World Journal of Pharmaceutical Science and Research
www.wjpsronline.com
Research Article
ISSN: 2583-6579
SJIF Impact Factor: 5.111
Year - 2025
Volume: 4; Issue: 1
Page: 486-512

USE OF URSODEOXYCHOLIC ACID CONJUGATES AS A CARRIER OF 5-AMINOSALICYLIC ACID TO DELIVER TO THE COLONIC REGION: COMPARISON WITH SALAZOSULFAPYRIDINE IN FED RATS

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Article Received: 20 December 2024 | | ***Article Revised: 08 January 2025*** | | ***Article Accepted: 31 January 2025***

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How to cite this Article: Yorinobu Maeda, Ryuji Kyan, Akira Shigenaga, Masaya Ohta, Takeshi Goromaru and Teruo Murakami (2025). USE OF URSODEOXYCHOLIC ACID CONJUGATES AS A CARRIER OF 5-AMINOSALICYLIC ACID TO DELIVER TO THE COLONIC REGION: COMPARISON WITH SALAZOSULFAPYRIDINE IN FED RATS. World Journal of Pharmaceutical Science and Research, 4(1), 486-512. https://doi.org/10.5281/zenodo.14936826

ABSTRACT

5-Aminosalicylic acid (5-ASA) is an anti-inflammatory drug widely used to treat ulcerative colitis, an inflammatory bowel disease in the colonic region. The targetability of 5-ASA to the colonic region after oral administration was compared between salazosulfapyridine, a prodrug of 5-ASA azo bonded with sulfapyridine, and 5-ASA combined with ursodeoxycholic acid (UDCA) conjugates in fed rats. The following 5-ASA-UDCA derivatives were synthetized: 5-ASA-UDCA, 5'-ASA-UDCA-3-glucuronide (5-ASA-UDCA-3G), 5'-ASA-UDCA-3-sulfate (5-ASA-UDCA-3S), and 5'-ASA-UDCA-3,7-disulfate (5-ASA-UDCA-DS). These 5-ASA-UDCA and 5-ASA-UDCA conjugates were stable in the biological samples such as plasma, intestinal mucosa, liver, and feces' homogenates. In contrast, they were split and released 5-ASA by cholylglycine (bile acid) hydrolase, whereas the hydrolysis rates of 5-ASA-UDCA-DS and 5-ASA-UDCA-3S were slow. In rats' everted gut sac study, 5-ASA-UDCA, but not other 5-ASA-UDCA conjugates, showed site-specific active membrane permeability in the lower ileum. In fed rats, these 5-ASA prodrugs were administered orally and mucosal concentrations of 5-ASA and N-acetyl metabolite (AC-5-ASA) were measured along the gastrointestinal tract 8 h after administration. 5-ASA-UDCA and 5-ASA-UDCA-3G, as well as salazosulfapyridine, showed higher total concentrations of 5-ASA and AC-5-ASA in the colonic-region membranes. The colonic distribution of 5-ASA from 5-ASA-UDCA-DS and 5-ASA-UDCA-3S was low. Taken together, 5-ASA-UDCA-3G, being not absorbed actively in the ileum in rats, was thought to be a feasible prodrug in the targeted delivery of 5-ASA to the colonic region, although further study is necessary.

KEYWORDS: 5-Aminosalicylic Acid, Prodrugs, Ursodeoxycholic Acid Conjugates, cholylglycine hydrolase, Colonic Delivery, Ulcerative Colitis.

