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Synthesis and *In Vitro* Properties of Prednisolone 21-Sulfate Sodium as a Colon-Specific Prodrug of Prednisolone

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Colon-specific delivery of glucocorticoids is highly desirable for the efficient treatment of inflammatory bowel disease. We synthesized prednisolone 21-sulfate sodium (PDS) as a colon-specific prodrug of prednisolone (PD) and investigated its properties using rats as test animals. We expected that introduction of sulfate ester as a sodium salt might increase the hydrophilicity and restrict the absorption in the GI tract. If PDS is stable and nonabsorbable in the upper intestine, it will be delivered to the colon as an intact form, where it hydrolyze by the sulfatase to release PD. Compared with PD, the solubility of PDS increased and the apparent partition coefficient decreased greatly. PDS was stable on incubation with pH 1.2 and 6.8 buffer solutions and with the contents of the stomach and small intestine. On incubation with the cecal contents, PDS decreased to 9.6% of the dose in 10 h producing PD. The amount of PD increased to give a maximum 54% of the dose and decreased. As a control, when PD was incubated with the cecal contents, it decreased to 29% of the dose in 8 h, which implied that reduction of PD proceeded under such conditions. These results suggested that hydrolysis of PDS took place to produce and accumulate PD, which decreased by reduction as the incubation period extended. Our results suggested that PDS can be a promising colon-specific prodrug of PD, and sulfate ester group might serve as a potential colon-specific promoiety, especially for the drugs which are resistant to reduction in the colon.

Key words: Colon-specific prodrugs of glucocorticoid, Prednisolone, Sulfatase

INTRODUCTION

Delivery of orally administered drugs specifically to the colon is highly desirable for the efficient treatment of diseases developed locally at the colon. Being delivered specifically to the site of action with limited systemic absorption, only a small dose is needed, which subsequently results in reduced side effects. Although glucocorticoids have been used most frequently for inflammatory bowel disease (IBD), long-term administration is not recommended to avoid the serious side effects caused by the systemically absorbed drugs (Crotty and Jewel, 1992). They are well absorbed in the upper intestine and only a limited fraction of the dose is delivered to the inflammatory site in the distal ileum or colon. For this reason, development of colon-specific prodrugs of glucocorticoids has been the targets of many studies (Fedorak *et al.*, 1995; Mcleod *et al.*, 1993; 1994, Friend and Chang, 1984; 1985). A colon-specific prodrug should be stable and nonabsorbable in the upper intestine so that it could be delivered to the colon as an intact form, and the linkage between the drug and promoeity should dissociate selectively in the colon to release the active drug. Therefore, selection of a suitable promoiety is critical for the design of a colon-specific prodrug.

Huijghebaert *et al.* (1984) reported that microbial estrone sulfatase activity in the intestinal contents of rats was high in the cecum, but very low in the small intestine. Schwenk *et al.* (1982) reported that estrone sulfate was not significantly influenced by the intestinal first-pass effects after instillation into the lumina of intact jejunal loops of rats, which indicated that sulfate esters might be stable in the small intestine. Van Eldere *et al.* (1994) reported on the isolation and identification of intestinal steroid-desulfating bacteria from rats and humans. These reports might indicate that the sulfate linkage is stable in the small intestine and hydrolyzed by sulfatases originated from

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microbes in the colon.

Introduction of sulfate ester as a sodium salt will increase the hydrophilicity of the compound and consequently limit transcellular absorption by way of lipid membrane permeation. On this regards, we thought that sulfate ester might serve as a potential colon-specific promoiety. We synthesized prednisolone 21-sulfate sodium (PDS) and investigated its *in vitro* properties as a colon-specific prodrug of prednisolone (PD) using rats as test animals. We expected that if PDS were stable and nonabsorbable in the upper intestine, a large fraction of the orally administered dose would be delivered to the colon as an intact form. Hydrolysis of the sulfate ester by the sulfatase in the colon would release PD, which should be available for the treatment of IBD.

MATERIALS AND METHODS

Chemicals and animals

Prednisolone (PD), sulfatrioxide triethylamine complex (STT) and sulfatrioxide pyridine complex (STP) were purchased from Sigma Chemical Co. (St. Louis, MO) and were used as received. Solvents for NMR and HPLC were obtained from Merck Inc. (Damstadt, Germany). All other chemicals were reagent grade, commercially available products. Male Sprague-Dawley rats weighing 200-250 g were received from Hyochang Science (Daeku, Korea) and maintained on a stock diet and water ad libitum. The animals were fasted overnight (16 h) prior to the sacrifice for the experiment.

