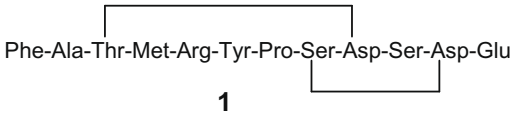
Keywords:
Ester linkage
Marinostatin (MST)
Protease inhibitor
Regioselective esterification

ABSTRACT

Marinostatin (MST) (**1**) isolated from a marine organism 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]. We synthesized MST by a regioselective esterification procedure employing two sets of orthogonally removable protecting groups at the side-chains of Asp and Ser/Thr. We optimized the esterification conditions to preferentially form the intramolecular ester linkages without any significant aspartimide (Asi) formation at Asp[9] and Asp[11]. The inhibitory potency of the synthetic MST against subtilisin (*Ki*, 0.6 nM) was comparable with a reported value for native MST (1.5 nM).

MST (**1**), a 12-residue peptide isolated from the marine bacterium *Pseudoalteromonas sagamiensis*, contains two ester linkages that are formed between the β-hydroxy and β-carboxyl groups, Thr[3]-Asp[9] and Ser[8]-Asp[11] (Fig. 1).[1] MST (**1**) is a serine protease inhibitor with a strong potency against subtilisin, chymotrypsin and elastase at an enzyme-inhibitor ratio of 1:1, but not against trypsin.[2] Its inhibitory potential is largely attributable to the hydrogen bond linking the backbone NH proton of Arg[5] and the carbonyl oxygen atom of the ester linkage of Thr[3]-Asp[9], which stabilizes the scissile bond of Met[4] (P1)-Arg[5] (P1′) for proteases.[3] In general, it is known that serine protease inhibitors have a rigid hydrophobic core derived from cross-connecting disulfide bridges in order to be able to directly bind to the catalytic sites of proteases. In the case of MST, however, the characteristic ester linkages may take the place of disulfide bridges to make it such a small-sized protease inhibitor for the cardinal inhibitory activity. Due to the interesting features of MST structure and biological activity, we tried to develop a protocol, which would allow us to synthesize not only the natural product to confirm its reported primary structure including ester linkages, but also its analogues possessing protease specificities that are different from those of the natural product.

The synthetic plan followed the retrosynthetic analysis shown in Scheme 1. MST (**1**) can be obtained by sequential intramolecular esterification of the linear peptide **3** followed by a final HF deprotection reaction[4] of the resulting diester **2**. To perform regioselective esterification, two sets of orthogonally removable side-chain protecting groups were required for Ser/Thr and Asp on **3**. The *t*Bu group was introduced to the side-chain functional groups of Thr[3] and Asp[9], and the *t*-butyldimethylsilyl (TBS) and 2-chlorotrityl [Trt(2-Cl)] groups were introduced to those of Ser[8] and Asp[11], respectively. The other side-chain-protecting groups and the N/C-terminal ones must remain unchanged in peptide synthesis using both Boc and Fmoc chemistry techniques and must be removed by HF treatment. Considering this, the 3-pentyl (Pen) group[5] was introduced to the phenolic hydroxyl group of Tyr[6], since it is compatible with Boc and Fmoc chemistry but is readily cleavable by the standard HF procedure without the formation of any significant amount of alkyltyrosine rearrangement product. The linear MST molecule was assembled by solid-phase peptide synthesis (SPPS) with Fmoc chemistry onto the C-terminal dipeptide **4**, which was attached to a Trt(2-Cl) resin through the β-carboxyl group of Asp[11] posterior to remove the *t*Bu group (Scheme 2). Peptide elongation was carried out with an ABI 433A peptide synthesizer using FastMoc® protocols of coupling with Fmoc-amino acid/HCTU/

