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Synthesis, characterization and biological activities of thymopentin ethyl ester

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The ethyl ester derivative of thymopentin was synthesized and characterized. The biological activities of thymopentin were evaluated by mouse spleen lymphocyte proliferation test and superoxide dismutase activity assay. Compared with thymopentin, the synthesized ethyl ester showed more potent immunoregulation activity in the MTT assay and anti-oxidation activity in the immune-suppressed rat model induced by hydrocortisone. The half life of the ester derivate in rat plasma determined by HPLC was slightly longer than that of thymopentin. The ester derivate gained advantages in activity and stability compared to thymopentin.

1. Introduction

Thymopentin (TP5), the synthetic pentapeptide Arg-Lys-Asp-Val-Tyr, corresponds to residues 32–36 of the thymic hormone thymopoietin. It has pleiotropic actions including selective induction of early T-cell differentiation and regulation of neuromuscular transmission. Furthermore, TP5 has been shown to have immunoregulatory actions on peripheral T cells (Goldstein et al. 1979). TP5 is freely soluble in water and quickly degraded by proteases and aminopeptidases in plasma. Some investigations had been made to increase its stability and efficacy (Pan et al. 2005; Heavner et al. 1986; Geiger et al. 1983). To our knowledge, no ester derivates of thymopentin were reported.

The present investigation described the synthesis of an ethyl ester derivative of thymopentin (TP-ET). The synthesized derivative was characterized by various spectroscopic techniques such as UV spectrophotometry, mass spectroscopy etc. The derivative was assessed for its *in vitro* immunomodulating activity using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] colorimetric assay. The ethyl ester showed anti-oxidation activity in immune-suppressed rat model induced by hydrocortisone. The half life of ethyl ester was determined by reversed phase high performance liquid chromatography (RP-HPLC).

2. Investigations and results

2.1. Characterization of thymopentin ethyl ester

Thymopentin ethyl ester was a white powder, freely soluble in methanol and ethanol, sparingly soluble in water

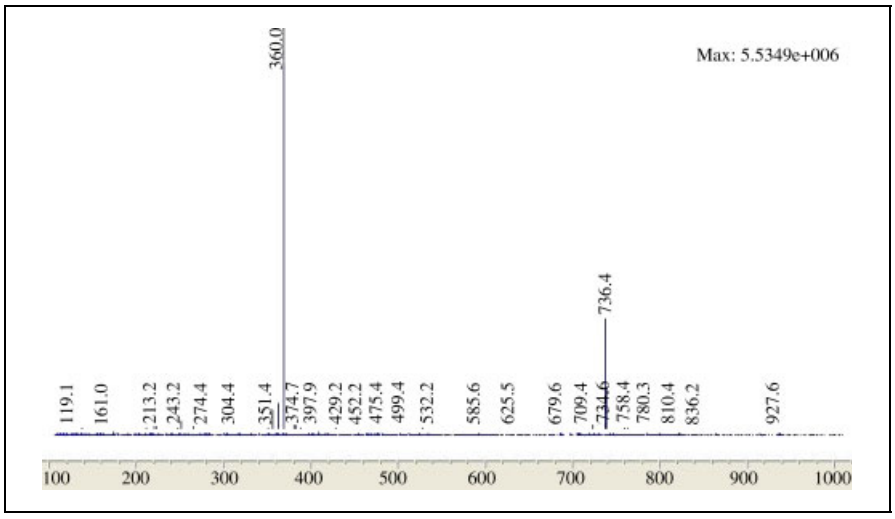

Fig. 1:
LC-MS analysis of thymopentin ethyl ester *m/z* 736.4 $[MW + H]^+$, *m/z* 368.8 $[MW + 2H]^{2+}$