INTRODUCTION

Ulcerative colitis (UC) and Crohn's disease (CD) are inflammatory bowel diseases of the intestine exhibiting similar symptoms and leading to digestive disorders and inflammation in the digestive system. UC is the colonic inflammation that affects the rectum only or progresses proximally to involve part of or the entire colon. In contrast, CD affects all parts of the gastrointestinal tract from mouth to anus, in which the ileum and colon are common.[1-3] 5-Aminosalicylic acid (5-ASA), alsoof mild-to-moderate UC. We recently examined the intestinal membrane distribution of unformulated pure 5-ASA by measuring concentrations of 5-ASA and its N-acetyl metabolite of 5-ASA (AC-5-ASA) along the whole intestine, in which various 5-ASA preparations (suspension with different pHs, or solution) were administered at a dose of 30 mg/kg as 5-ASA to fed rats. In that study, it was found that the dissolved 5-ASA in the small intestine is readily distributed into the intestinal membrane, and the remaining unabsorbed 5-ASA in the small intestine is delivered to the distal intestine and distributed into the colonic membrane. It was thought that the membrane distribution of 5-ASA in the small intestine is restricted by the pH-dependent low solubility of 5-ASA and saturable membrane permeability and that the more 5-ASA is delivered to the distal intestine at the higher oral dose of 5-ASA [4]. In the Biopharmaceutical Classification System (BCS), 5-ASA is classified as a BCS IV drug with low solubility and permeability.[5,6] The high oral doses of 5-ASA such as 2 to 4 g/day are reportedly more effective than lower doses in treating patients with mild-to-moderate active UC.[7]

Various delivery systems of 5-ASA have been developed to efficiently deliver to the colonic region by preventing the release in the small intestine and promoting the release in the proximal colon. Delivery systems of 5-ASA involve time-dependent-release formulations such as Pentasa® (5-ASA granules), pH-dependent-formulations such as Asacol®, and diazo-bonded prodrugs such as salazosulfapyridine (SASP; also called sulfasalazine).[8-11] Topical preparations of 5-ASA such as suppositories, enemas and forms of 5-ASA-relating medicine are also clinically available.[8,12]

In the present study, various 5-ASA bounded with ursodeoxycholic acid (UDCA) or its comjugates were synthesized and their efficacies as colon-targeting carriers of 5-ASA were compared with salazosulfapyridine. Salazosulfapyridine is a prodrug of 5-ASA diazo-bonded with sulfapyridine, a sulfonamide, which is split into 5-ASA and sulfapyridine by bacterial azo reductase in the intestine.[13,14] After oral administration of these 5-ASA-related drugs at 30 mg/kg as 5-ASA to fed rats, the membrane concentrations of 5-ASA and AC-5-ASA along the whole intestine were determined 8 h after administration. Previously, we used *p*-aminobenzoic acid (PABA)-UDCA conjugate to evaluate the overgrowth of intestinal bacteria in rats and humans. In that study, PABA-UDCA and PABA-UDCA-disulfate (PABA-UDCA-DS) were split by bacterial bile acid (cholylglycine) hydrolase readily, and the overgrowth of intestinal bacteria was evaluated by measuring the urinary excretion rate of PABA in rats and humans.[15-18] These results may imply the usefulness of UDCA and/or UDCA-DS as effective carriers of 5-ASA. In the present study, the following 5-ASA-UDCA conjugates were synthesized and their efficacy as colon-targeted 5-ASA prodrugs were examined: 5-ASA-UDCA, 5'-ASA-UDCA-3-glucuronide (5-ASA-UDCA-3G), 5'-ASA-UDCA-3-sulfate (5-ASA-UDCA-3S), and 5'-ASA-UDCA-3,7-disulfate (5-ASA-UDCA-DS). Figure 1 shows the chemical structures of salazosulfapyridine, 5-ASA-UDCA, and 5-ASA-UDCA-conjugates used in the present study. In Figure 2, the fate of 5-ASA in the intestine after oral administration of 5-ASA, 5-ASA-UDCA, and 5-ASA-UDCA conjugates observed in the previous and present study is schematically presented.[18]

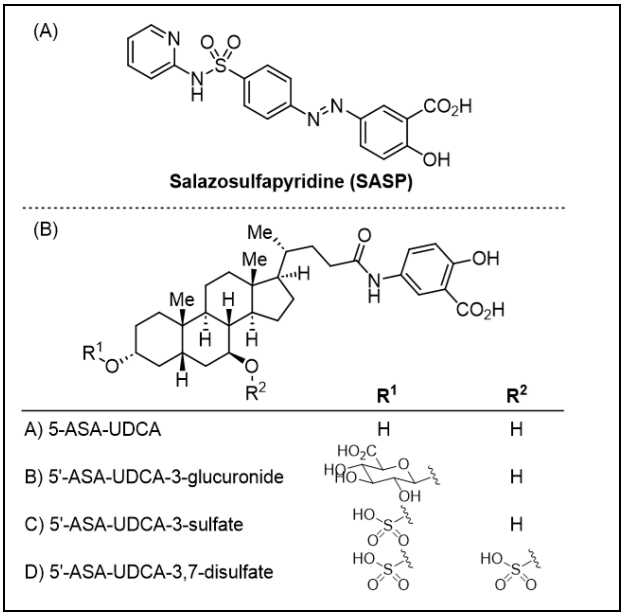

Figure 1: Chemical structures of (A) Salazosulfapyridine and (B) 5-ASA-UDCA-derivatives.

