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Spermined dextran, a cationized polymer, as absorption enhancer for pulmonary application of peptide drugs

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Received October 11, 2007, accepted November 1, 2007

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Pharmazie 63: 180–184 (2008) *doi: 10.1691/ph.2008.7323*

Sperminated dextrans (SD) having different average molecular weights (MWs; 10, 40 and 70 kDa) and numbers of amino groups were prepared as cationized polymers for use as absorption enhancers. The absorption enhancing effects on the pulmonary absorption of insulin in rats and the permeation of FITC-dextran (MW 4,400, FD4) through calu-3 cell(human airway epithelial cell)monolayers by SD were evaluated. SD significantly enhanced the pulmonary absorption of insulin SD and the permeation of FD4 through calu-3 cells. The enhancing effects on the absorption insulin and permeation of FD4 through calu-3 cells increased with an increase in the molecular weigh of SD over the range 10–70 kDa. SD may interact directly with the luminal surface of mucus membranes via an ion-ion interaction and then induce signals that open tight junctions resulting in intercellular permeation of water soluble drugs. SD may be useful as an absorption enhancer for pulmonary delivery of peptide and protein drugs.

1. Introduction

Pulmonary administration of peptide and protein drugs is a useful way to avoid the problems associated with parenteral formulations, such as tissue invasion, and also to improve patient compliance, since the pulmonary alveoli have a large surface area and a rich blood flow (Patton 1996; Gonda 2006). The use of absorption enhancers and proteolytic enzyme inhibitors and suitably designed formulations are all useful approaches to increase the bioavailability of peptide and protein drugs in novel pulmonary delivery systems (Wearley 1991; Sakagami and Byron 2005). The absorption enhancers, which increase the permeability of drugs through the epithelial membranes without causing any tissue damage, are especially useful for the delivery of peptide and protein drugs (Davis and Illum 2003; Thanou et al. 2001). Surfactants, bile salts and fatty acids have been evaluated as absorption enhancers and, although most of them exhibit permeation-enhancing effects, they also produce membrane damage (Marttin et al. 1995; Merkus et al. 2003). Therefore, safe absorption enhancers are needed as suitable pulmonary delivery systems for peptide and protein drugs.

It has recently been reported that cationic polymers, including chitosan and its derivatives, poly-L-arginine and aminated gelatins are able to improve the absorption of peptide and protein drugs through mucosal membranes while causing negligible damage to these membranes (Davis and Illum 2003; Thanou et al. 2001; Natsume et al. 1999; Wang et al. 2002). Since the cationic polymers can interact with the luminal surface of mucus membranes directly by an ion-ion interaction and thereby increase the intercellular permeation of water soluble drugs, the charge density of the polymers should influence their effects. In our previous reports, ethylenediaminated gelatins and sperminated gelatins (SG) having different numbers of amino groups were prepared and the absorption-enhancing effects on the nasal absorption of insulin and 5(6)-carboxyfluorescein (5-CF) were examined in rats (Seki et al. 2007). The results obtained suggest that the effects depend on the amino group content of the sperminated polymers.

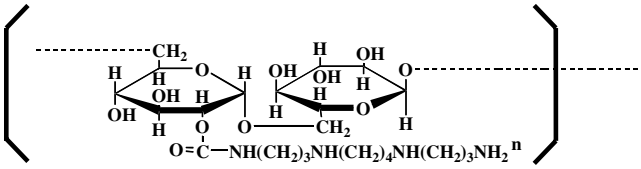
In this study, sperminated dextrans (SD) having different average molecular weights (MWs; 10, 40 and 70 kDa) and numbers of amino groups were prepared and their enhancing effects on the pulmonary absorption of insulin in rats were compared with that of SD. In addition, the permeation characteristics of FITC-dextran (MW 4400, FD-4) through calu-3 cell(human airway epithelial cell)monolayer were examined with reference to the enhancing mechanism of SD.

2. Investigations and results

2.1. Structure and amino group content of the sperminated dextrans

The Table shows the primary and total amino group contents (mmol/g polymer) of sperminated dextrans (SDs)

with different average molecular weights (MWs; 10, 40 and 70 kDa) prepared in this study. The primary amino group contents of SDs are the number of TNBS reactive primary amino groups. Native dextran (70 kDa; ND) has no charged groups. The amino group contents of SDs were not significantly different among the different average molecular weights of SDs. For example, the amino group content of SD-70 (5.27 mmol/g polymer) means that one spermine is bound per two glucose units.



