# Effects of spacer homologous and heterologous combinations on enzyme immunoassay for paeonimetabolin I, a major metabolite of paeoniflorin

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## Abstract

In the course of developing enzyme immunoassay for paeonimetabolin I, a metabolite of paeoniflorin, we investigated the effects of spacer homologous and heterologous haptens on the sensitivity and specificity in the assay. Three paeonimetabolin-I derivatives possessing spacers of different length at C-8 were employed as haptens for the preparation of antisera and labeled antigens. These were 8-(carboxymethylthio) paeonimetabolin I (CMP), 8-(2′-carboxyethylthio) paeonimetabolin I (CEP) and 8-(5′-carboxypentylthio) paeonimetabolin I (CPP). The *N*-succinimide esters of the respective carboxythio derivatives were coupled with β-galactosidase (β-Gal) and bovine serum albumin (BSA) to give enzyme-labeled antigens and BSA conjugates (immunogens). Antisera **1CEP** and **2CPP** were prepared by immunization of rabbits with the CEP- and CPP-BSA conjugates. The sensitivity was higher in heterologous combinations using the labeled antigens linked with a shorter spacer arm than that used for antibody production. The cross reactivities of **2CPP** with the paeonimetabolin-I related compounds were higher than those of **1CEP**.

**Key words** enzyme immunoassay, paeoniflorin, paeonimetabolin I, paeony root.

**Abbreviations** BSA, bovine serum albumin ; CEP, 8-(2′-carboxyethylthio) paeonimetabolin I ; CMP, 8-(carboxymethylthio) paeonimetabolin I ; CPP, 8-(5′-carboxypentylthio) paeonimetabolin I ; HOSu, *N*-hydroxysuccinimide ; EDC, 1-Ethyl-3-(3-diethylaminopropyl) carbodiimide HCl ; EIA, enzyme immunoassay ; β-Gal, β-galactosidase ; PF, paeoniflorin ; PM-I, paeonimetabolin I ; NMR, nuclear magnetic resonance.

## Introduction

It is often found that the use of the same hapten for preparation of both immunogen and labeled antigen, *i.e.* homologous enzyme immunoassay (EIA), does not provide satisfactory sensitivity,[1,2] because the binding affinity of the labeled antigen (with a spacer) to the antibody is higher than that of the unlabeled antigen (without a spacer). The use of an immunogen consisting of a hapten with a longer spacer than that used for labeled antigen, was reported to be advantageous for increasing sensitivity of the EIA.[3-5] The longer spacers provide binding sites, large enough to accommodate both labeled and unlabeled antigens, on the antibody molecule. This heterology presumably reduces the difference in affinity of antibody to labeled and unlabeled antigens, giving the unlabeled antigen a better chance for competition.

In a previous paper, we reported an EIA method for determination of paeonimetabolin I (PM-I ; **2**), a major metabolite of paeoniflorin (PF ; **1**) by human

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intestinal bacteria.[6] The method was a spacer homologous combination, in which 8-(2'-carboxyethylthio)paeonimetabolin I (CEP ; **4**), a PM-I derivative possessing a carboxyethylthio group at C-8, was used as a hapten for preparing both immunogen and labeled antigen.

In this paper, we wish to report the synthesis of other haptens with shorter and longer spacers than the previous one (**4**) for preparing the immunogens and labeled antigens. Furthermore, we investigated the effects of spacer homologous and heterologous combinations between the labeled antigens and the antisera prepared, on the sensitivity and specificity of EIA in comparison with our previous findings.

## Materials and Methods

*Apparatus* : Ultraviolet spectra were measured with a Shimadzu UV-260 spectrophotometer. Fluorometry was performed on a Shimadzu RF-5000 spectrofluorometer (Kyoto, Japan). Purification of CPP (**5**) was performed by preparative HPLC, Gilson Model 201/202 (Medical Electronics, Inc., Villiers-Le-Bel, France). Proton and carbon-13 nuclear magnetic resonance ($^1$H-and $^{13}$C-NMR) spectra were measured with Varian Gemini 300 ($^1$H, 300 MHz ; $^{13}$C, 75.5 MHz) and Varian Unity plus 500 ($^1$H, 500 MHz ; $^{13}$C, 125 MHz) NMR spectrometers. Tetramethylsilane was used as an internal standard. $^1$H-$^1$H shift correlation spectroscopy (COSY), $^1$H-$^{13}$C COSY and heteronuclear multiple quantum coherence (HMQC) experiments were performed with the usual pulse sequence and data processing was obtained with the standard Varian software.

*Chromatography* : Thin-layer chromatography was performed on silica gel plates (0.25 mm thickness, Kieselgel $F_{254}$, E. Merck, Darmstadt, Germany) and spots were detected under UV light or by spraying with $H_2SO_4$-anisaldehyde reagent followed by heating. Column chromatography was performed on Diaion (ion exchange resin, Mitsubishi Chemical Corporation, Tokyo, Japan) and Lichroprep RP18 (40-63 $\mu$m ; E. Merck).