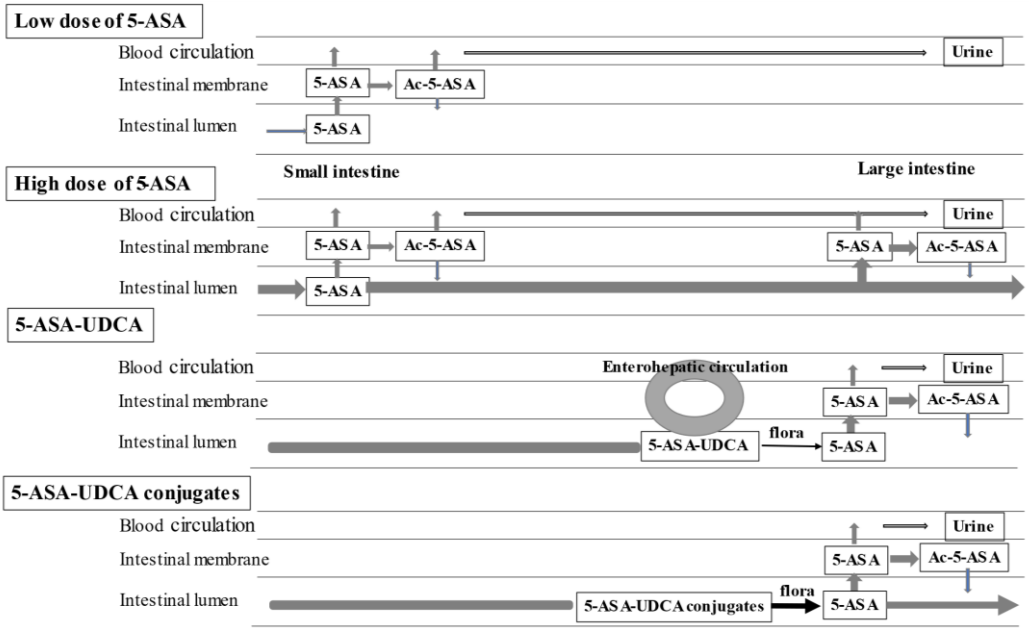

Figure 2: Intestinal mucosal distribution of 5-ASA after oral administration of 5-ASA at a low dose (A), 5-ASA at a high dose (B), 5-ASA-UDCA at a high dose as 5-ASA (C), and appropriate 5-ASA-UDCA conjugates at a high dose as 5-ASA (D) in fed rats.

MATERIALS AND METHODS

Materials

5-ASA and UDCA were purchased from FUJIFILM Wako Chemicals, Japan. Salazosulfapyridin (SASP; Salazopyrin® Tablet) was purchased from Pfzer Co. Ltd. Tokyo, Japan. The acetone powder of cholylglycine hydrolase [from *C. perfringens* (welchii)] was from Sigma Chemical Co. (St. Louis, MO). All reagents and solvents used were reagent grade and were purchased from FUJIFILM Wako Chemicals, Japan. Thin-layer chromatography (TLC) of 5-ASA-UDCA conjugates was carried out on precoated silica-gel plates (Kieselgel 60 F_{254} plate, Merck). Plates were

developed in a solvent system of benzene: dioxane: acetic acid; 15: 5: 2 (v/v/v). After development, the spot was detected using shortwave ultraviolet light (254 nm), and the spot was visualized by spraying the plates with phosphomolybdic acid (5% in ethanol) and subsequent heating at 100℃ for 2 min.