Instruments

IR spectra were recorded on a Bomem MB 100 FT-IR spectrophotometer (Bomem). ^{1}H-NMR spectra were taken on a Varian AS 500 spectrometer and the chemical shifts were in ppm downfield from tetramethylsilane. Elemental analysis was carried out by an Elemental Analyzer System (Profile HV-3). Melting points were taken on a Mel Temp II (Laboratory Devices, Holliston, MA) and were uncorrected. A Polytron PT 3100 homogenizer, an Eppendorf Centrifuge 5415C (Hamburg, Germany) and a Taitec microincubator M-36 (Japan) were used.

Buffer solutions

Buffer A: hydrochloric acid buffer (0.2 M hydrochloric acid and 0.2 M potassium chloride were mixed to give pH 1.2).

Buffer B: Isotonic phosphate buffer (0.1 M sod. phosphate dibasic and 0.15 M sod. phosphate monobasic were mixed to give pH 6.8).

Buffer C: Isotonic acetate buffer (0.15 M sod. acetate and 0.3 M acetic acid were mixed to give pH 4.5).

Buffer D: 0.067 M phosphate buffer (0.067 M sod. phosphate monobasic and 0.107 M phosphoric acid were mixed to give pH 4.5)

HPLC analysis

The HPLC system consisted of Model 305, 306 pumps, a 117 variable UV detector, a Model 234 autoinjector, a Model 805 manometric module, and a Model 811C dynamic mixer from Gilson. The mobile phase consisted of acetonitrile/buffer D (3/7) solution, which was filtered through 0.45 μm membrane filter before use. A Lichrospher 100 RP-18 column (250×4.6 mm, 5 μm) was eluted with the mobile phase at a flow rate of 1.0 mL/min. The eluate was monitored by measuring the absorption at 248 nm at sensitivity of AUFS 0.01. The Gilson 712 software was employed for the data analysis. The retention time of PD and PDS was 9.95 min and 6.12 min, respectively.

Calibration of PD and PDS in various biological media

Stock solution of PD or PDS was prepared in methanol (50 μg/mL). A male Sprague-Dawley rat was anesthetized by diethyl ether and a midline incision was made and various segments of gastrointestinal tract were obtained. The contents of the stomach, proximal small intestine (PSI) or distal small intestine (DSI) and cecum were homogenized separately and diluted with buffer solution C (10 w/v%). To a 100 μL portion of the above homogenates, were added 20, 40, 100, 200 μL of the stock solution of PD or PDS and appropriate volume of methanol to make the final volume of 1 mL, which provided standard solutions of PD and PDS in concentration of 1, 2, 5 or 10 μg/mL, respectively, in various biological specimen.

Standard or blank solution (1 mL) was mixed on a vortex mixer for 2 min, centrifuged at 10,000 rpm for 5 min and filtered through a membrane filter (0.45 μm). The filtrate (20 μL) was analyzed by HPLC as described in previous section and a calibration curve was constructed from the results.

Preparation of prednisolone 21-sulfate triethylammonium

To the solution of PD (3.60 g, 10.0 mmol) in 64 mL of anhydrous benzene and pyridine (1/1), STT (3.80 g, 17.0 mmol) was added in portions with stirring at 56-60°C for 20 min. The reaction mixture was evaporated under reduced pressure to remove the solvent. The residue, prednisolone 21-sulfate triethylammonium, was used for the preparation of PDS without further purification.

Preparation of prednisolone 21-sulfate sodium

Prednisolone 21-sulfate triethylammonium dissolved in minmum amount of distilled water was added to a solution

of 10% NaCl with mechanical stirring for 1 h. The resulting precipitates, PDS, were collected by suction filtration and recrystallized from absolute ethanol. Overall yield was 60-80%. mp 120°C (decomp.); IR (nujol) 3450 (OH), 1675 (C=O), 1640 (C=C), 1600 (C=C), 1259 (S=O), 1034 (S=O) cm^{-1}; ^{1}H-NMR (D_2O) δ 0.78 (s, 3H, C-18), 1.30 (s, 3H, C-19), 4.89 (ABq, 2H, C-21), 5.94 (s, 1H, C-4), 6.21 (d, 1H, C-1), 7.42 (d, 1H, C-2); Anal. Calcd for $C_{21}H_{27}SO_8Na$: C, 54.55: H, 5.84; S, 6.93. Found: C, 54.24; H, 6.20; S, 6.72.

pH stability

A solution of PDS (100 μg/mL) was incubated in buffer A and buffer B at 37°C for 10 h. At a predetermined time interval, a 20 μL portion of the solution was removed, and the concentration of PDS or PD was analyzed by HPLC as described previously.