*Chemicals* : The goat antiserum to rabbit IgG was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and Sepharose 6B from Pharmacia Biotech (Uppsala, Sweden). BSA was purchased from Sigma Chemicals (St. Louis, USA). Complete Freund's adjuvant was a product of Difco Co. (Detroit, USA). Mercaptocarbonic acids, *N*-hydroxysuccinimide and 4-methylumbelliferyl galactoside were purchased from Wako Pure Chemical Industries Ltd. $\beta$-Galactosidase (EC 3.2.1.23) from *Escherichia coli* was obtained from Boehringer Co. (Mannheim, Germany). Paeoniflorin (**1**) was isolated from the dried roots of *Paeonia albiflora* Pall.[7,8] (*7R*)-and (*7S*)-PM-I (**2**) were prepared as reported previously[9] with some modifications. Buffer I was 20 mM phosphate buffered saline (pH 7.3) containing 0.1 % BSA, 0.1 % $NaN_3$ and 0.001 % $MgCl_2$, and buffer II was 20 mM phosphate buffered saline (pH 7.3) containing 0.1 % $NaN_3$ and 0.001 % $MgCl_2$.

*Bacterium* : *Lactobacillus brevis* was provided by Professor Tomotari Mitsuoka (University of Tokyo, Japan).

*Preparation of 8-(carboxymethylthio)paeonimetabolin I (CMP ; 3)* : Compound **3** was prepared as reported previously,[6] using paeoniflorin (**1**, 1.2 g, 2.5 mmol) and mercaptoacetic acid (500 mg, 5.4 mmol), to give a colorless oily residue of 92 mg. $^1$H-NMR (500 MHz, $CD_3OD$) $\delta_H$ : 5.33 [1H, s, H-9 (*7S*)] and 5.39 [1H, s, H-9 (*7R*)] in a 2 : 1 ratio. $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta$c : 21.7 [q, C-10 (*7S*)], 21.8 [q, C-10 (*7R*)], 31.8 [t, C-5 (*7S*)], 32.1[t, C-5 (*7R*)], 32.6 [t, C-8 (*7S, 7R*)], 37.1 [t, S-$CH_2$ (*7S*)], 37.4 [t, S-$CH_2$ (*7R*)], 43.9 [d, C-7 (*7S*)], 44.6 [d, C-7 (*7R*)], 48.1 [t, C-2 (*7S*)], 48.2 [t, C-2 (*7R*)], 49.1 [d, C-4 (*7S*)], 49.4 [d, C-4 (*7R*)], 79.3 [s, C-1 (*7S*)], 79.9 [s, C-1 (*7R*)], 102.06 [d, C-9 (*7S*)], 102.1 [d, C-9 (*7R*)], 102.6 [s, C-6 (*7S*)], 102.9 [s, C-6 (*7R*)], 178.6 [s, COOH (*7S*)], 178.7 [s, COOH (*7R*)], 212.9 [s, C-3 (*7R*)], 214.2 [s, C-3 (*7S*)].

*Preparation of 8-(2'-carboxyethylthio)paeonimetabolin I (CEP ; 4)* : Compound **4** was prepared as reported previously,[6] to give an oily residue (50 mg), which was a mixture of the (*7R*)-and (*7S*)-isomers. The ratio was approximately 1 : 1 on the basis of the $^1$H-NMR spectral data.

*Preparation of 8-(5'-carboxypentylthio)paeonimetabolin I (CPP ; 5)* : 6-Mercaptohexanoic acid was prepared according to the literature,[10] and the structure was confirmed by $^1$H- and $^{13}$C-NMR spectra. 6-

Mercaptohexanoic acid, thus obtained, (700 mg, 4.7 mmol) and paeoniflorin (**1**, 1 g) were incubated anaerobically with a bacterial suspension (50 ml) of *Lactobacillus brevis* in 0.1 M phosphate buffer (pH 7.3) at 37°C for 4 hours. Chromatography of the metabolites on a Diaion (1 g) column gave an oily residue (1.31 g), which was applied to a column of silica gel (100 g, 26×3.2 cm) using benzene-acetone (8 : 2) as an eluting solvent. The eluate (900 mg) was further applied to a reversed phase column (RP18, 8 g, 15×0.5 cm) and the column was eluted with $H_2O$-acetonitrile (7 : 3). Fractions were collected to give a mixture (150 mg). HPLC was performed on an octadecyl silica column (ODS-AP-323 : YMC Co. Ltd., Kyoto, Japan) for purification of the mixture under the following conditions : mobile phase, 0.1 % TFA and $H_2O$-$CH_3CN$ (10 %-70 %) with a linear gradient elution ; flow rate, 2.5 ml/min ; pressure, 66 bar ; UV detection, 230 nm. A colorless oily residue (50 mg) was obtained. The compound was a mixture of (*7R*)- and (*7S*)-8-(5'-carboxypentylthio) paeonimetabolin I (PCP ; **5**) : $^1$H-NMR (500 MHz, $CD_3OD$) $\delta_H$ : 5.33 [1H, s, H-9 (*7S*)] and 5.38 [1H, s, H-9 (*7R*)] in a 2 : 1 ratio. $^{13}$C-NMR (125 MHz, $CD_3OD$) $\delta$c : 21.7 [q, C-10 (*7S*)], 21.8 [q, C-10 (*7R*)], 25.5 [t, C-3'(*7R, 7S*)], 29.1 [t, C-4'(*7S*)], 29.16 [t, C-4' (*7R*)], 30.1 [t, C-2'(*7S*)], 30.2 [t, C-2'(*7R*)], 31.5 [t, C-5 (*7R*)], 31.8 [t, C-5 (*7S*)], 32.0 [t, C-8 (*7S, 7R*)], 32.6 [t, $\underline{C}H_2$-COO (*7S*)], 33.1 [t, $\underline{C}H_2$-COO (*7R*)], 34.6 [t, S-$CH_2$ (*7S, 7R*)], 44.4 [d, C-7 (*7S*)], 45.1 [d, C-7 (*7R*)], 48.1 [t, C-2 (*7R*)], 48.9 [t, C-2 (*7S*)], 49.2 [d, C-4 (*7S*)], 49.7 [d, C-4 (*7R*)], 79.2 [s, C-1 (*7S*)], 79.3 [s, C-1(*7R*)], 101.9 [d, C-9(*7S*)], 102.6 [d, C-9(*7R*)], 102.5 [s, C-6 (*7S*)], 102.9 [s, C-6 (*7R*)], 175.8 [s, COOH (*7S, 7R*)], 212.7 [s, C-3 (*7R*)], 213.8 [s, C-3 (*7S*)].