Synthesis of 5-ASA-UDCA

The 5-ASA-UDCA was synthesized according to the modified method of Bergstöm and Norman.[19] Briefly, UDCA (1 g, 2.55 mmol) was dissolved in 15 mL of dioxane and 0.57 mL tri-*n*-butylamine was added. To the cold solution, 0.33 mL of ethyl chloroformate was added and the contents were kept at 10℃ for 15 min. A solution of 5-ASA (510 mg, 3.33 mmol) in 4 mL 1 N sodium hydroxide was then added to the suspension and the clear solution obtained was stirred for 1 h at room temperature. The contents were poured into 60 mL ice-cold water and acidified with 1 N hydrochloric acid to pH 1. The grey-colored precipitate was filtered and washed thoroughly with water. The product (1,031 mg) was poured over a column of silica gel (60 g) and eluted with chloroform followed by increasing proportions of methanol. Elution with chloroform (0.5 L) and chloroform-methanol mixture (9:1 v/v, 0.5 L) yielded unreacted UDCA. Further elution of the column with chloroform-methanol mixture (1:1 v/v, 1 L) yielded 950 mg (71% overall yield) of a light grey solid and re-crystallized from methanol–ethyl acetate to cream-colored microscopic crystals. It was a single spot on TLC with Rf of 0.56 when developed with a mixture of benzene, dioxane, and acetic acid (15: 5: 2, v/v/v).

Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL JNM-400S at 400 MHz (JEOL Ltd., Tokyo, Japan). Chemical shifts are reported relative to Me_4Si (δ 0.00). NMR spectra were measured with DMSO-d_6 (δ 2.49). The carbon nuclear magnetic resonance (^{13}C- NMR) spectroscopic data were recorded with a JEOL JNM-400S at 101 MHz. Chemical shifts are reported relative to DMSO-d_6 (δ 39.7). Mass spectra were recorded on Waters XEVO G2-XS QToF mass spectrometer.

Synthesis of 5'-ASA-UDCA-3-glucuronide

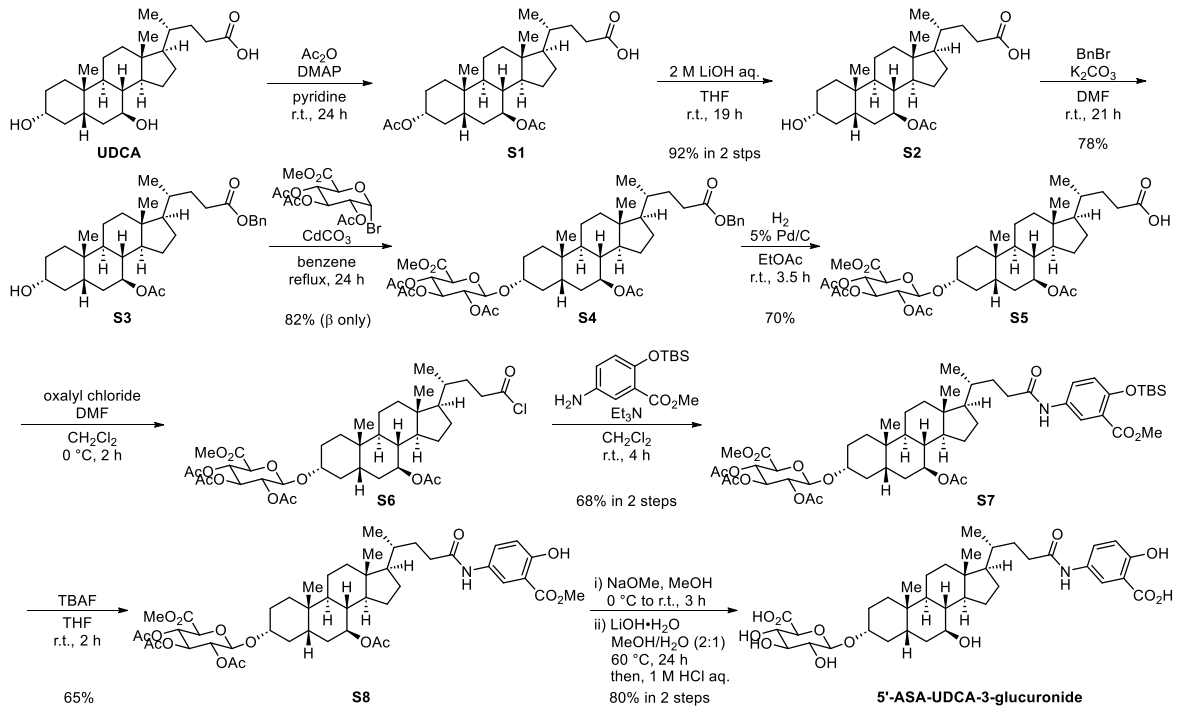

Figure 3: Synthesis of 5'-ASA-UDCA-3-glucuronide from UDCA.