Apparent partition coefficient

To a solution (10 mL) of PD or PDS (100 μg/mL) in buffer B presaturated with 1-octanol, was added 10 mL of 1-octanol presaturated with buffer B. The mixture was shaken for 10 h and left for 4 h at 37°C. The concentration of PD or PDS in the aqueous phase was analyzed by HPLC as described previously. The apparent partition coefficients were calculated by employing the equation $(C_o-C_w)/C_w$, where C_o and C_w represent the initial and equilibrium concentration of the drug in aqueous phase, respectively.

Incubation of PDS with the contents of the stomach or small intestine

Contents of the stomach or small intestine were diluted to half concentration with buffer C for the stomach and with buffer B for the small intestine. To a 0.2 g portion of each sample, 0.8 mL of PDS solution (100 μg equivalent of PD) in buffer B was added and the mixture was incubated under nitrogen at 37°C. At appropriate time intervals, it was centrifuged at 5,000 rpm for 3 min. To a 0.1 mL portion of the supernatant, 0.9 mL of methanol was added, vortexed for 2 min and centrifuged for 5 min at 10,000 rpm. The concentration of PDS or PD in a 20 μL portion of the supernatant was determined by HPLC as described previously.

Incubation of PDS with the cecal contents

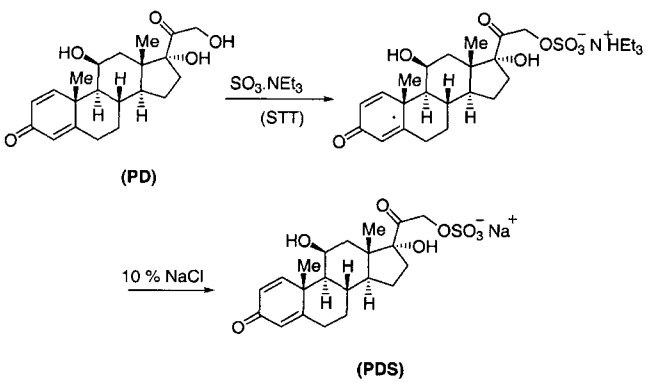
The cecal contents were collected in a glove box, which was previously displaced by nitrogen. To a 0.1 g portion of the cecal contents, 0.9 mL of PDS solution (100 μg equivalent of PD) in buffer B was added and the mixture was incubated at 37°C. At appropriate time intervals, the amount of PDS or PD in the medium was determined by HPLC as described previously.

RESULTS AND DISCUSSION

For the design of colon-specific prodrugs, hydrophilic small molecules (Jung, *et al.*, 2000; 2001; Ryde, 1992; Istran, *et al.*, 1991) or polymers (Kopeckova and Kopecek, 1990; Larsen and Johansen, 1985; Brown *et al.*, 1983) are used as the promoiety to prevent absorption, and the linkage between the drug and the promoiety is adopted which would dissociate selectively in the colon. This is generally accomplished by the enzymes originated from the microbes in the colon (Faigle, 1993; Rubinstein, 1990).

Base on literature reports (Huijghebaert *et al.*, 1984; Schwenk *et al.*, 1982; Van Eldere *et al.*, 1994), we expected that PDS would possess the characteristics required for a colon-specific prodrug. Conversion of a hydroxyl group in PD to sulfate ester sodium would increase the water solubility and prevent the absorption in the GI tract. The sulfatase in the colon is microbial origine and it would be most abundant in the colon where the microbial count is high. Hydrolysis of the sulfate ester of PDS and subsequent release of PD would take place specifically in the colon.

