Two [Met(0)]\textsuperscript{6} deacetyl-thymosin $\beta_4$ analogs containing Phe(4F) or Tyr(Me) at position 12 were synthesized by the manual solid-phase method, and their anti-inflammatory effect on carrageenin-induced edema in the mouse paw was studied. Fluorination of the para-position of Phe\textsubscript{12} resulted in a marked anti-inflammatory effect on carrageenin-induced edema in the mouse paw compared with that of our synthetic [Met(0)]\textsuperscript{6}deacetyl-thymosin $\beta_4$, but the other analog, [Met(0)]\textsuperscript{6}, Tyr(Me)$\textsuperscript{12}$deacetyl-thymosin $\beta_4$, showed a marked reduction of the anti-inflammatory effect.

Key words: Anti-inflammatory effect, [Met(0)]\textsuperscript{6}deacetyl-thymosin $\beta_4$ analogue synthesis, Carrageenin-induced edema, Mouse paw

Introduction

Thymosin $\beta_4$ consists of 43 amino acid residues (Fig. 1) with a molecular weight of 4963 and an isoelectric point of 5.1.\textsuperscript{1} The N-terminus of the peptide is blocked by an acetyl group. This peptide exhibits important activities in the regulation and differentiation of thymus-dependent lymphocytes. Recently, Young et al.\textsuperscript{2} reported that [Met(0)]\textsuperscript{6}thymosin $\beta_4$ (Met(0), methionine sulfoxide) is generated by monocytes in the presence of glucocorticoids and acts as a signal to inhibit an inflammatory response, and [Met(0)]\textsuperscript{6}thymosin $\beta_4$ was a potent inhibitor of carrageenin-induced edema in the mouse paw. [Met(0)]\textsuperscript{6}thymosin $\beta_4$ may have value in anti-inflammatory drug therapy, with great potential advantages over existing nonsteroidal drugs that alleviate the distressing symptoms of inflammation without preventing the tissue damage. Therefore, [Met(0)]\textsuperscript{6}thymosin $\beta_4$ might be able to reproduce the considerable benefits of glucocorticoids originally seen in rheumatoid arthritis, but without the subsequent disabling steroid toxicity.

In our preceding paper,\textsuperscript{3} we concluded that the acetyl group at the N-terminal Ser residue of thymosin $\beta_4$ is not required for immunological activity. We have also reported\textsuperscript{4} that our synthetic [Phe(4F)$\textsuperscript{12}$] deacetyl-thymosin $\beta_4$ (Phe(4F), para-fluorophenylalanine) which has the strong electron-withdrawing fluoride atom on the para position of the aromatic ring of deacetyl-thymosin $\beta_4$, showed stronger immunological activity than that of our synthetic deacetyl-thymosin $\beta_4$. These results prompted us to synthesize two deacetyl-thymosin $\beta_4$ analogs: [Met(0)]\textsuperscript{6}, Phe(4F)$\textsuperscript{12}$deacetyl-thymosin $\beta_4$, which has an electron-withdrawing atom (F) at the 12 position of [Met(0)]\textsuperscript{6}thymosin $\beta_4$; and [Met(0)]\textsuperscript{6}, Tyr-(Me)$\textsuperscript{12}$deacetyl-thymosin $\beta_4$, which has an electron-donating group (-OCH$_3$) at the 12 position of [Met(0)]\textsuperscript{6}deacetyl-thymosin $\beta_4$.

This paper presents the syntheses of [Met(0)]\textsuperscript{6}, Phe(4F)$\textsuperscript{12}$deacetyl-thymosin $\beta_4$ and [Met(0)]\textsuperscript{6}, Tyr-(Me)$\textsuperscript{12}$deacetyl-thymosin $\beta_4$, and an examination of the comparative anti-inflammatory effect of these analogs on carrageenin-induced edema in the mouse paw.

Materials and methods

9-Fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives and Fmoc-Ser(tert-buty)-phenylacetoamido-methyl (Pam) resin (0.64 mmol/g, 100–200 mesh) were purchased from Kokusan Chemical Works Ltd. (Japan), Watanabe Chemical Industries Ltd. (Japan), Peptide Institute Inc. (Japan) and Sigma Chemical Co. (USA). Thin-layer chromatography (TLC) was effected with silica gel (Kieselgel 60 F$_{254}$; Merck) on pre-
coated aluminum sheets using n-BuOH-acetic acid-H$_2$O as a solvent system. Analytical high-performance liquid chromatography (HPLC) and amino acid analysis were conducted with a Shimadzu LC-6A and a Hitachi 835A, respectively. The fast atom bombardment mass spectrometry (FAB-MS) spectrum was obtained on a UG analytical 2-AB-2SEQ spectrometer equipped with the 11–250J data system.