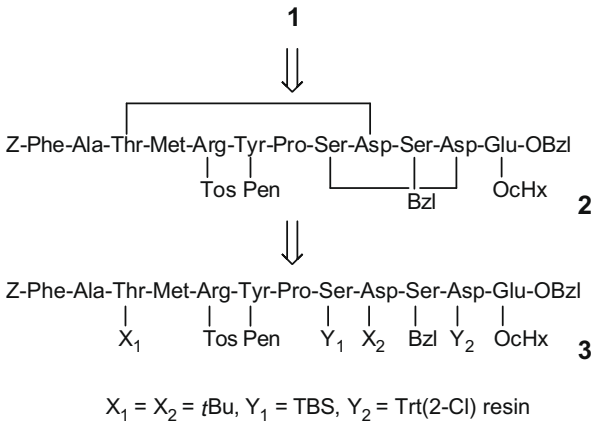


1

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

* Corresponding author. Tel.: +81 72 643 4411; fax: +81 72 643 4422.
E-mail address: yuji@peptide.co.jp (Y. Nishiuchi).

0040-4039/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tetlet.2009.02.213

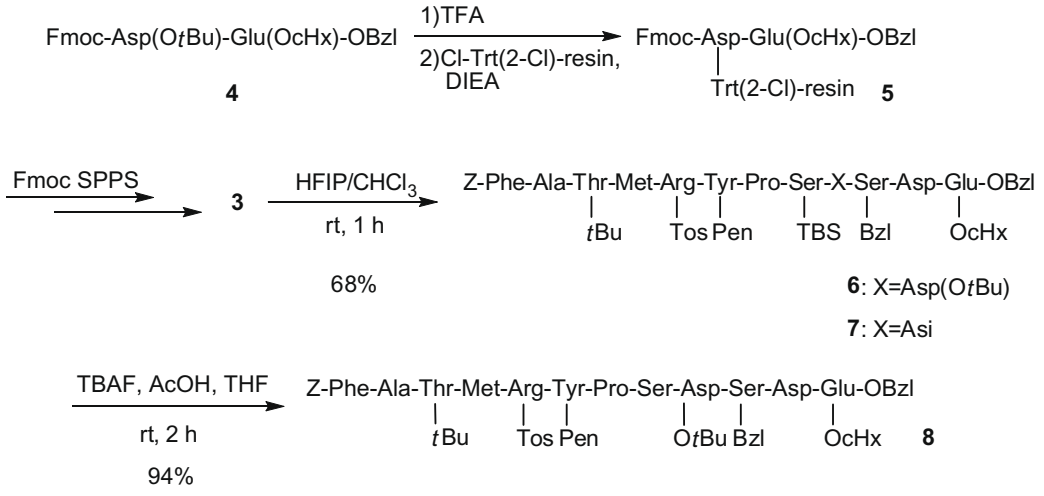


Scheme 1. Retrosynthetic analysis of **1**.

Table 2
Effects of solvents and peptide concentrations on the esterification reaction between Ser[8] and Asp[11] mediated by the MNBA method

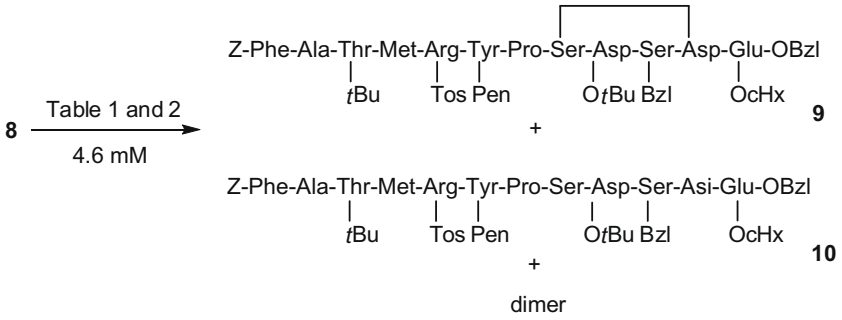
Entry	Solvent	Peptide concentration (10^{-3}M)	Ratio of products **9**:**10**:dimer[a]
5	CH_2Cl_2	4.6	66:1:33
6	CH_2Cl_2	1.0	69:0:31
7	CH_2Cl_2/DMF (3:1)	4.6	84:4:12
8	DMF	4.6	74:22:4
9	CH_2Cl_2	4.6[b]	94:3:3

[a] Dimer refers to cyclic and linear dimerized peptides.
[b] Pseudo-high dilution procedure involving progressive addition of the linear peptide 8 to the reaction mixture for 3.5 h.