We tried STP or STT to convert the primary hydroxyl of PD to sulfate ester group. Sulfation is known to proceed preferably in a non-polar solvent and so we used anhydrous benzene. Pyridine was added to help dissolution of the reactants. Sulfation did not take place by STP, but it proceeded readily by STT in good yield (Scheme 1). The reaction affected greatly by the ratio of the reactant and reaction time. Use of 1.5-1.7 molar excess of STT provided optimum result. We terminated the reaction when most of PD converted to PDS by monitoring the reaction process by TLC because prolongation of the reaction time resulted in sulfation of the secondary hydroxyl group also. IR spectrum showed two strong absorption peaks originated from (-S=O) asymmetric and symmetric stretching of sulfonic acid ester at 1259 and 1034 cm^{-1}, respectively. Compared with PD, the most characteristic change in ^{1}H-NMR spectrum of PDS was the downfield shift of the two



Scheme 1. Preparation of prednisolone 21-sulfate sodium

protons on C-21 to 0.4 ppm by the introduction of the sulfate group. Solubility of PDS increased greatly (46.2 mg/mL). The apparent partition coefficient of PD and PDS was 21.3 and 0.11, respectively, which suggested that absorption of PDS in the upper intestine via transcellular passive diffusion might be restricted. To determine whether PDS would be chemically stable during the transit through the upper intestine, it was incubated for 10 h at 37°C in buffer solutions of pH 1.2 and pH 6.8, which represent the pH of the stomach and upper intestine, respectively. Neither the concentration of PDS changed nor PD was detected for the whole incubation period. When PDS (100 μg) was incubated with the contents of the stomach or small intestine, PDS was not hydrolyzed, showing no change in the concentration of PDS during 24 h incubation period. These results suggested that PDS might be chemically and enzymatically stable during the transit through the upper intestine, which are the necessary properties as a colon-specific prodrug. To assess the degree of prodrug activation in the human colon, we used the cecal contents of rats because the bacterial counts in the rat cecum are as high as those in the human colon. When PDS (100 μg) was incubated with the cecal contents (10%, 1 mL), PD was produced. The concentration of PD increased to give a maximum 38% of the dose at 4 h and decreased (Fig. 1), which indicated that conversion of PD took place once it released from PDS. To clarify this, PD was incubated with the cecal contents under the identical condition, and observed that the amount of PD decreased progressively to 29% of the dose at 8 h (Fig. 2). Considering that reduction is one of the common reactions of the microbes in the large intestine, it seemed that reduction of the A ring of the steroid has taken place because the reduced forms are not detectable by the UV detector of the HPLC system. The amount of PD in the medium is related to the relative rates of sulfate hydrolysis of PDS and reduction of the produced PD. In scheme 2, the proposed metabolic pathways of PDS in the large intestine of rats are shown. The rate of hydrolysis of PDS (1) should be faster than that of reduction (2 and 3), otherwise PD may not be detected in the medium. We investigated the effect of the concentration of the cecal contents on the relative rates of hydrolysis and reduction. When PDS (100 μg) was incubated with 10% cecal contents (1 mL), the amount of PDS decreased to 68, 43, 22 and 12% of the initial dose at 2, 4, 6 and 8 h, respectively (Fig. 3). If PD were the only product of PDS, the amount of PD is expected to be 32, 57, 78, and 88% of the initial dose at 2, 4, 6 and 8 h, respectively. The amount of PD was 20, 32, 38, 18% of the dose, which was less than the decreased PDS. As the concentration of the cecal contents decreased to 5 or 3%, the rate of PDS conversion decreased accordingly. The level of PD also decreased as the concentration of the cecal contents decreased during the earlier period of incubation. As the incubation period extended, the level of PD was not in parallel order with the concentration (Fig. 3, 4 and 5). When the concentration of the cecal contents

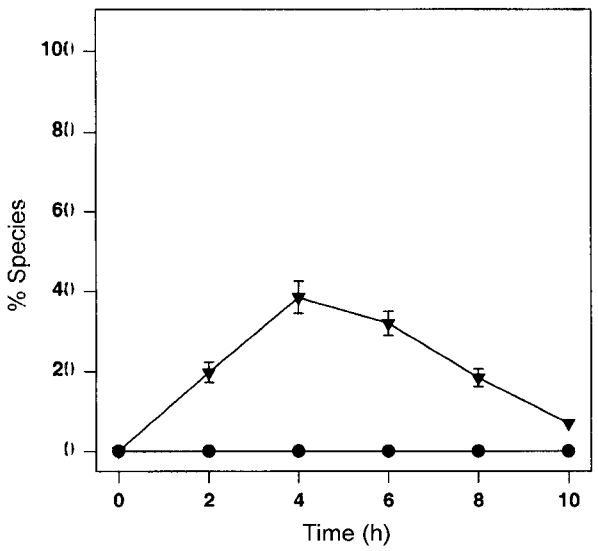


Fig. 1 Incubation of PDS (equivalent to 100 μg PD) in 1 mL of 10% intestinal contents of rats in pH 6.8 isotonic phosphate buffer at 37°C. ●; contents of the stomach and small intestine, ▼; contents of the cecum. Data are mean±SE (n=8).