*Preparation of N-hydroxysuccinimide esters of CMP, CEP and CPP (6, 7 and 8)* : According to the previously reported method,[6] a solution of CMP (**3**, 30 mg, 0.1 mmol) and *N*-hydroxysuccinimide (28.8 mg, 0.2 mmol) in DMF (0.5 ml) was stirred for 30 min in an ice bath. 1-Ethyl-3-(3-diethylaminopropyl) carbodiimide HCl (48 mg, 0.2 mmol) was then added and the reaction mixture was stirred for 24 hours at room temperature. The mixture was poured into cold water saturated with NaCl (15 ml), then extracted with $CHCl_3$ (40 ml×3) . The $CHCl_3$ solution was washed with water saturated with NaCl, dried over $MgSO_4$ and evaporated *in vacuo* to give an oily residue (**6**, 72 mg), then stored at 4°C. Compounds CEP (**4**) and CPP (**5**) were worked up as described above with two equimolar amounts of *N*-hydroxysuccinimide to give chromatographycally homogeneous CEP ester (**7**, 200 mg) and CPP ester (**8**, 54 mg), respectively.

*(7R)-and (7S)-CMP ester mixture (6)* : $^1$H-NMR (500 MHz, $CD_3OD$) $\delta$H : 5.33 [1H, s, H-9 (*7S*)] and 5.35 [1H, s, H-9 (*7R*)] in a ratio of 1 : 1. $^{13}$C-NMR (125 MHz, $CD_3OD$) of the succinimidyl residue, $\delta$c : 26.5 [t, $CH_2$×2 (*7R, 7S*)] and 171.5 [q, CO-×2 (*7S*)], 171.7 [q, CO-×2 (*7R*)].

*(7R)-and (7S)-CEP ester mixture (7)* : The compound was identified by comparing the $^1$H-and $^{13}$C-NMR spectral data with those in the literature.[6]

*(7R)-and (7S)-CPP ester mixture (8)* : $^1$H-NMR (500 MHz, $CD_3OD$) $\delta_H$ : 5.33 [1H, s, H-9 (*7S*)] and 5.38 [1H, s, H-9 (*7R*)] in a 2 : 1 ratio. $^{13}$C-NMR (125 MHz, $CD_3OD$) of the succinimidyl residue, $\delta$c : 26.4 [t, $CH_2$×2 (*7S, 7R*)], 171.7 [q, CO-×2 (*7S*)], 171.8 [q, CO-×2 (*7R*)].

*Preparation of enzyme-labeled antigens* : CMP ester (**6**, 39 $\mu$g in 100 $\mu$l DMF, 97 nmol) was added to a solution of $\beta$-galactosidase (5 mg, 9.7 nmol) in 50 mM phosphate buffer (pH 7.3, 1 ml). The mixture was stirred at 4°C overnight, and chromatographed on a Sepharose-6B column (50×1.5 cm) using 200 ml of 4 mM phosphate buffer (pH 7.3) containing 20 mM NaCl, 0.2 mM $MgCl_2$ and 3 mM $NaN_3$ as an eluent. Fractions indicating $\beta$-galactosidase activity, using 0.1 mM 4-methylumbelliferyl galactoside as substrate, were collected and stored at 4°C until use. The fractions were used as a CMP-labeled antigen (**9**). This was checked by SDS-PAGE (12 % gel), using $\beta$-galactosidase (mw, 517,000) as a reference protein and Coomassie Brilliant blue for staining.

Similarly, CEP ester (**7**) and CPP ester (**8**) were coupled with $\beta$-galactosidase with a hapten/enzyme molar ratio of 10 : 1 to give CEP- and CPP-labeled antigens (**10** and **11**), respectively.

*Preparation of a CEP-BSA conjugate (12)* : The conjugate was prepared as described in our previous paper.[6]

*Preparation of a CPP-BSA conjugate (13)* : A

solution of BSA (33 mg, 0.5 $\mu$mol) in 50 mM phosphate buffer (pH 7.3, 2 ml) was added to a solution of CPP ester (**8**, 9 mg, 22 $\mu$mol) in pyridine (1 ml) and stirred at 4°C for 24 hours. The mixture was successively dialyzed at the same temperature for 5 days against 10, 5, 2 % pyridine-$H_2O$ and $H_2O$ in this order. The undialyzable materials were then lyophilized to give a CPP-BSA conjugate (**13**). This was checked by SDS-PAGE (12 % gel), using BSA as a reference and Coomassie Brilliant blue for staining.