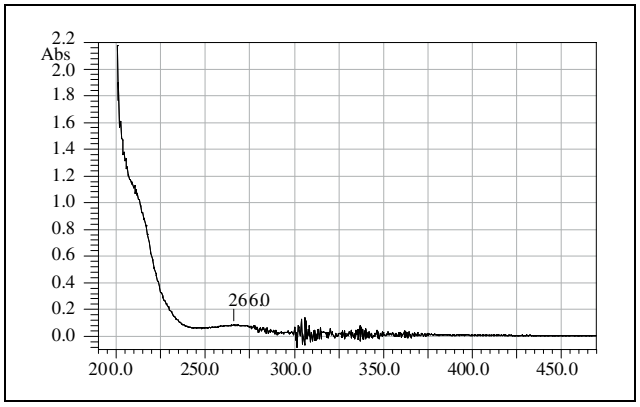

Fig. 2: UV spectrum of thymopentin ethyl ester

(< 5 mg/ml), insoluble in ethyl acetate, chloroform and petroleum ether.

HPLC-ESI-MS assay was performed by Agilent 1100 HPLC-MS (Fig. 1). The molecular weight of thymopentin diethyl ester was calculated as 735, the detected $[MW + H]^+$ 736.4, $[MW + 2H]^{2+}$ 368.8. The UV spectrum (TP-ET 20 μg/ml methanol solution, Fig. 2) was in consistency with TP5. The spectra data meant that the synthesized compound was thymopentin diethyl ester.

2.2. Half-life of ethyl ester in rat plasma

Under the HPLC conditions mentioned below, complete seperation was obtained between the drug and the plasma (TP-ET: Fig. 3; TP-5: Fig. 4). The drug concentration (C) was calculated based on the peaks areas. The linear regression between logarithm of drug concentration (Log C) and time (t) was made to calculate the half lives of TP5 and TP-ET (Fig. 5). The calculated results showed the half life of TP5 was 1.3 min and TP-ET 2.0 min. The half life of ester derivate was a little longer than thymopentin.

2.3. Lymphocyte proliferation test

The results of lymphocyte proliferation test for mouse spleen were shown in Fig. 6. Under the three tested con-

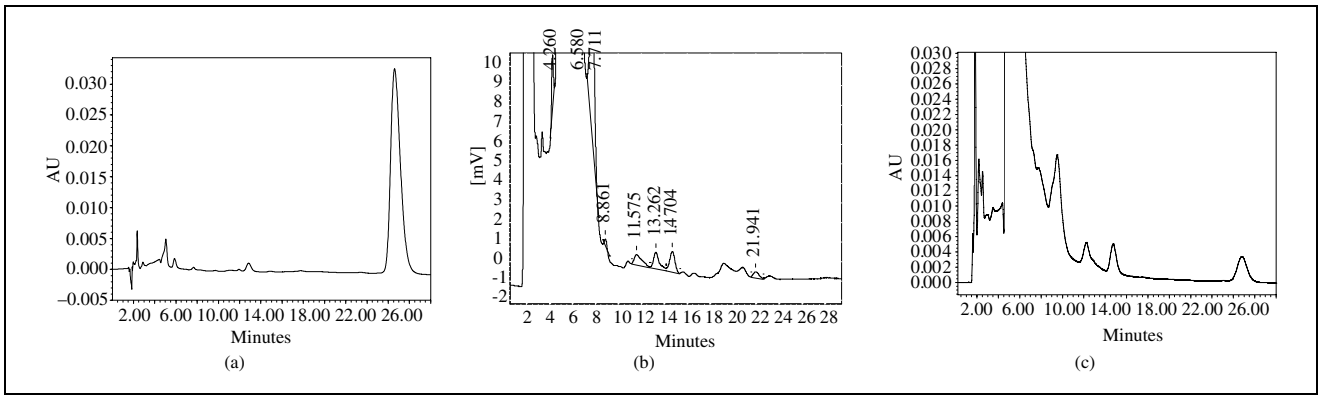

Fig. 3: Separation chromatography of thymopentin ethyl ester and plasma
(a) TP-ET; (b) blank plasma; (c) TP-ET in plasma