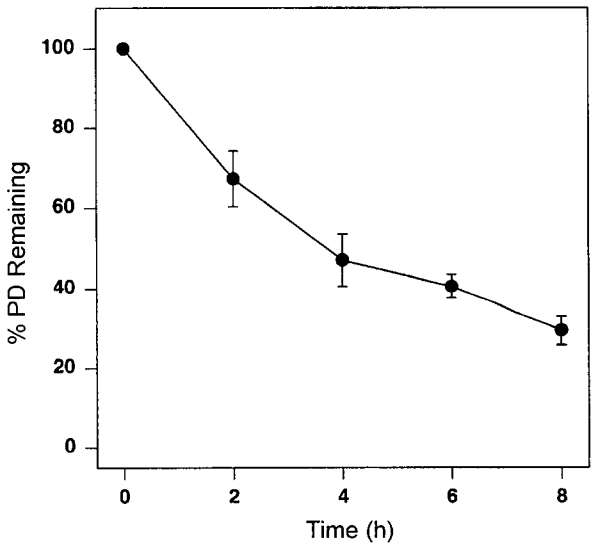
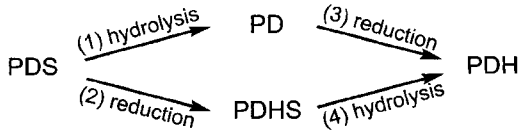


Fig. 2. Incubation of PD (1 mg) in 10 mL of 10% cecal contents of rats in pH 6.8 isotonic phosphate buffer at 37°C. Data are mean±SE (n=4).



Scheme 2. Proposed metabolic pathways of prednisolone 21-sulfate sodium

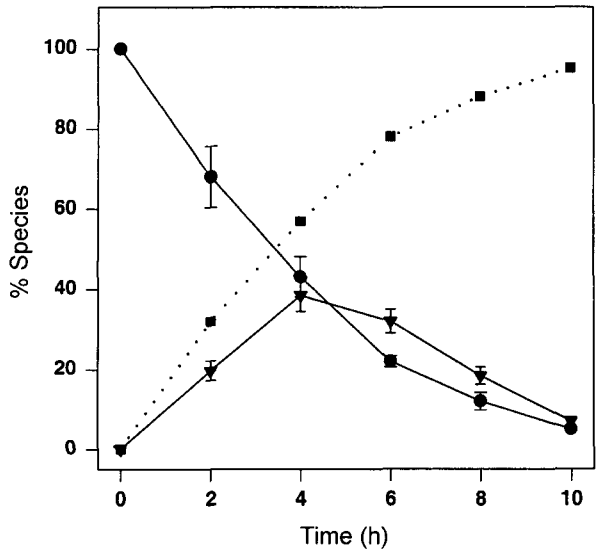


Fig. 3. Incubation of PDS (equivalent to 1 mg PD) in 10 mL of 10% cecal contents of rats in pH 6.8 isotonic phosphate buffer at 37°C. ●; PD released, ▼ ; PDS remaining, (■); predicted amount of PD. Data are mean±SE (n=8).

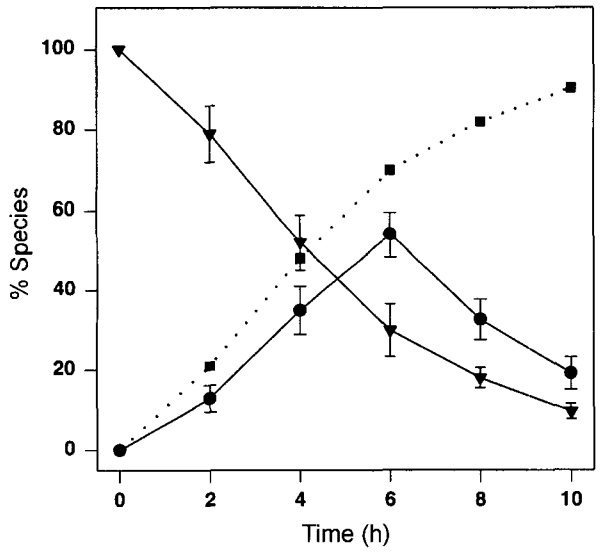


Fig. 4. Incubation of PDS (equivalent to 1 mg PD) in 10 mL of 5% cecal contents of rats in pH 6.8 isotonic phosphate buffer at 37°C. ●; PD released, ▼ ; PDS remaining, (■); predicted amount of PD. Data are mean±SE (n=8).