Sperminated dextran (SD)

2.2. Effect of sperminated dextrans on the pulmonary absorption of insulin in rats

The effects of different concentrations and MWs of SDs on the pulmonary absorption of insulin were examined in rats. The absorption of insulin was detected by the change in plasma glucose levels. Fig. 1 (a–b) shows the effects of SD (0.1%) with different molecular weights SD (SD-10 (10 kDa), SD-04 (40 kDa) and SD-70 (70 kDa)) on the plasma glucose levels after pulmonary administration of insulin. The plasma glucose levels fell to a degree that depended on the molecular weight of SD over the range 10–70 kDa (Fig. 2 (a)). Fig. 1 (b) shows the relationships between the molecular weights of SD over the range 10–70 kDa and D% after pulmonary administration of insulin. D% increased with the increase in the molecular weigh of SD over the range 10–70 kDa. A high correlation was found between the molecular weights of SD over the range 10–70 kDa and D%.

Figure 2 (a–b) shows the effects of different concentrations (0.02–0.2%) of SD-70 (MW 70 kDa) on the plasma glucose levels after pulmonary administration of insulin. In the case of insulin solution without enhancers, the plasma glucose levels were similar to those following PBS application without insulin. The glucose levels showing a continuous increase may be due to surgical damage and/or the effect of the anesthetic on the rats. ND had no enhancing effects on the insulin absorption. The plasma glucose levels after treatment with SD-70 at the concentrations of 0.02–0.2% fell significantly compared with that without enhancer. Figure 2 (b) shows the relationships between the concentrations of SD-70 and the D% as indexes of the pharmacological effect

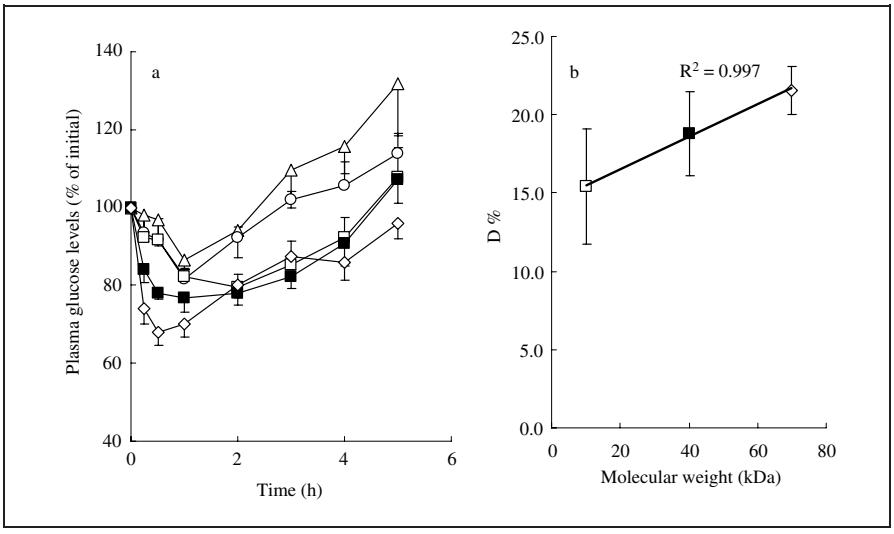


Fig. 1 (a–b):
Effects of different molecular weights of SD (SD-10 (10 kDa), SD-04 (40 kDa) and SD-70 (70 kDa)) (0.1%) on the absorption of insulin in rats. (a) Plasma glucose levels after pulmonary administration of insulin (10 IU/kg). (b) Relationships between the concentrations of SD-70 and the D% as indexes of the pharmacological effects after pulmonary administration of insulin (10 IU/kg).
□ PBS, ○ insulin without enhancer, □ insulin with SD-10, ■ insulin with SD-40, □ insulin with SD-70, The line in (b) is the regression line ($r = 0.997$, $p < 0.05$). Each data set is the mean ± SEM ($n = 4$)