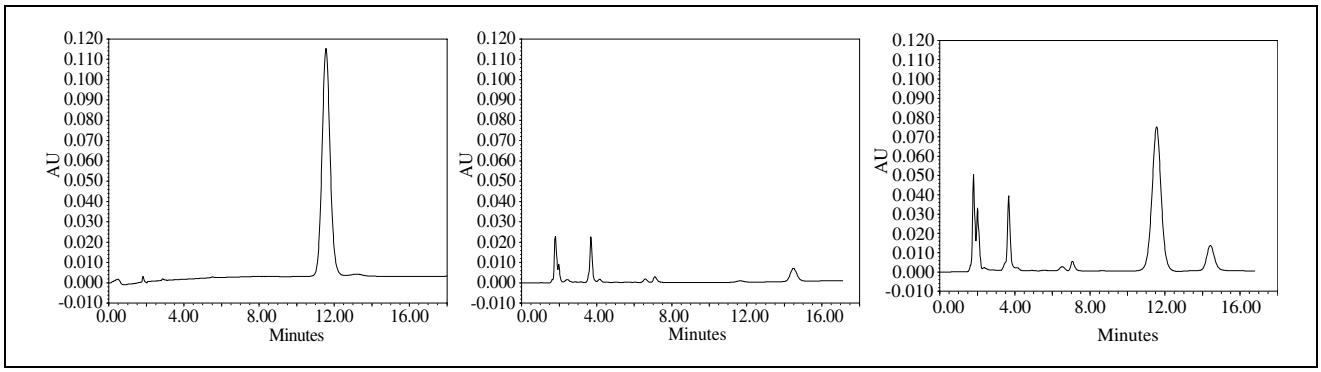

Fig. 4: Separation chromatography of thymopentin and plasma
(a) TP5; (b) blank plasma; (c) TP5 in plasma

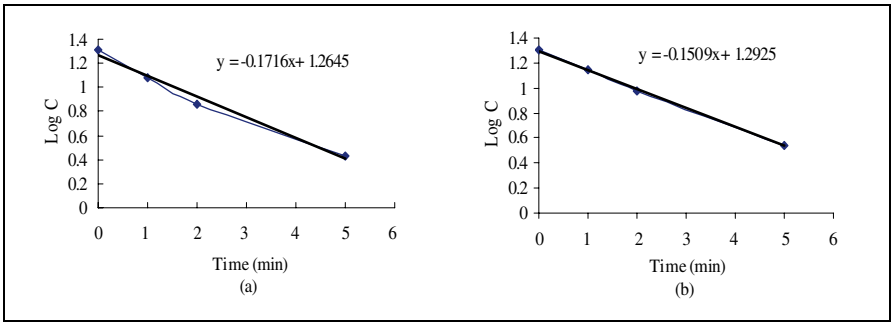

Fig. 5:
Half lives of TP5 and TP-ET in rat plasma
(a) TP5 (b)TP-ET

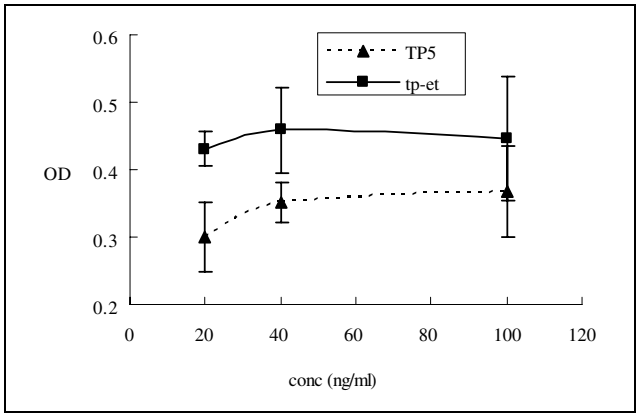

Fig. 6: Results of TP5 and TP-ET in lymphocyte proliferation test

centrations, TP-ET showed more potent lymphocyte proliferation activity than TP5. TP-5 and TP-ET were tested in three different concentrations (20, 40, or 100 ng/ml). TP-ET showed increased lymphocyte proliferation activity in three concentrations ($P < 0.05$).