was 10, 5 and 3%, the maximum level of PD was 38, 54 and 12% of the dose at 4, 6, and 10 h, respectively. The level of PD was the greatest when 5% cecal contents were used. These results suggested that hydrolysis of PDS dominates in earlier period of incubation to produce and accumulate PD, which subsequently decreased by the reduction. As the incubation period extends, the amount of PDS, the rate of hydrolysis and consequently the production of PD decreases, which lowers the level of

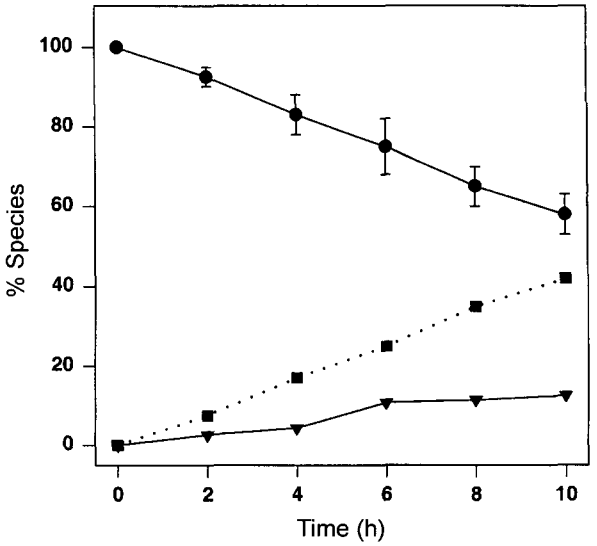


Fig. 5. Incubation of PDS (equivalent to 1 mg PD) in 10 mL of 3% cecal contents of rats in pH 6.8 isotonic phosphate buffer at 37°C. ●; PD released, ▼; PDS remaining, (■); predicted amount of PD. Data are mean±SE (n=8).

PD as the reduction proceeds dominantly. Because anti-inflammatory action of glucocorticoids diminishes if A ring of the steroid is reduced (Schimmer and Parker 2001), the therapeutic potential of colon-specifically delivered glucocorticoid will be affected by the vulnerability of the drug to reduction by the microbes in the large intestine.

There are several reports that stated that certain enzymes produced by the gut microflora are inducible with diet. For instance, Shiau *et al.* (1983) reported that β-glucuronidase increased in rats fed fibre-free diet and significantly decreased in those fed 15% fibre diets. Mallett *et al.* (1986) studied on the influence of wheat bran on some reductive and hydrolytic activity of rat cecal flora and reported that wheat bran significantly decreased enzyme activities for nitro and nitrate reduction per gram of cecal contents but increased β-glucosidase activity. Nakamura *et al.* (1989) reported that the blood concentration of salicylic acid increased in rabbits fasted for 24 h compared to the fully fed ones following intracecal administration of salicyluric acid. We investigated the effect of feeding condition on the metabolism of PDS by the cecal contents. In a series of experiments, we incubated PDS with the cecal contents collected from rats which were fully fed prior to the sacrifice and compared the results with the ones which were fasted overnight (16 h). Conversion of PDS occurred to the same extend for both groups, but the concentration of PD was lower from the fed group than from the fasted group (n=16). Until further information is available, no conclusive explanation could be drawn from the present results whether and how the activity of certain enzymes of the microbes in the GI tract are affected by

the feeding condition.

To conclude, PDS was prepared in good yield by the simple processes from PD. Even though reduction of the released PD was observed to some extend by the cecal contents, *in vitro* properties of PDS revealed its potential as colon-specific prodrug of PD. Our results suggested that sulfate ester as a sodium salt might serve as a promising colon-specific promoiety, and utilization of this moiety will lead to the development of promising colon-specific prodrugs, especially for the glucocorticoids which are resistant to reduction in the colon. Further studies are in progress.

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