### Solid-phase peptide synthesis

Peptide synthesis was performed manually by the stepwise solid-phase method with a hand-made peptide synthesizer, using the base-labile Fmoc group for protecting the $\beta$-amino groups, and such acid-labile groups as tert-butyl for the hydroxy and carboxy groups, tert-butyloxycarbonyl for the $\beta$-amino group of Lys, and sulfoxide for Met. The peptide was assembled on Fmoc-Ser(tert-butyl)-Pam resin. The Fmoc group was removed with 30% piperidine in N,N$_2$-dimethylformamide (DMF).

Elongation of the peptide chain was carried out by the dicyclohexylcarbodiimide-1-hydroxybenzotriazole (DCC-HOBT) method in CH$_2$Cl$_2$-DMF (1:1) or N-methyl-2-pyrrolidone. The coupling reaction and deprotection of the Fmoc group were monitored by the ninhydrin test. The general procedure for each synthetic cycle (starting material, 0.64 mmol/g Fmoc-Ser(tert-butyl)-Pam resin (400 mg)) was: (1) CH$_2$Cl$_2$ wash (twice); (2) DMF wash (twice); (3) deprotect in DMF-piperidine (7:3) for 20 min; (4) DMF wash (twice); (5) dioxane-water (2:1) wash (twice); (6) DMF wash (three times); (7) CH$_2$Cl$_2$ wash (three times); (8) addition of 3 equivalents (eq) Fmoc-amino acid, HOBT, and DCC in CH$_2$Cl$_2$; (9) reaction for 120 min; (11) recoupling if necessary by repeating steps 7–10; (12) DMF wash (three times); (13) isopropanol wash (three times); and (14) CH$_2$Cl$_2$ wash (four times). Whenever the ninhydrin test was still slightly positive, even after three couplings, the remaining unreacted amino groups were acetylated with 0.4 M acetyl-imidazole in DMF (1x, 30 min), and (step 15) DMF wash (twice). The protected resin thus obtained (200 mg) was treated with 2 M tetrafluoroboronic acid-thioanisole in trifluoroacetic acid (TFA) (7 ml) in the presence of m-cresol (218 µl, 100 eq) and ethane-1,2-diol (524 µl, 300 eq) at 4°C for 90 min.

After the deprotection, the resin was removed by filtration and the filtrate was evaporated under reduced pressure, and the residue was solidified by addition of anhydrous ether to give a crude peptide. The resulting powder was dissolved in H$_2$O (6 ml). The solution was treated with Amberlite CG-4B (acetate form, approximately 3 g) for 30 min, and filtered by suction and evaporated *in vacuo*. The residue was dissolved in a small amount of 1% acetic acid and then applied to a column of Sephadex G-25 (2.3 x 96 cm), which was eluted with the same solvent. Individual fractions (5 ml each) were collected and absorbancy at 260 nm was determined for each fraction. The fractions corresponding to the front main peak were combined and the solvent was removed by lyophilization. The peptide was further purified by semi-preparative PR-HPLC. The semi-preparative PR-HPLC was performed on a Nucleosil C18 column (250 x 10 mm I.D.; 7 µm particle size; Marcherey Nagel). Solvent A was 0.05% TFA in water and solvent B was 60% acetonitrile in solvent A. A linear gradient was applied from 10 to 50% B during 50 min, at a flow rate of 3.0 ml/min. Detection of the peptide was set at 230 nm. The major peak was lyophilized to give the purified product. Overall yields of the two peptides were 5.9% ([Met(0)$_6$, Phe-(4F)$_{12}$]deacetyl-thymosin $\beta_4$) and 6.1% ([Met(0)$_6$, Tyr[Me]$_{12}$]deacetyl-thymosin $\beta_4$), respectively, based on the C-terminal Ser loaded on the resin. Homogeneity of the peptides was checked by TLC, amino acid analysis after 6N HCl hydrolysis, The physicochemical data of the synthetic analogs are presented in Tables 1 and 2.

### Bioassay

Carrageenin-induced inflammation was initiated in BALB/C mice as described elsewhere. One of the three analogs, [Met(0)$_6$]deacetyl-thymosin $\beta_4$,
(obtained from protected deacetyl-thymosin $\beta_4$ without reduction treatment), and [Met(0)$^6$, Phe(4F)$^{12}$]deacetyl-thymosin $\beta_4$ and [Met(0)$^6$, Tyr(Me)$^{12}$]deacetyl-thymosin $\beta_4$, was administered 30 min before (intraperitoneal injection), coincident with (intra-paw injection) and 6 h after (intraperitoneal injection) carrageenin injection into the right hind footpad. The change in footpad thickness between right and left hindlimbs was assessed using dial calipers by two observers ‘blinded’ to the treatment status of the mice. Control carrageenin-injected mice received phosphate-buffered saline at similar times. We judged our synthetic peptide had a positive effect when we found more than 80% of footpad swelling was suppressed (Tables 3 and 4).