*Determination of the number of CPP residues linked to a BSA molecule* : The protein content of the conjugate and the number of compound **5** residues per BSA molecule were determined according to the method of Inoue.[11]

*Preparation of antiserum 1CEP* : Procedures were followed up as described in our previous paper.[6]

*Preparation of antiserum 2CPP* : A purified CPP-BSA conjugate (**13**, 3 mg) was dissolved in sterile saline (1.5 ml) and emulsified with complete Freund's adjuvant (1.5 ml). The emulsion was injected into three domestic albino female rabbits subcutaneously at multiple sites on the back and legs. Five boosters (2 mg each) of the immunogen, dissolved in sterile saline (2.5 ml) and emulsified with 2.5 ml of incomplete Freund's adjuvant (2.5 ml), were carried out an interval of once every two weeks for 2 months and monthly after that. The blood was occasionally collected from the ear vein. The sera were obtained by centrifugation at 3000×g for 15 min, after standing for 2 hours at room temperature, then stored at -20°C until use. The titer of sera was measured using the immunoassay procedures described below. Two rabbits were shown to have the highest titer sera after 6 months of the first immunization.

*Antiserum dilution curve* : Mixtures of the appropriately diluted labeled antigen (**9**, **10** or **11** in 50 $\mu$l of buffer II) and the various dilutions of anti-PM-I antisera (**1CEP** and **2CPP** in 100 $\mu$l of buffer I) were incubated at room temperature for 2 hours. Ten-fold diluted solution of goat antiserum to rabbit IgG (50 $\mu$l) and 100-fold diluted normal rabbit serum (20 $\mu$l) were added to the reaction mixture. The mixture was allowed to stand overnight at 4°C. The immunoprecipitate formed was washed twice with buffer I (1 ml, each), then centrifuged at 3000×g for 20 min. After decantation of the supernatant, the immunoprecipitate was used for measuring the enzyme activity. The procedures were performed to estimate the % binding ($B_0$/T) of the labeled antigen with antibodies in the various dilutions of antisera, in which $B_0$ and T represent the bound enzyme activity and the total enzyme activity, respectively.

*Assay procedure* : A sample (100 $\mu$l in buffer I, plasma or serum) was added to a mixture of the 10000-fold diluted anti-PM-I antiserum (**1CEP** or **2CPP** ; 100 $\mu$l in buffer I) and labeled antigen (**9**, **10** or **11** ; 50 $\mu$l in buffer II). The mixture was incubated at room temperature for 2 hours and 10-fold diluted solution of goat antiserum to rabbit IgG (50 $\mu$l) and 100-fold diluted normal rabbit serum (20 $\mu$l) were added to the reaction mixture. The mixture was then allowed to stand overnight at 4°C. After addition of buffer I (1 ml), the resulting mixture was treated by the same procedure as described above to measure the enzymatic activity of the immunoprecipitate. The procedures were performed to estimate the % binding (B/Bo) of the labeled antigen with antibodies in the presence of various amounts of an unlabeled antigen, PM-I (**2**). B and Bo represent the bound enzyme activities in the presence and the absence of an unlabeled antigen, respectively.

*Measurement of β-D-galactosidase activity* : The immunoprecipitate was incubated with 150 $\mu$l of 0.1 mM 4-methylumbelliferyl galactoside at 30°C for 30 min. The reaction was stopped by the addition of 3 ml of 100 mM glycine-NaOH buffer (pH 10.3) and 7-hydroxy-4-methylumbelliferone released was measured spectrofluorometrically at wavelengths of 365 nm for excitation and 448 nm for emission.

*Precision of the assay* : Variations in the intra- and inter-day assays were calculated using test samples of PM-I (**2**) at high, moderate and low concentrations.

*Specificity of the assay* : The cross-reactions among PM-I [**2** ; as a mixture of (*7R*)- and (*7S*)-isomers] and related compounds were examined using antisera **1CEP** and **2CPP** with the CMP-labeled antigen (**9**) under the assay conditions described above.

## Results and Discussion

In our previous paper,[6] an enzyme immunoassay of paeonimetabolin I (PM-I ; **2**), a major metabolite of paeoniflorin (PF ; **1**) from *Paeonia albiflora* roots, was reported, where 8-(2'-carboxyethylthio) paeonimetabolin I (CEP ; **4**) was adopted as a hapten for preparing both bovine serum albumin conjugate (BSA-conjugate, immunogen) and $\beta$-galactosidase conjugate (labeled antigen). Although the method was sensitive and specific for PM-I (**2**), the sensitivity is anticipated to increase more when the spacer attached to the labeled antigen and the immunogen differs slightly (*i. e.* heterologous EIA).[3,5]

In the present study, a modification of the previous method was performed to investigate the influence of such spacers on the sensitivity and specificity of EIA and to establish a more sensitive and reproducible method capable of measuring PM-I (**2**) levels in the biological fluids after administration of PF (**1**). PF (**1**) has been reported to have some pharmacological actions,[12,13] especially its significant effect on attenuating-aging induced learning deficits and spatial work memory deficits produced by scopolamine.[14,15]

The study was accomplished by preparing the carboxymethylthio, carboxyethylthio and carboxypentylthio PM-I derivatives, as haptens possessing short, medium and long spacers to prepare the BSA-conjugate and labeled antigen. Three labeled antigens (**9**-**11**) were prepared and two types of antisera **1CEP** and **2CPP** were obtained by immunization of rabbits with the BSA-conjugates linked with the medium and long spacers, respectively. The immunoassay was performed with homologous and heterologous combinations and the effects of spacers in combination were investigated on sensitivity, specificity and precision in EIA.