The 5'-ASA-UDCA-3-glucuronide was synthesized using a reported method with extensive modifications.[20,21] To a solution of UDCA (4.00 g, 10.0 mmol) in dry pyridine (20.0 mL) was added DMAP (0.12 g, 1.00 mmol) and acetic anhydride (9.45 mL, 100 mmol) at 0 °C under N_2 atmosphere. After being stirred at room temperature for 24 h, the reaction mixture was added CH_2Cl_2 and acidified with 2 M aqueous solution of HCl. The mixture was extracted with CH_2Cl_2, washed with 2 M aqueous solution of HCl and dried over $MgSO_4$. Concentration under reduced pressure gave a crude product of 3α,7β-diacetoxy-5β-cholanoic acid (**S1**) as a colorless amorphous solid. The crude product was used in the next step without further purification.

To a solution of the crude product of **S1** (10.0 mmol) in THF (50.0 mL) was added 2 M aqueous solution of LiOH (50.0 mL, 100 mmol) at room temperature. After being stirred at room temperature for 19 h, the reaction mixture was acidified with 1 M aqueous solution of HCl and added EtOAc. The mixture was extracted with EtOAc, washed with brine and dried over Na_2SO_4. Concentration under reduced pressure followed by flash column chromatography over silica with *n*-hexane-EtOAc (1:1) gave 7β-acetoxy-3α-hydroxy-5β-cholanoic acid (**S2**) (4.02 g, 92% yield in 2 steps) as a white solid. This compound was used immediately in the next step.

To a solution of the 7β-acetoxy-3α-hydroxy-5β-cholanoic acid (**S2**) (4.02 g, 9.25 mmol) in DMF (18.5 mL) was added K_2CO_3 (1.60 g, 11.6 mmol) at room temperature. After being stirred at room temperature for 30 min, the suspension was added benzyl bromide (1.37 mmol, 11.6 mmol). After being stirred at room temperature for 21 h, the reaction mixture was quenched with water and added *n*-hexane-EtOAc (4:1). The mixture was extracted with *n*-hexane-EtOAc (4:1), washed with water, brine and dried over Na_2SO_4. Concentration under reduced pressure followed by flash column chromatography over silica with *n*-hexane-EtOAc (3:1) gave benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate (**S3**) (3.77 g, 78% yield) as a white solid.

To a solution of the benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate (**S3**) (1.85 g, 3.53 mmol) in dry benzene (35.3 mL) was added $CdCO_3$ (3.04 g, 17.6 mmol) and acetobromo-α-D-glucuronic acid methyl ester (2.80 g, 7.05 mmol) at room temperature under Ar atmosphere. After being stirred at reflux for 24 h, the reaction mixture was filtered through a pad of celite, washed with EtOAc. Concentration under reduced pressure followed by flash column chromatography over silica with *n*-hexane-EtOAc (3:1) gave benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate 3-1'β-glucuronide methyl ester-triacetate (**S4**) (2.44 g, 82% yield) as a white amorphous solid, which was observed only b-isomer for anomeric position in glucuronide by ^{1}H-NMR.

To a solution of the benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate 3-1'β-glucuronide methyl ester-triacetate (**S4**) (2.44 g, 2.90 mmol) in EtOAc (29.0 mL) was added 5% Pd/C (0.24 g, 10 wt%) at room temperature. After being stirred at room temperature for 4 h under H_2 atmosphere, the reaction mixture was filtered through a pad of celite, washed with EtOAc. Concentration under reduced pressure followed by trituration with Et_2O gave 7β-acetoxy-3α-hydroxy-5β-cholanoic acid 3-1'β-glucuronide methyl ester-triacetate (**S5**) (1.52 g, 70% yield) as a white solid.