2.4. SOD activity determination

Superoxide dismutase (SOD) activity in plasma was measured according to the SOD kit instruction. The SOD values in immune suppressed rats following subcutaneous injection of TP-ET at three different doses of 0.1 mg/kg, 0.5 mg/kg and 2.5 mg/kg and TP5 0.5 mg/kg for 14 consecutive days are shown in Fig. 7.

The normal group and the model group (immune suppressed rats) received saline solution subcutaneously. Immune suppressed rats in the TP5 control group received TP5 solution (0.5 mg/kg), those in the TP-ET high, medium and low dose groups received TP-ET solution (2.5 mg/kg, 0.5 mg/kg, 0.1 mg/kg). SOD values of the TP5 control group were higher than those of the model group ($P < 0.05$); SOD values of the TP5 control group were equivalent to those of the TP-ET group with low doses ($P > 0.05$), but lower than those in the TP-ET group with medium and high doses ($P < 0.05$).

The results showed that the SOD values of the immune suppressed rats were significantly lowered as compared with those of the normal control rats, indicating that the immunosuppression model was stably established. The SOD values of the immune suppressed rats after subcutaneous administration of TP-ET were significantly increased when compared with those of the immune suppressed model control group.

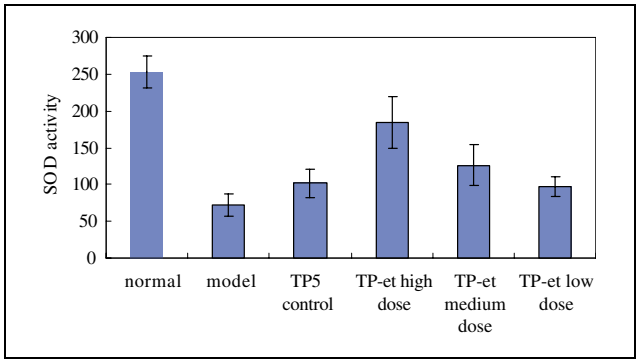

Fig. 7: Effect of TP-ET on SOD of immune suppressed rats induced by hydrocortisone

3. Discussion

To synthesize ethyl ester of thymopentin by thionyl chloride method, it was necessary to prepare absolute ethanol from ethanol (analytical reagent) by sodium. The reaction between thionyl chloride and absolute ethanol at 0 °C produced sulfite chloride, the latter esterified the peptide and a peptide ester was obtained.

The half-lives of peptides are often very limited under *in vivo* conditions, thereby not favoring their systemic administration as drugs (Loffet 2002). Therefore, the use of plasma as a partly predictive *in vitro* system helps to characterize the expected metabolization induced by amino- (Taylor 1993) and carboxypeptidases (Sanderson et al. 1996) or other enzymes present in the circulation (Rose et al. 2004; Yeo et al. 2004). Esterification might change the affinity of the peptide to aminopeptidases and carboxypeptidases, and thereby change the stability in plasma. So the half lives of thymopentin and its ethyl ester in rat plasma were determined by HPLC.In order to distinguish the plasma stability of the ester derivate from that of thymopentin, the plasma was diluted with an equivalent amount of saline solution (Anderson and Kim 1986).

Earlier studies had established that thymopentin was less potent than thymopoietin (Goldstein et al. 1979). Thymopoietin binds to the receptor glycoprotein by two regions corresponding to amino acids 32–36 (TP-5) and 38–45, the latter comprising mostly of hydrophobic amino acids Val-Glu-Leu-Tyr-Leu-Gln-Ser-Leu-Tyr (Haevner et al. 1985). So a deduction might be drawn that TP5 modified by a hydrophobic alkyl chain might be easier to bind to the thymopoietin receptor glycoprotein, and lead to an enhanced potency.

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical (O^{2-}) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2), and as such, provides an important defense against the toxicity of superoxide radical. The present study showed that the SOD activity was significantly increased, indicating that TP-ET was effective and the action was dependent on the doses.