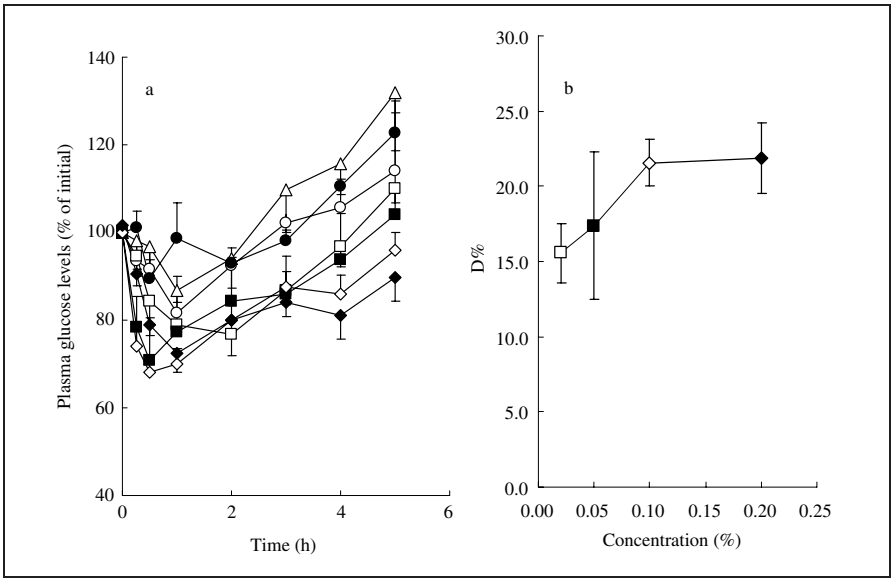


Fig. 2 (a–b):
Effect of different concentrations (0.02–0.2%) of SD-70 (MW 70 kDa) on the absorption of insulin in rats. (a) Plasma glucose levels after pulmonary administration of insulin (10 IU/kg). (b) Relationships between the concentrations of SD-70 and the D% as indexes of the pharmacological effects after pulmonary administration of insulin (10 IU/kg).
□ Control (PBS), ○ insulin without enhancer, ● insulin with 0.2% ND, □ insulin (10 IU/kg) with 0.02% SD-70, ■ insulin with 0.05% SD-70, □ insulin with 0.1% SD-70, □ insulin with 0.2% SD-70 Each data set is the mean ± SEM ($n = 4$)

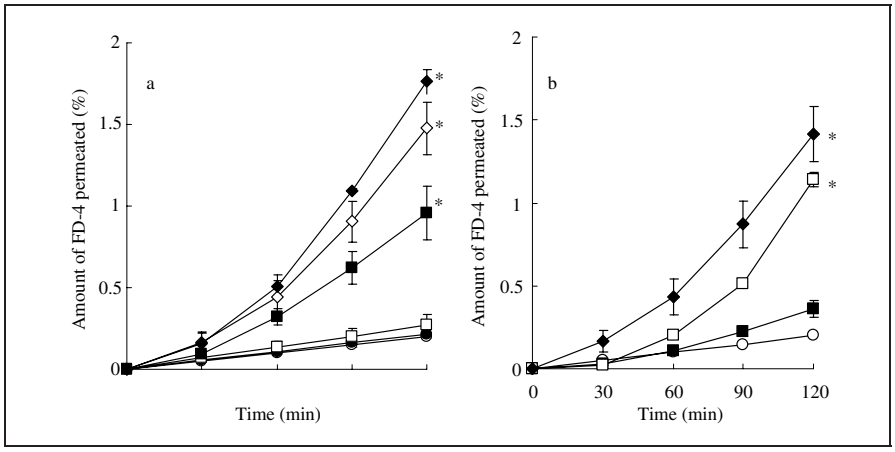


Fig. 3 (a–b):
Effects of different molecular weights of SD (SD-10 (10 kDa), SD-04 (40 kDa) (0.1%) and SD-70 (70 kDa)) on the absorption of insulin in rats. (a) Plasma glucose levels after pulmonary administration of insulin (10 IU/kg). (b) Relationships between the concentrations of SD-70 and the D% as indexes of the pharmacological effects after pulmonary administration of insulin (10 IU/kg). □ PBS, ○ insulin without enhancer, □ insulin with SD-10, ■ insulin with SD-40, □ insulin with SD-70 0.1% The line in (b) is the regression curve ($r = 0.997$, $p < 0.05$). Each data set is the mean ± SEM ($n = 4$)

after pulmonary administration of insulin. D% increased with the increase in concentration over the range 0.02–0.1% SD-70 and became constant at over 0.1% SD-70.