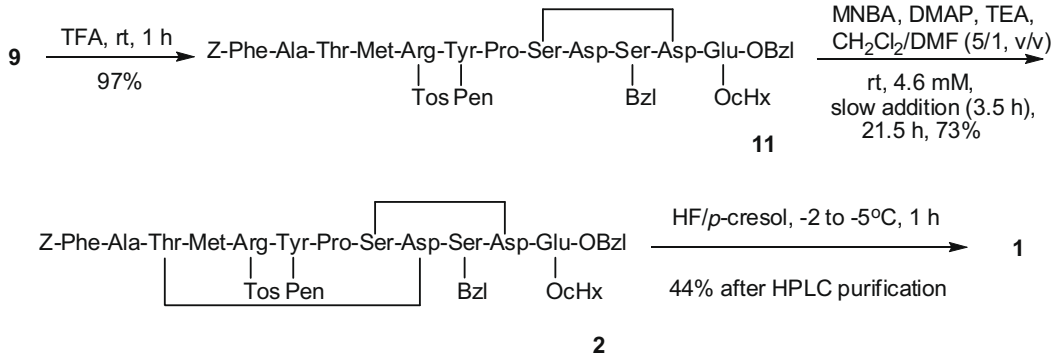
Scheme 2. Synthesis of the protected linear peptide **8**.

Table 1
Effects of coupling reagents on the esterification reaction between Ser[8] and Asp[11] performed in CH_2Cl_2 at the peptide concentration of 4.6×10^{-3} M



Entry	Coupling reagents	Additives	Base	Ratio of products **9**:**10**:dimer[a]
1	PyBOP (3.0 equiv)	—	DIEA (5.0 equiv)	9: 91: 0
2	TCB-Cl[b] (1.2 equiv)	DMAP (0.5 equiv)	TEA (2.2 equiv)	62: 4: 34
3	MNBA (1.2 equiv)	DMAP (0.5 equiv)	TEA (5.0 equiv)	66: 1: 33
4	DIC[c] (1.2 equiv)	DMAP (0.5 equiv)	—	61: 5: 34

[a] Dimer refers to cyclic and linear dimerized peptides.
[b] Trichlorobenzoyl chloride.
[c] Diisopropylcarbodiimide.



Scheme 3. Synthetic route for **1**.

6-Cl-HOBt/DIEA (4:4:4:8) in NMP. After completion of the chain assembly, the peptide resin **3** was treated with HFIP/chloroform (1:4, v/v) for 1 h to afford **6**. However, **6** was found to be accompanied by the aspartimide (Asi)-peptide **7** between Asp^9 and Ser^{10} (≈10%) during chain elongation using repeated Fmoc deprotection performed by 20% piperidine/NMP (2.5 min × 4). The Asi formation could be significantly reduced (<2%) by substituting 20% morpholine/NMP for 20% piperidine/NMP in the Fmoc deprotection reaction although prolonged deprotection steps (5 min × 4) were required for complete removal of the Fmoc group. Removal of the TBS group at Ser^8 on **6** was achieved by treatment with TBAF/AcOH in THF to give **8** having free hydroxyl and carboxyl groups at Ser^8 and Asp^{11}, respectively.