Fig. 1 shows the synthetic scheme of PM-I derivatives (**3**, **4** and **5**) with the different spacers at C-8 position by anaerobic incubation of PF (**1**) with a suspension of *Lactobacillus brevis* in the presence of mercaptoacetic acid, 3-mercaptopropionic acid and 6-mercaptohexanoic acid, respectively. Compounds **3**, **4** and **5** thus prepared were repeatedly purified by column chromatography to give the respective colorless oils as a mixture of (*7S*)- and (*7R*)-isomers, in yields of 29 %, 15 % and 13.5 %, respectively.

The $^1$H-NMR spectrum of compound **4** was identical to that reported previously.[9] In addition, compounds **3** and **5** exhibited signals quite similar to those in compound **4** except for signals due to carboxymethylthio and carboxypentylthio groups introduced at C-8, respectively. The spectra exhibited a broad singlet signal of an -S-$CH_2$- group at $\delta_H$ 3.16 in compound **3**

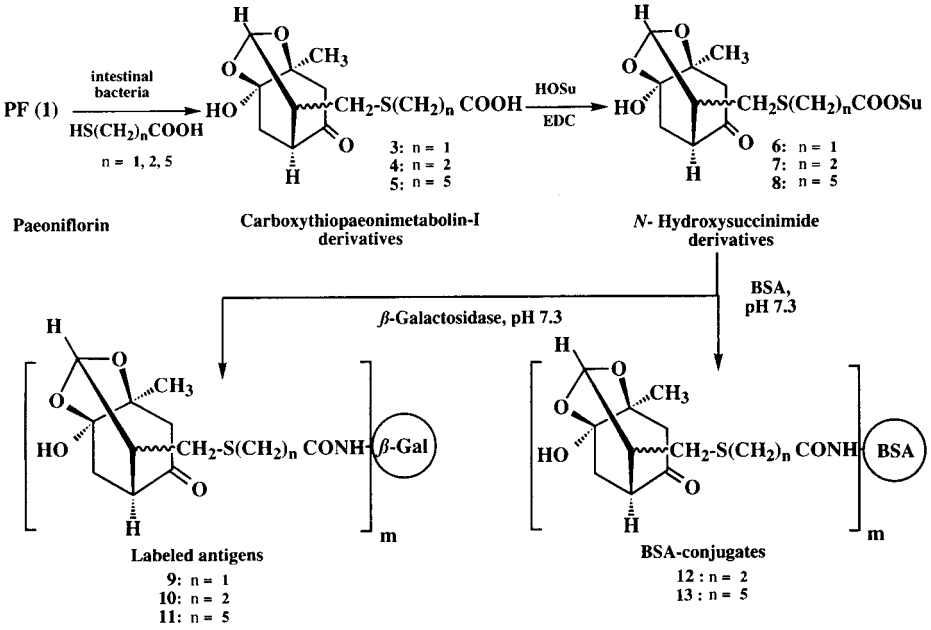



Fig. 1 Synthetic scheme of BSA-conjugates and labeled antigens

and multiple signals of a $-(CH_2)_3-$ group at $\delta_H$ 1.40-1.65 in compound **5**. The $^{13}C$-NMR spectrum of compound **4** was identical to that reported previously.[9] The relative ratios of (*7R*)- and (*7S*)-isomers in compounds **3** and **5** were determined by their signal intensities in the $^1H$- and $^{13}C$-NMR spectra (see Materials and Methods). The structures of compounds **3** and **5** were further verified by measuring their HMBC and HMQC spectra. By treating with *N*-hydroxysuccinimide (HOSu) and 1-ethyl-3-(3-diethylaminopropyl) carbodiimide HCl (EDC), compounds **3**, **4** and **5** were converted to their succinimidyl esters **6**, **7** and 8, respectively. The $^1H$-NMR spectra of compounds **6**, **7** and 8 exhibited signals of the two methylene protons adjacent to the carbonyl of a succinimidyl group as a singlet at $\delta_H$ 2.86 (4H) and the $^{13}C$-NMR spectra of these compounds exhibited characteristic carbonyl signals due to the presence of the succinimidyl group (see Materials and Methods).