2.3. Effect of sperminated polymers on the permeation of FD4 through calu-3 cell monolayers

The effects of different concentrations and MWs of SD on the permeation of FD4 through the calu-3 cell monolayers were examined. Figure 3 (a) shows the permeation profiles of FD4 at different concentrations (0.02 - 0.2%) of SD-70. SD-70 increased the FD4 permeation with the increase in the concentration over the range 0.05–0.2% of SD-70, while ND (0.2%) had no enhancing effects. Figure 3 (b) shows the permeation profiles of FD4 with SD (0.1%) having different SD molecular weights (SD-10, (10 kDa), SD-40 (40 kDa) and SD-70 (70 kDa)). FD4 permeation increased with the increase in the molecular weight of SD over the range 10–70 kDa.
Figure 4 shows the effects of different concentrations of SD-70 on the change in TEER values of the calu-3 cell monolayers during the permeation experiments. The TEER values were reduced with the increase in the concentration over the range 0.05–0.2% of SD-70, suggesting the opening of the tight junctions during this period in the permeation experiments.

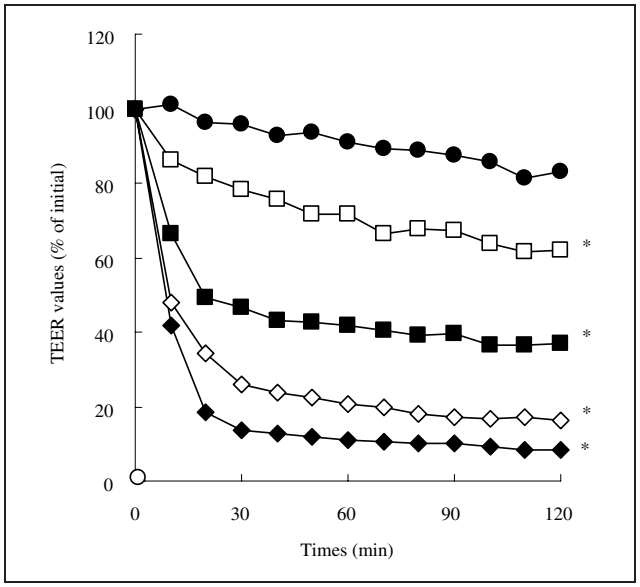


Fig. 4: Effects of different concentrations of SD-70 on the change in TEER values of the calu-3 cell monolayers during the permeation experiments.
○ Control, ● with 0.2% ND, □ with 0.02% SD, ■ with SD 0.05%, □ with 0.1% SD, □ with 0.2% SD
Each data set is the mean ± SEM ($n = 5–6$). *Significant difference in the Dunnett test ($p < 0.01$)

Figure 4 shows the effects of different concentrations of SD-70 on the change in TEER values of the calu-3 cell monolayers during the permeation experiments. The TEER values were reduced with the increase in the concentration over the range 0.05–0.2% of SD-70, suggesting the opening of the tight junctions during this period in the permeation experiments.

3. Discussion

We previously reported a study in rats showing that cationized gelatins with different numbers of amino groups using gelatin and different amounts of ethylenediamine exhibited enhancing effects on the nasal absorption of insulin which depended on the amino group content (Seki et al. 2005). Natsume et al. examined the effect of MW and charge density on the enhancing effect of cationic polymers and found that the charge density was the most important parameter as far as the enhancement of the nasal absorption of FD4 was concerned (Natsume et al. 1999; Miyamoto et al., 2001). Spermine, a polyamine having four amino groups, could be useful in cationized polymers to increase the charge density. The sperminated gelatin, SG, was a more efficient absorption enhancer than the ethylenediaminated gelatins as far as the nasal absorption of insulin in rats was concerned (Seki et al. 2007). However, although gelatin is safe and widely used as a biomaterial, limited numbers of functional groups are able to induce the cationic moiety in the molecule. In this study, we prepared SD as a new type of cationized polymer and examined its enhancing effects on the pulmon-