The solubility and lipophilicity for TP-ET were distinguished from TP5. TP-ET showed more potent in lymphocyte proliferation and antioxidation action in rat immunosuppression model compared with TP5. The half life of TP-ET in rat plasma was a little longer than TP5. The potent activity of TP-ET might be attributed to its increased lipophilicity.

4. Experimental

4.1. Materials

Thymopentin (purity 99.89%) was supplied by Sinopeptide pharmaceutical company (Zhengjiang, China). Thionyl chloride ($SOCl_2$), anhydrous ethanol and 1, 10-phenanthroline were purchased form Beijing chemical reagent company, MTT and concanavalin A (Con A) were purchased from Sigma Co., HPLC-grade acetonitrile was obtained from Fisher Co., Trifluoroacetic acid (TFA) was purchased from Fluka. All other reagents used were of analytical grade. SOD kit was obtained from Nanjing Jiancheng bioengineering institute (Jiangsu, China). BAL B/c mice (20 ± 2 g) and Sprague–Dawley rats (180 ± 20 g) from the Experimental Animal Center of Beijing Institute of Pharmacology and Toxicology (BIPT) were used. Principles in good laboratory animal care were followed and all animal experiments complied with the requirements of the National Act on the use of experimental animals (People's Republic of China).

4.2. Synthesis and purification of thymopentin ethyl ester

Absolute ethanol: 2 g sodium and 100 ml ethanol (analytical grade) were placed in a dry 250 ml round bottomed flask fitted with a reflux condenser (protected from air with a drying tube filled with $CaCl_2$). After refluxing for 0.5 h, 4 g diethyl phthalate were added. Continued refluxing for 10 min and then distilled the ethanol.

2 ml $SOCl_2$ were dissolved in 20 ml of absolute ethanol. The mixture was stirred at 0 °C for 0.5 h. TP5 (200 mg) was added and stirring was continued for 4 h at 0 °C. The temperature was allowed to come back to room temperature spontaneously and stirring was continued until the TP-5 disappeared (Huang and Chen 1985). Thin-layer chromatography (TLC) was used to detect whether the reaction was completed. The solvent system used for TLC was the upper phase of a 1-butanol : acetic acid : water (4 : 1 : 5, by volume) mixture (Tischio et al. 1979). Ninhydrin TLC reagent spray was employed to visualize the peptides on the TLC plate. The synthetic pathway of thymopentin ethyl ester is shown in the Scheme.

Scheme

Arg-Lys-Asp-Val-Tyr + 2 EtOH
$\xrightarrow{SOCl_2}$ Arg-Lys-Asp(OCH_2CH_3)-Val-Tyr (OCH_2CH_3)

The reaction mixture was concentrated *in vacuo* to a solid. The crude product was reconstituted and purified using an Agela SS-1 Flash column chromatography system (Agela technologies, China). Solvent systems: S1: 0.05% aqueous TFA, S2: 70% acetonitrile + 0.05% TFA, linear gradient from 0–50% of S2 for 40 min, flow rate 5.0 ml/min, UV absorbance at 254 nm or 280 nm was used to monitor the elution. The elution was collected sequentially every one minute within a loop of 40 min. Analytical HPLC was conducted using a Waters Chromatograph (Waters Co., USA) with a C18 reversed phase column (5 μm, 4.6 mm × 250 mm, 100 Å, Agela technologies, China). Mobile phase was the same as the above. Acetonitrile and TFA in the useful fraction were removed under vacuum, followed by lyophilization. The dry product was reconstituted and analyzed once again by RP-HPLC with an Agela C18 reversed phase column (5 μm, 150 mm × 4.6 mm, 100 Å) and UV detection at 220 nm and 266 nm for the identity and purity determination. The mobile phase and the elution gradient were the same as the above. The ester was found to be > 98% pure and was further characterized using UV spectrum (GBC scientific instrument company,Australia), LC-ESI-MS (Agilent 1100 Quadrupole LC/MS mass spectrometer, USA). Moreover, the purified ethyl ester was then used for cellular and animal experiments and plasma half life determination.