Compounds **6**, **7** and 8 were coupled with $\beta$-galactosidase ($\beta$-Gal) and BSA in phosphate buffer (pH 7.3) to give labeled antigens (**9-11**) and BSA conjugates (**12** and **13**). Compounds **9-11** were used for EIA after purification on a Sepharose 6B column, while the BSA coujugates (**12** and **13**) were used for immunization after dialysis. About 8 molecules of compound **5** were incorporated into a BSA molecule in CPP-BSA (**13**), as calculated by Inoue's method.[11]

CEP-BSA (**12**) and CPP-BSA (**13**) conjugates were immunized in rabbits as described in Materials and Methods to give antisera **1CEP** and **2CPP**, respectively. EIA was carried out with homologous and heterologous combinations of labeled antigens to antisera. The EIA reaction was performed using goat anti-rabbit antiserum (IgG), as the second antibody, and the bound enzymatic activity in the immunoprecipitate was determined fluorometrically with 4-methylumbelliferyl galactoside as a substrate.

Fig. 2 shows the dilution curves of antisera where the percent binding ($B_0/T$) of three types of labeled antigens (**9-11**) was plotted against various dilutions of antisera **1CEP** and **2CPP** on semilogarith-

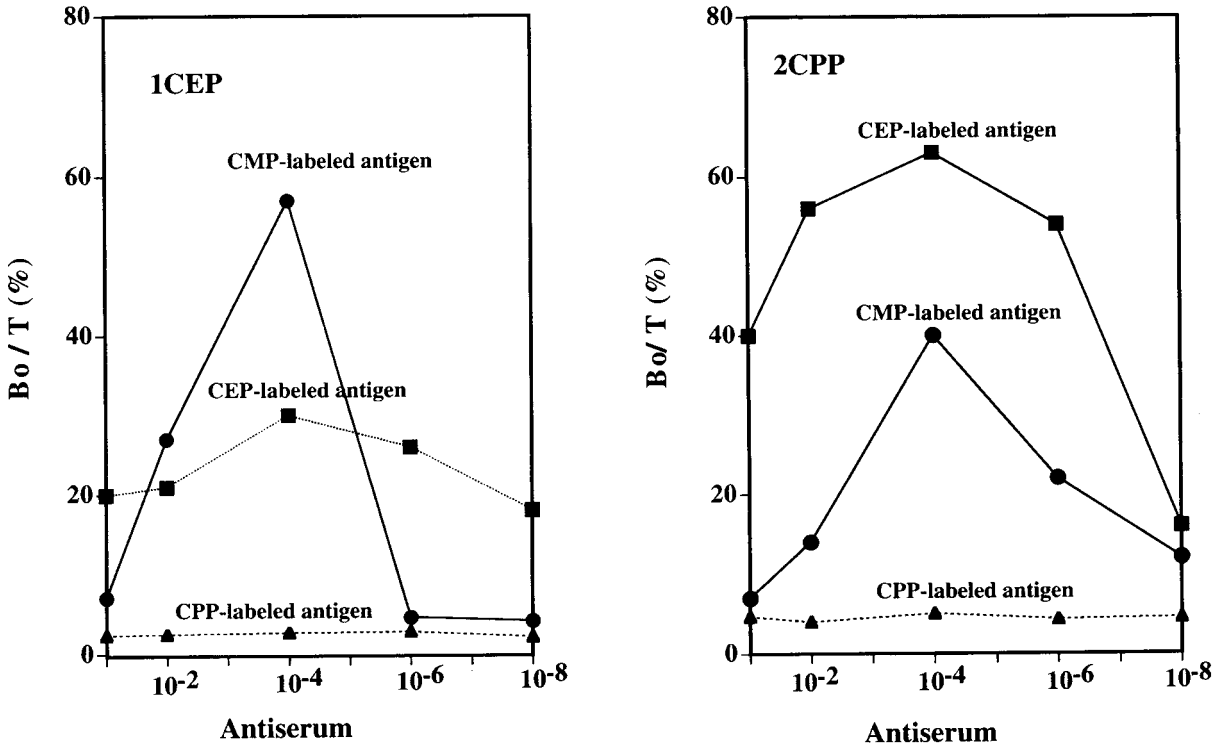



Fig. 2 Binding curves of antisera **1CEP** and **2CPP** using CMP-labeled (●), CEP-labeled (■) and CPP-labeled (▲) antigens
$B_0/T$ represents the percent of bound enzyme activity ($B_0$) of the CMP-, CEP- or CPP-labeled antigen with antiserum **1CEP** or **2CPP** to the total enzyme activity (T) of each labeled antigen

mic scale. For antiserum **1CEP**, a $10^4$-fold dilution of it showed relatively high binding to the CMP-labeled antigen (**9**) (58 % in maximum) but less to the CEP-labeled antigen (**10**) (30 %) and the CPP-labeled antigen (**11**) (less than 5 %). For antiserum **2CPP**, a $10^4$-fold dilution of it resulted in high binding to both CMP- and CEP-labeled antigens (**9** and **10**) (40 % and 60 %, respectively) but not to the CPP-labeled antigen (**11**). These results indicated that the highest affinity of antisera **1CEP** and **2CPP** was observed for the CMP- and CEP-labeled antigens (**9** and **10**), respectively.

Typical atandard curves for PM-I EIA were obtained with antisera **1CEP** and **2CPP** using the CMP-labeled antigen (**9**) (Fig. 3). The curves were plotted as linearized logit-log against various amounts of unlabeled PM-I (**2**) [approximately 1 : 1 mixture of (*7R*)- and (*7S*)-isomers] in a range of 0.5 ng-500 ng/tube and the ratio of bound enzyme activity at the midpoint showed 30 ng/tube for **1CEP** and 10 ng for **2CPP**, respectively.