To a solution of the 7β-acetoxy-3α-hydroxy-5β-cholanoic acid 3-1'β-glucuronide methyl ester-triacetate (**S5**) (2.88 g, 3.83 mmol) in CH_2Cl_2 (19.2 mL) was added oxalyl chloride (0.66 mL, 7.66 mmol) and a catalytic amount of dry DMF (one drop) at 0°C under Ar atmosphere. After being stirred at 0°C for 2 h, the solvent was removed under reduced pressure followed by drying under vacuo for 30 min gave a crude product of 7β-acetoxy-3α-hydroxy-5β-

cholanoyl chloride 3-1'β-glucuronide methyl ester-triacetate (**S6**) as a colorless amorphous solid. The crude product was used immediately in the next step without further purification.

To a solution of the crude product of 7β-acetoxy-3α-hydroxy-5β-cholanoyl chloride 3-1'β-glucuronide methyl ester-triacetate (**S6**) (3.83 mmol) in CH_2Cl_2 (33.3 mL) was added Et_3N (0.80 mL, 5.75 mmol) and a solution of methyl 5-amino-2-*O*-(*tert*-butyldimethylsilyl)salicylate (2.16 g, 7.66 mmol) in CH_2Cl_2 (5.00 mL) dropwise at 0 °C under Ar atmosphere. After being stirred at room temperature for 4.5 h, the reaction mixture was quenched with saturated aqueous solution of $NaHCO_3$. The mixture was extracted with CH_2Cl_2, washed with water and dried over Na_2SO_4. Concentration under reduced pressure followed by flash column chromatography over silica with *n*-hexane-EtOAc (2:1) gave *N*-(4''-(*tert*-butyldimethylsilyl)oxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholan- 24-amide 3-1'β-glucuronide methyl ester-triacetate (**S7**) (2.59 g, 68% yield in 2 steps) as a brown amorphous solid.

To a solution of the crude product of *N*-(4''-(*tert*-butyldimethylsilyl)oxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholan- 24-amide 3-1'β-glucuronide methyl ester-triacetate (**S7**) (2.54 g, 2.51 mmol) in THF (12.5 mL) was added 1.0 M THF solution of tetrabutylammonium fluoride (5.02 mL, 5.02 mmol) at room temperature under Ar atmosphere. After being stirred at room temperature for 2 h, the reaction mixture was added water and diluted with EtOAc. The mixture was extracted with EtOAc, washed with water and dried over Na_2SO_4. Concentration under reduced pressure followed by flash column chromatography over silica with *n*-hexane-EtOAc (2:1) gave the brown amorphous solid, which was triturated with EtOAc gave *N*-(4''-hydroxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholano-24-amide 3-1'β-glucuronide methyl ester-triacetate (**S8**) (1.46 g, 65% yield) as a white solid.

To a suspension of *N*-(4''-hydroxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholano-24-amide 3-1'β-glucuronide methyl ester-triacetate (**S8**) (1.40 g, 1.56 mmol) in MeOH (31.2 mL) was added sodium methoxide (25.3 mg, 0.47 mmol) at 0 °C. After being stirred at room temperature for 3 h, the reaction mixture was added water (15.6 mL), lithium hydroxide monohydrate (1.35 g, 31.2 mmol) at room temperature. After being stirred at 60 °C for 24 h, the reaction mixture was acidified with 1 M aqueous solution of HCl, and then the precipitate appeared. The precipitate was collected and washed with water, dried under vacuo gave 5'-ASA-UDCA-3-glucuronide (*N*-(3''-carboxy-4''-hydroxyphenyl)-7β-acetoxy-3α-hydroxy-5β-cholano-24-amide 3-1'β-glucuronide, 0.88 g, 80% yield in 2 steps) as an off-white solid. 5'-ASA-UDCA-3-glucuronide were a single spot on TLC, Rf, 0.14 (EtOAc:MeOH: water; 6:2:1, v/v/v).(Figure 3)