To examine the conditions for preferentially forming the intramolecular ester linkage with Ser^8-Asp^{11}, the linear peptide **8** in hand was subjected to esterification that was performed in CH_2Cl_2 at a peptide concentration of 4.6×10^{-3} M with the aid of various coupling reagents (Table 1). This reaction more or less accompanied Asi formation at Asp^{11}, regardless of the coupling reagent type, in addition to intra- and/or intermolecular esterification. The PyBOP method (entry 1) almost quantitatively converted the Asp^{11}-peptide **8** to the Asi^{11}-peptide **10**, whereas the 2-methyl-6-nitrobenzoic anhydride (MNBA)/DMAP method developed by Shiina et al.[6] was observed to be accompanied by a small amount of Asi^{11} formation. However, the latter resulted in the ratio of the cyclic monomer **9** and the cyclic/linear dimer to be 2 to 1 since intramolecular esterification did not occur predominantly (entry 3). We therefore tried to optimize the conditions to preferentially form the intramolecular ester linkage when using the MNBA/DMAP method by changing the solvent and the peptide concentration as shown in Table 2. Increasing the concentration of DMF in the reaction mixture increased the extent of Asi^{11} formation (entries 7 and 8), while its formation remained at a minor level as long as the reaction was performed in CH_2Cl_2, regardless of the peptide concentration (entry 5, 6). In addition, no significant changes with the ratio of **9** and the cyclic/linear dimer were observed even by lowering the peptide concentration in CH_2Cl_2 to 1×10^{-3} M (entry 6). Therefore, a *pseudo*-high dilution procedure involving progressive addition of the linear peptide **8** to the reaction mixture containing MNBA/DMAP in CH_2Cl_2 was employed to accelerate the intramolecular esterification. This could be successfully performed to preferentially produce the cyclic monomer **9** without any significant amounts of side products such as the cyclic/linear dimer or Asi^{11}-peptide **10** (entry 9).

After removal of the *t*Bu groups at Thr^3 and Asp^9 by treating **9** with TFA, the second ester linkage with Thr^3-Asp^9 was built up to obtain the fully protected MST (**2**) by employing the procedure same as that for the first one with Ser^8-Asp^{11}, except for the reaction solvent (Scheme 3). A mixture of CH_2Cl_2 and DMF (v/v, 5:1) was needed to dissolve **11** due to its low solubility in CH_2Cl_2. The increment of Asi^9 formation associated with the use of DMF could be kept to a minimum by reducing the DMF concentration as much as possible. Thus, the *pseudo*-high dilution procedure performed in CH_2Cl_2/DMF (v/v, 5/1) predominantly led to intramolecular esterification with Thr^3-Asp^9 to afford the diester-peptide **2** in a 73% yield with the proportions of the products being 90/2/8 for **2**/Asi^9-peptide/cyclic or linear dimer. The diester-peptide **2** was treated with anhydrous HF in the presence of *p*-cresol (v/v, 8/2) at −2 °C to −5 °C for 1 h to remove all the protecting groups and the product **1** was obtained in a 44% yield after purification by RP–HPLC.[7] Spectral and analytical data of synthetic MST were in good agreement with those of the literature data,[1,8]although we had no opportunity to directly compare the synthetic peptide with the natural one by an analytical procedure using RP-, ion exchange-HPLC or capillary zone electrophoresis. As for inhibitory activity against subtilisin, the *K*i value was measured using Suc-Ala-Ala-Pro-Phe-MCA as a substrate and its potency (*K*i, 0.6 nM) was comparable with the reported value for native MST (1.5 nM).[1,9,10]

In conclusion, we have achieved the first total synthesis of MST (**1**) by regioselective formation of the intramolecular ester linkages, Thr^3-Asp^9 and Ser^8-Asp^{11}, with the aid of MNBA-mediated esterification as a key step. By applying the structural motif of MST, the preparation of analogues is in progress for rationally designing protease inhibitory specificities that are different from those of the natural product.

Acknowledgement

This work was supported partly by the Program for Promotion of Fundamental Studies in Health Science of National Institute of Biomedical Innovation (NIBL).