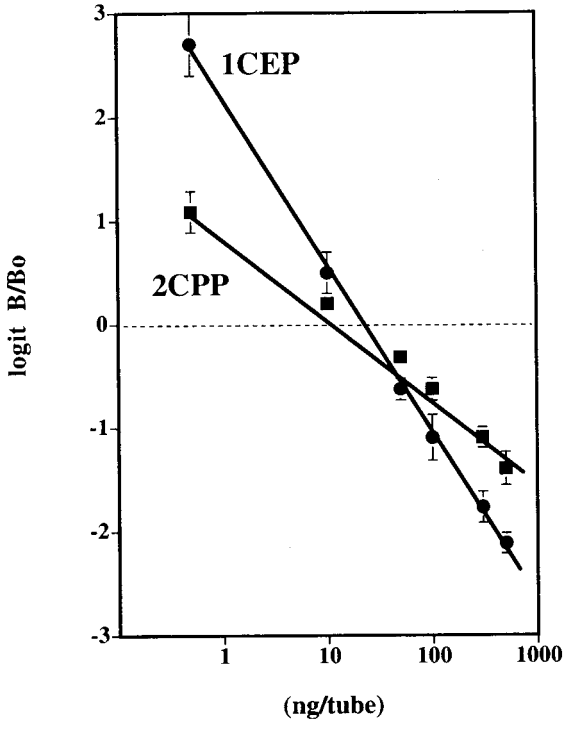



Fig. 3 Standard curves for PM-I, using antisera **1CEP** (●) and **2CPP** (■) with CMP-labeled antigen
$B/B_0$ represents the percent of bound enzyme activity, where B and $B_0$ are the bound enzyme activities in the presence and the absence of unlabeled antigen (PM-I). Each point represents the mean±S.D. (n=5)

Table I Concentrations of PM-I (ng/tube) at 50 % inhibition of the binding of the enzyme-labeled antigen

| Antisera | Labeled antigens | | |
|---|---|---|---|
| | CMP | CEP | CPP |
| **1CEP** | 30 | >100 | >1000 |
| **2CPP** | 10 | 100 | >1000 |

To examine the effects of spacer homologous and heterologous combinations on sensitivity in EIA, concentrations of PM-I (**2**) at the logit $B/B_0$=0, that reduce the binding of the labeled antigen to antibody by 50 %, were compared using antisera **1CEP** and **2CPP**. Three heterologous combinations, using the labeled antigens linked with a shorter spacer than that used for antiserum production, showed higher sensitivity (30, 10 and 100 ng PM-I/tube) than the homologous combinations [**1CEP** with the CEP-labeled antigen (**10**) and **2CPP** with the CPP-labeled antigen (**11**)] (Table I). On the other hand, the reverse heterologous combination [**1CEP** with the CPP-labeled antigen (**11**)] were insensitive.

The fact that sensitivity in the three heterologous combinations was higher than in the homologous ones may be due to the mismatch of the structure between the BSA conjugate and the labeled antigen. Providing that a relatively longer spacer is used for the preparation of BSA conjugate (immunogen), larger binding sites may be given on antibodies, leading to better competition between the labeled antigen and the unlabeled antigen, PM-I (**2**). The unexpected insensitive results obtained with the homologous combination such as **2CPP** with the CPP-labeled antigen (**11**), could be explained by the steric interaction between the antibody binding site and the CPP-labeled antigen (**11**). In the reverse heterologous combination, the size of the antibody binding sites formed with the CEP-BSA conjugate (**12**) may be inadequate to accommodate the CPP-labeled antigen (**11**) and the unlabeled antigen, PM-I (**2**), leading to insensitive results.[2,3]

For examination of the precision of assay, the intra- and inter-day assays[17] were carried out for the constructed standard curves. The coefficients of variance at concentrations of 500, 50 and 0.5 ng PM-I/tube were 8.1-14.1 % for intra-day assay and 6.5-11.7 % for inter-day assay.

Fig. 4 shows the standard curve of EIA in the presence of plasma, where a slight change was noted compared to that obtained in the absence of it, possibly due to the interference of some components in the plasma. In the presence of plasma, the coefficients of variance at 500, 50 and 0.5 ng PM-I/tube were 3.6-13 % for intra-day assay and 7.5-15 % for inter-day assay, respectively.

Table II shows the cross reactivities of two antisera **1CEP** and **2CPP** using the CMP - labeled antigen (**9**). The cross reactivities of antiserum **1CEP** against various PM-I related compounds were similar to those reported previously.[6] On the other hand, a little cross reactivity of antiserum **2CPP** was found with some of PM-I-related compounds, such as benzoylpaeoniflorin (**15**), oxypaeoniflorin (**16**) and paeoniflorigenone (**14**) (1 %, 0.7 % and 0.5 %, respectively) (Fig. 5), suggesting the role of less specific antibodies in the heterologous EIA.[18, 19]

From the above findings, we concluded that spacer heterology between the BSA-conjugate and the labeled antigen had considerable effects on EIA sensitivity and specificity. In this paper, we obtained higher sensitivity in heterologous combinations than in homologous ones using the newly prepared antisera and the labeled antigens. Comparing with the results in our previous paper,[6] the sensitivity was similar, but the higher precision and the more reproducible results were obtained both in buffer and plasma samples using the heterologous combination (Fig. 4), that will enable us to apply this method to determine concentrations of PM-I (**2**) in the plasma and serum after administration of PF (**1**) to animals or humans.