Table: Amino group content of the sperminated dextrans (SD)

	MW[a] (kDa)	primary amino group content[b] (NH_2-) (mmol/g polymer)	total amino group content (NH_2−, NH=) (mmol/g polymer)
SD-10	10	1.94 ± 0.20	5.82
SD-40	40	1.89 ± 0.16	5.66
SD-70	70	1.76 ± 0.26	5.29

a Molecular weight of dextran used for the preparations of the sperminated dextran.
b The primary amino group content (PA) was expressed as the amount of TNBS-reactive amino groups in 1 g polymers (mean + SEM, n = 5)

ary absorption of insulin in rats. Dextran has many hydroxyl groups which can react with spermine. SD prepared using *N*,N′-carbonyldiimidazole as a activator had a higher charge density than SG and the charge density could be controlled by the amount of *N*,*N*′-carbonyldiimidazole used (Table). The amino group content of SD prepared with MWs of 10, 40 and 70 kDa was three fold that of SG (Seki et al. 2007) and this value means that one spermine is bound per two glucose units in the dextran backbone.

The enhancing effects of SD were evaluated in terms of the pulmonary absorption of insulin and the permeation of FD4 through calu-3 cell monolayers. Although calu-3 is a human bronchial epithelial cell line, it is available as a model airway epithelial cell to evaluate the pulmonary and nasal absorption of drugs (Florea et al., 2005; Nagendry et al. 2005). In both the *in vivo* and *in vitro* experimental systems, the enhancing effects of absorption insulin and permeation of FD4 through calu-3 cell increased with the increase of the molecular weighs of SD at the range 10–70 kDa.

The enhancing effects of SD were evaluated in terms of the pulmonary absorption of insulin and the permeation of FD4 through calu-3 cell monolayers.

Since the epithelial cell surface has a positive charge, cationized polymers can interact with it. We previously investigated the interaction of sperminated polymer with the epithelial cell surface by measurement of the zeta potential of red blood cells (Seki et al. Submitted, 2007). Since the luminal surface of the mucus membrane involving sialic acid is also negatively charged, SD may bind to it after pulmonary application. SD may interact with the mucus membrane surface depending on its molecular weigths. This binding may trigger the enhancement, as the first step in the reaction mechanism.

Condensation of drugs on mucus membranes, inhibition of proteolysis and opening of tight junctions to increase paracellular permeation are all possible mechanisms of absorption enhancers used for peptide drugs (Muranishi 1990). In our previous report about the enhancing effects of SG in caco-2 cell monolayers, the enhancing mechanism of SG for insulin permeation involves an increase in the number of permeation pathways in the tight junctions (Seki et al. 2006). The tight junctions, acting as a permeation barrier, could be converted to permeation pathways by the opening-effect of the cationized polymers. The TEER values of monolayers were reduced and the enhancing of FD-4 permeation was related to the calu-3 cell monolayers treated with SD (Figs. 3 and 4). This result suggests that SD can also open the tight junctions to increase the paracellular permeation of FD4. SD could interact with the luminal surface of mucus membranes directly by an ion-ion interaction and then induce signals that would open tight junctions resulting in intercellular permeation of water soluble drugs.

In conclusions, in this study, SD having different molecular weights were prepared and, in both the *in vivo* and *in vitro* experimental systems, enhancing effects on the pulmonary absorption of insulin in rats and the permeation of FD4 through the calu-3 cell monolayers were produced by treatment with SD. SD may interact directly with the luminal surface of mucus membranes by an ion-ion interaction, producing signals that would open tight junctions resulting in the intercellular permeation of water-soluble drugs. SD may be useful as an absorption enhancer for pulmonary delivery of peptide and protein drugs.

4. Experimental

4.1. Materials

Dextrans (MWs; 10, 40 and 70 kDa), FITC-dextran (FD-4), spermine, glucosamine hydrochloride and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride were purchased from Sigma Chemical Co. (St Louis, MA, USA). Recombinant human insulin (26 IU/mg), glucose B-test kits, *N*,*N*′-carbonyldiimidazole and spermine tetrahydrochloride were purchased from Wako Pure Chemical Industries (Osaka, Japan). Glycine and 2,4,6-trinitrobenzenesulfonic acid (TNBS) were purchased from Kanto Kagaku (Tokyo, Japan) and Nacalai Tesque (Kyoto, Japan), respectively. All other chemicals were of reagent grade and used as received.