**Results and discussion**

In our preceding paper, we reported that Phe$^{12}$ residue of deacetyl-thymosin $\beta_4$ is one of the structural essentials for immunological activity on the impaired blastogenic response of uremic T lymphocytes. One of our synthetic analogs, $^3,4$ [Phe(4F)$^{12}$]deacetyl-thymosin $\beta_4$, exhibited stronger immunological activity than that of our synthetic deacetyl-thymosin $\beta_4$. Recently, Young et al. $^2$ reported that [Met(0)$^6$]thymosin $\beta_4$ was a potent inhibitor of carrageenin-induced edema in the mouse paw. These results prompted us to synthesize two analogs, one of which has a fluorine atom on the para-position of Phe$^{12}$ and the other has methoxy group on the para-position of Phe$^{12}$ in [Met(0)$^6$]deacetyl-thymosin $\beta_4$.

Anti-inflammatory effects of the synthetic [Met(0)$^6$]deacetyl-thymosin $\beta_4$, [Met(0)$^6$, Phe(4F)$^{12}$]deacetyl-thymosin $\beta_4$ and [Met(0)$^6$, Tyr(Me)$^{12}$]deacetyl-thymosin $\beta_4$ were examined by carrageenin-induced inflammation test using BALB/c mice. $^2$ The in vivo anti-inflammatory effect of the synthetic peptides on carrageenin-induced edema in mouse paw.

| Peptide | Dose (μg/kg) | Suppression effect of mouse edema$^*$ |
|---------|-------------|-------------------------------------|
| [Met(0)$^6$]deacetyl-thymosin $\beta_4$ | 20 | ++* |
| [Met(0)$^6$, Phe(4F)$^{12}$deacetyl-thymosin $\beta_4$ | 10 | +++* |
| [Met(0)$^6$, Phe(4F)$^{12}$deacetyl-thymosin $\beta_4$ | 20 | ++++* |
| [Met(0)$^6$, Tyr(Me)$^{12}$deacetyl-thymosin $\beta_4$ | 20 | ++* |
| [Met(0)$^6$, Tyr(Me)$^{12}$deacetyl-thymosin $\beta_4$ | 40 | +* |

Met(0), methionine sulfoxide; Phe(4F), para-fluorophenylalanine.

$^a$ Control carrageenin-injected mice received phosphate-buffered saline at similar times.

$^b$ Order of suppression effect on mouse edema was as follows: + < + < ++ < +++ < ++++.
carrageenin-induced edema in the mouse paw is presented in Table 3. Interestingly, our synthetic [Met(0)\textsuperscript{6}, Phe(4F)\textsuperscript{12}]deacetyl-thymosin \(\beta_4\) showed stronger anti-inflammatory activity than that of our synthetic [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\). In this study, the strong electron-withdrawing fluorine atom on the para-position of the aromatic ring results in an analog that possesses stronger activity than that of [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\). On the contrary, another analog, [Met(0)\textsuperscript{6}, Tyr(Me)\textsuperscript{12}]deacetyl-thymosin \(\beta_4\), which has an electron-donating group -OCH\(_3\) on the para-position of the aromatic ring, showed a much weaker anti-inflammatory effect than that of our synthetic [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\). Our synthetic [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\) at a concentration of 20 \(\mu\)g induced suppression equivalent to the suppression induced by 0.5 mg/kg dexamethasone, a potent anti-inflammatory steroid, and one of our two analogs, [Met(0)\textsuperscript{6}, Phe(4F)\textsuperscript{12}]deacetyl-thymosin \(\beta_4\), showed the same suppression activity at the concentration of 5 \(\mu\)g induced suppression equivalent to the suppression induced by 0.5 mg/kg dexamethasone, which means that suppression activity of this analog is about fourfold stronger than that of [Met(0)\textsuperscript{6}]deacetyl-thymosine \(\beta_4\) (data not shown). These results seem to suggest that aromaticity at the 12 position of [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\) plays significant roles for anti-inflammatory activity on carrageenin-induced edema in the mouse paw, and modification of the Phe residue of thymosin \(\beta_4\) could produce more potent analogs capable of anti-inflammatory effects on inflammatory diseases.

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| Peptide | Relative potency (molar basis) |
|---------|-------------------------------|
| [Met(0)\textsuperscript{6}]deacetyl-thymosin \(\beta_4\) | 1.00 |
| [Met(0)\textsuperscript{6}, Phe(4F)\textsuperscript{12}]deacetyl-thymosin \(\beta_4\) | 2.52 |
| [Met(0)\textsuperscript{6}, Tyr(Me)\textsuperscript{12}]deacetyl-thymosin \(\beta_4\) | 0.11 |

Met(0), methionine sulfoxide; Phe(4F), para-fluorophenylalanine.