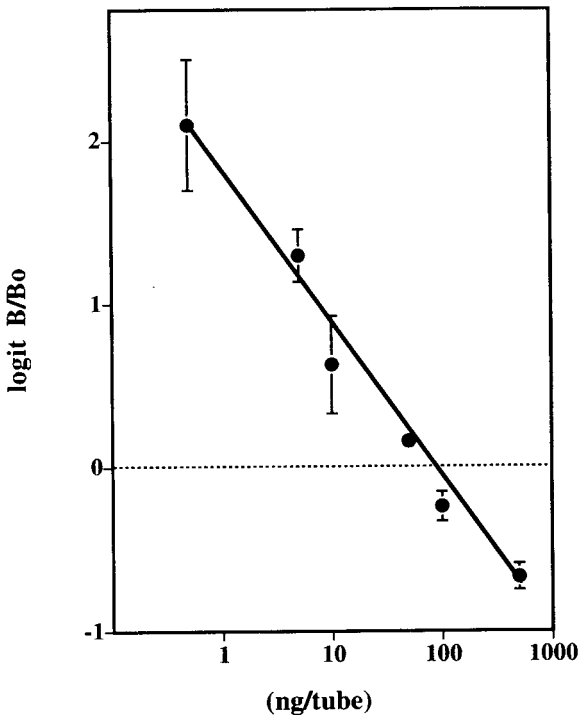
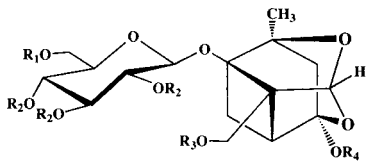



Fig. 4 Standard curves for PM-I in the presence of 95 % rat plasma using antiserum **1CEP** (●) with a CMP-labeled antigen
$B/B_0$ is the percent of bound enzyme activity. Each point represents the mean±S.D. (n=4)

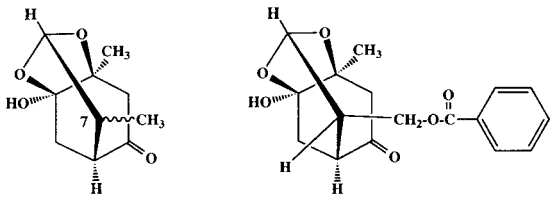
Table II Specificity of antisera

| Compound | Antisera Cross reaction %[a] | |
|---|---|---|
| | **1CEP** | **2CPP** |
| (7*R*)-, (7*S*)-PM-I (**2**) | 100 | 100 |
| (7*S*)-PM-I | 120 | 120 |
| (7*R*)-PM-I | 110 | 90 |
| Paeoniflorin (**1**) | 0.02 | 0.3 |
| Paeoniflorigenon (**14**) | 0.03 | 0.5 |
| Benzoylpaeoniflorin (**15**) | 0.01 | 1.0 |
| Oxypaeoniflorin (**16**) | 0.02 | 0.7 |
| Pentaacetylpaeoniflorin (**17**) | 0.02 | 0.02 |

a: the ratio of the amount of PM-I to that of the above compound at 50 % inhibition of the binding of the enzyme-labeled antigen.



**1** : $R_1$= $R_2$= $R_4$= H, $R_3$= Bz
**15** : $R_1$= $R_3$= Bz, $R_2$= $R_4$= H
**16** : $R_1$= $R_2$= $R_4$ =H, $R_3$= *p*-HO-Phe
**17** : $R_1$= $R_2$= Ac, $R_3$ = Bz, $R_4$= Ac



**2** **14**

Fig. 5 Structures of PF (**1**), PM-I (**2**) and related compounds

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## 和文抄録

Paeoniflorinの代謝物であるpaeonimetabolin Iの酵素免疫測定法(EIA)の開発研究において，各種スペーサーの異なるハプテンを合成し，それらの組み合わせによるEIA感度や特異性を検討した。ハプテンとしてはpaeonimetabolin IのC-8に長さの異なるスペーサーを連結した化合物を腸内細菌による反応を利用して合成し，免疫原や標識抗原の作製に用いた。すなわち，8-(carboxymethylthio) paeonimetabolin I (CMP), 8-(2′-carboxyethylthio) paeonimetabolin I (CEP), 8-(5′-carboxypenthylthio) paeonimetabolin I (CPP)をそれぞれ*N*-succinimideエステルに誘導し，$\beta$-galactosidase, ウシ血清アルブミン (BSA) と結合させ，標識抗原，免疫原とした。抗血清**1CEP, 2CPP**はCEP-BSA, CPP-BSA抱合体（免疫原）をウサギに投与して作製した。免疫原（BSA抱合体）に用いたものより短いスペーサーを持つ標識抗原（$\beta$-Gal抱合体）を利用した異種の組み合わせにおいて測定感度が高いこと，抗血清**2CPP**は**1CEP**よりpaeonimetabolin I関連化合物との交叉性がやや高いことがわかった。

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