4.2. Synthesis of sperminated dextrans

Dextran (MWs; 10, 40 and 70 kDa; 50 mg) and *N*,*N*′-carbonyldiimidazole (75 or 225 mg) were dissolved in dimethyl sulfoxide (DMSO, 5.0 mL) and then the solution was mixed with a spermine solution in DMSO (1.87 g in 43 mL) (Kanatani et al. 2006). The resulting solution was kept for 20 h at 34 °C. The resulting SDs (SD-10 (MW 10), SD-40 (MW-40) and SD-70 (MW-70)), were purified by dialysis for 72 h then isolated in powder form by lyophilization.

In order to determine the amino group content of SDs, a 1 mL solution of SDs (0.50 mg/mL) in phosphate buffered saline (PBS, pH 7.4) was mixed with 1.0 mL sodium bicarbonate solution (4.0%) and 1.0 mL TNBS solution (0.10%). The mixture was kept at 40 °C for 2 h protected from light and then the absorbance of the solution at 415 nm was determined (Seki et al. 2005, 2006). Calibration curves were prepared using glucosamine for β-alanine. The primary and total amino group contents were expressed as the amount of TNBS-reactive amino groups in 1 g SDs.

4.3. Pulmonary administration of insulin to rats

Animal experiments were carried out in accordance with the Guiding Principles for the Care and Use of Experimental Animals, Hokkaido Pharmaceutical University (2006). Male Wistar rats (Sankyo LaboService Co.), weighing 200–240 g, were fasted for 18–24 h before the experiments, but had free access to water. The rats were anesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 50 mg/kg. Insulin solution (40 IU/mL, in PBS), with or without SD was administered into the bronchus using a MicroSprayer™ (Penn Century Inc., PA, USA) at a dose of 10 IU/250 μL/kg. Blood samples (each 0.1 mL) were withdrawn from the jugular vein 10 min before administration and at predetermined times after dosing for up to 5 h. After centrifugation of the blood samples at 8.000 g for 5 min, the plasma was isolated and the plasma glucose concentration was determined using a Glucose B-test kit (glucose oxidase method). In order to quantify the enhancing effect on insulin absorption, we calculated the D% value defined by the following Eq. (1) (Seki et al. 2007).

$$D\% = \frac{AUC_{G,\,PBS} - AUC_{G,\,Insulin}}{AUC_{G,\,PBS}} \times 100 \qquad (1)$$

where $AUC_{G,PBS}$ and $AUC_{G,Insulin}$ are the area under the curves of the plasma glucose levels from 0 hr to 5 h after nasal administration of PBS and insulin solution, respectively.

4.4. Calu-3 cell monolayer permeation experiments

Calu-3 cells were purchased from American Type Culture Collection (ATCC), (Manassas VA, USA). Calu-3 cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 40 μg/mL gentamicin and 1% nonessential amino acids, in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells from passage number 43–63 were seeded (4.5×10^5 cell/cm^2) on polyester filter inserts (pore size 0.4 μm, area 0.33 cm^2, Transwell, Costar) and cultivated in the medium for 9–15 days before starting the drug transport experiments. Transepithelial electrical resistance (TEER) was measured using a Millicell®-ERS (Millipore, Mass., USA) before the transport experiments and the monolayers exhibiting 330–660 $\Omega \cdot cm^2$ TEER were used for the experiments. FD4 solution (50 mg/mL, in PBS), with or without SD, was applied on the apical side. Hanks balanced salt solution (HBSS, pH 7.4, 0.60 mL) was used as the basolateral side solution and this was changed every 30 min for 2 h. The TEER was also measured after the transport experiments to evaluate the change in the electrical conductance of the monolayers. The FD-4 concentration was determined using a spectrofluorometer (F-2000, Hitachi, Tokyo, Japan) at an excitation wavelength of 495 nm and an emission wavelength of 519 nm.

This research paper was presented during the 6th Conference on Retrometabolism Based Drug Design and Targing, June 3–6, 2007, Göd, Hungary.

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