4.3. Half life of ethyl ester in rat plasma

The samples for analysis were prepared in accordance with the methods reported previously (Amoscato et al. 1988). Eighteen 100 μl aliquots of plasma were added into vials and maintained at 37 °C for 30 min. Drug solution (100 μl, 200 μg/ml) was added to each vial and the contents were mixed and incubated at 37 °C for varying times. At time intervals of 0, 1, 2, 5, 10 and 20 min, aminopeptidase inhibitor 2% phenanthroline (20 μl) was added to each three vials. The vials were placed into an ice bath immediately. Acetonitrile 800 μl was added to each vial, vortexed for 30 s. The mixture was centrifuged at 15,000 × g for 10 min (Hermle Z160, Hermle Labortechnik, Germany), the supernant was collected and evaporated to dryness under nitrogen stream at 35 °C. The residue was reconstructed in 100 μl of methanol with 20 μl injected into the HPLC system.

HPLC assays were performed by Waters HPLC, Venusil MP C_{18} column (4.6 × 150 mm). Mobile phaseadopted as follows: methanol-phosphate buffer solution (pH 3.0, containing 0.2% triethylamine) 30: 70 for TP-ET (Fig. 3); methanol : phosphate buffer solution (pH 7.0) for TP-5 (Fig. 4). Injection volume 20 μl. Detection wavelength 220 nm,275 nm. Flow speed 1.0 ml/min.

4.4. Lymphocyte proliferation test

In vitro biological activity of TP-ET was performed by cell proliferation test (Heldt et al. 2006). Lymphocyte (5×10^6) of mouse spleen suspended in 0.1 ml of RPMI 1640 medium (Gibco Labs, Grand island, USA) with 10% heat inactivated fetal calf serum were set up in 96-wells cell culture cluster (Costar, USA). Three different doses (20, 40, or 100 ng/ml) of TP-ET and TP-5 were added to the microplates in 0.1 ml medium. For each dose of both drugs to be tested, six replicates were performed. The proliferation was performed with ConA (100 μg/ml) 10 μl. Cells were incubated for 48 h at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Six hours before the end of culturing, 20 μl MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) (5 mg/ml) were added to each well. Before the colorimetric assay, the microplates were centrifuged 15 min at 3000 revolutions per minute (Sigma 3K18). The formazan precipitates were solubilized by the addition of 200 μl of dimethyl sulfoxide (DMSO). After shaking 15 min at room temperature, absorbance was determined on a spectrophotometric microplate reader (Sunrise, Austria) at test wavelength of 570 nm and reference wavelength of 630 nm.

4.5. SOD activity assay

Male Sprague–Dawley rats (180 ± 20 g) from the Experimental Animal Center of Beijing Institute of Pharmacology and Toxicology (BIPT) were used for pharmacodynamic studies. All animal experiments complied with the requirements of the National Act on the use of experimental animals (People's Republic of China). 60 normal male Sprague–Dawley rats were randomly divided into 6 groups. At the first 7 days, rats except the normal group were intramuscularly primed with hydrocortisone at a dose of 500 mg/(kg · d), animals in the the normal group received 1 ml of saline solution (Xu et al. 2002; Huang et al. 2004). All the rats were fasted for 8 h before the experiments, but had free access to water. From the eighth day, the first group of immune suppressed rats was given saline solution subcutaneously as model control. The following formulations were administered to the groups from the second to the fifth subcutaneously for consecutive 14 days: (1) TP5 solution (0.5 mg/kg); (2) TP-ET solution (2.5 mg/kg); (3) TP-ET solution (0.5 mg/kg); (4) TP-ET solution (0.1 mg/kg). 6 h after the last administration, 100 μl of blood were collected via the orbit venous plexus into heparinized tubes and stored at 4 °C until analysis. For each assay, a volume of 15 μl plasma sample was used. All the processing and assay validation met the requirements of the kit instruction. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of SOD by 50% under the specified conditions.

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