References and notes

1. (a) Kanaori, K.; Kamei, K.; Taniguchi, M.; Koyama, T.; Yasui, T.; Takano, R.; Imada, C.; Tajima, K.; Hara, S. *Biochemistry* **2005**, *44*, 2462–2468; (b) Taniguchi, M.; Kamei, K.; Kanaori, K.; Koyama, T.; Yasui, T.; Takano, R.; Harada, S.; Tajima, K.; Imada, C.; Hara, S. *J. Pept. Res.* **2005**, *66*, 49–58.
2. Imada, C.; Maeda, M.; Hara, S.; Taga, N.; Simidu, U. *J. Appl. Bacteriol.* **1986**, *60*, 469–476.
3. Takano, R.; Imada, C.; Kamei, K.; Hara, S. *J. Biochem.* **1991**, *110*, 856–858.
4. (a) Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Jpn.* **1967**, *40*, 2164–2167; (b) Sakakibara, S.; Kishida, Y.; Nishizawa, R.; Shimonishi, Y. *Bull. Chem. Soc. Jpn.* **1968**, *41*, 438–441.
5. Bódi, J.; Nishiuchi, Y.; Nishio, H.; Inui, T.; Kimura, T. *Terahedron Lett.* **1998**, *39*, 7117–7120.
6. Shiina, I.; Ibuka, R.; Kubota, M. *Chem. Lett.* **2002**, 286–287.
7. The protected MST (**2**) (70 mg, 35 μmol) was treated with HF (2.0 ml) in the presence of *p*-cresol (0.50 ml) at −2 °C to −5 °C for 1 h to give a crude product of **1**, which was purified by RP-HPLC using a YMC-Pak ODS column (30 × 250 mm) at a flow rate 20 ml/min. The RP–HPLC run was eluted with an increasing gradient of CH_3CN in 0.1% TFA (10–30%, 80 min) to obtain **1** (21 mg, 44%).

8. Analytical and spectral data of **1**: Amino acid analysis (after hydrolysis with 6 N HCl at 110 °C for 22 h): 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); MALDI-TOF MS: 1383.38 ($[M+H]^+$) theoretical value, 1383.46.

^1H and ^{13}C NMR chemical shifts (ppm) [750 MHz, H_2O/D_2O (v/v, 9:1), 10 °C]

Residue	NH	Hα (Cα)	Hβ (Cβ)	Others (C)
Phe[1]		4.24 (56.22)	3.24, 3.09 (39.3)	2, 6H 7.26 (131.64) 3, 5H 7.35 (131.33) 4H 7.32 (130.24)
Ala[2]	8.64	4.47 (51.45)	1.35 (19.28)	
Thr[3]	8.72	4.58 (58.73)	5.52 (73.98)	Hγ 1.35 (19.28)
Met[4]	8.95	4.66 (54.28)	2.32, 1.81 (31.57)	Hγ 2.58, 2.44 (31.68)
Arg[5]	7.72	4.10 (56.55)	1.79, 1.70 (30.43)	Hγ 1.70, 1.53 (27.49) Hδ 3.26, 3.18 (42.92) NH 7.36 NH_2 6.94, 6.48
Tyr[6]	8.52	4.52 (54.70)	2.96, 2.86 (39.98)	2, 6H 7.11 (132.83) 3, 5H 6.83 (117.94)
Pro[7]		3.49 (63.52)	1.76, 1.63 (32.82)	Hγ 1.65, 1.56 (24.28) Hδ 3.62, 3.34 (49.48)
Ser[8]	7.19	4.66 (54.78)	4.92, 4.25 (66.24)	
Asp[9]	9.29	4.47 (54.65)	2.97, 2.97 (36.20)	
Ser[10]	8.59	4.55 (56.40)	3.84, 3.79 (62.13)	
Asp[11]	7.83	4.78 (51.60)	2.72, 2.63 (38.26)	
Glu[12]	8.36	4.23 (55.59)	2.14, 1.93 (28.35)	Hγ 2.41, 2.41 (32.70)

9. Green, N. M.; Work, E. *Biochem. J.* **1953**, *54*, 347–352.

10. The inhibitory activity of the synthetic MST (**1**) against subtilisin was measured according to a literature procedure.[1a] Briefly, the substrate (Suc-Ala-Ala-Pro-Phe-MCA) obtained from Peptide Institute, Inc. (Osaka, Japan) was dissolved in DMSO at concentration of 25 μM. Subtilisin Carlsberg (Calbiochem, Germany, 110 nM) was incubated with an appropriate amount of **1** 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 addition of 0.1 ml of the substrate solution. The release of 7-amino-4-methylcoumarin (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 **1** to calculate the *K*i value.[9]