Synthesis of 5'-ASA-UDCA-3,7-disulfate and 5'-ASA-UDCA-3-sulfate

The disulfated ester 2Na of 5-ASA-UDCA, 5-ASA-UDCA-DS, was prepared by modifying the method of Goto.[22] Briefly, to 5-ASA-UDCA (1.0 g, 1.90 mmol) solution in anhydrous pyridine (10 mL), ice-cold chlorosulphonic acid (1.0 mL, 8.58 mmol) solution in anhydrous pyridine (10 mL) was added, and the mixture solution was then heated overnight at 50°C. In the case of 5-ASA-UDCA-3S, ice-cold chlorosulphonic acid (0.25 mL, 2.15 mmol) solution in anhydrous pyridine (10 mL) was added to a solution of 5-ASA-UDCA (1.0 g, 1.90 mmol) in anhydrous pyridine (10 mL), and the mixture solution was heated for 2h at 50°C. The solution was poured into ice water, acidified with concentrated HCl, and extracted with *n*-butanol. The organic layer was washed with distilled water, dried over anhydrous Na_2SO_4, mixed with a few drops of concentrated NH_4OH, and evaporated in vacuo. 5-ASA-UDCA-DS yielded 948 mg (68% overall yield) of a purple solid and it was re-crystallized from 4 mL of 1 N NaOH methanol–

diethyl ether. The product of 5-ASA-UDCA-3S was poured over a column of silica gel (60 g) and eluted with chloroform followed by increasing proportions of methanol. Elution of the column with chloroform-methanol (4:1 v/v, 1 L) yielded 650 mg (56% overall yield) of a light grey solid, and it was re-crystallized from methanol–ethyl acetate to cream-colored microscopic crystals. The obtained 5-ASA-UDCA-DS and 5-ASA-UDCA-3S were a single spot on TLC with Rf values of 0.31 and 0.48, respectively when developed with a mixture of *n*-butanol, acetic acid and water (15: 5: 2, v/v/v).

Partition Coefficients

Partition coefficients of 5-ASA-UDCA-derivatives were determined in the same manner as reported previously.[23] Briefly, chloroform and 0.1 M phosphate buffer solutions (PBS: pH 4.0 and pH 6.5) were mutually saturated before experiments. 5-ASA-UDCA-derivatives were dissolved in PBS at 100 µg/mL each. The supernatant was filtrated through a syringe filter with 0.22 µm of pore size (Millipore, Tokyo, Japan). Four mL of each drug solution was added to 4 mL of chloroform, and the mixture was vigorously shaken for 30 min at 25 ◦C. After centrifugation at 3000 ×g for 10 min, the concentration of the compound in both phases was determined by HPLC after hydrolysis of 5-ASA-UDCA-derivatives with 6 N HCl in a boiling bath for 60 min.

Chemical and Enzymatic Stability

The chemical and enzymatic stability of 5-ASA-UDCA-derivatives and salazosulfapyridine were evaluated in the same manner as reported previously.[17,23] Briefly, the chemical stability of 5-ASA-UDCA-derivatives and salazosulfapyridine was measured in 0.2M Tris-HCl buffer solutions (pH 4.0 and pH 8.0). Enzymatic stability of 5-ASA-UDCA-derivatives and salazosulfapyridine at 0.1 mM was determined at 0, 1, 3, and 6 h after the start of incubation at 37 ◦C in the same manner as described previously using the following specimen: 10% plasma, 5 % small intestinal mucosal homogenates, 5 % liver homogenates, and 5 % colon feces. Each biological specimen was obtained from male SD rats and was prepared using pH 7.4, 25 mM Tris-HCl buffer. Concentrations of 5-ASA released from 5-ASA-UDCA-derivatives and salazosulfapyridine in the reaction mixture were determined periodically after the start of incubation, in which the further metabolic reaction after sampling was stopped by adding methanol at a volume ratio of 1:2. Male Sprague Dawley (SD) rats were purchased from Japan SLC, Inc. (Hamamatsu, Japan), and kept in stainless steel cages equipped with an automatic water supply and excrement flushing device. The animal study was performed according to the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Fukuyama University (2023-A-10).

Hydrolysis of 5-ASA-UDCA Derivatives by Cholylglycine Hydrolase

Incubation of 5-ASA-UDCA conjugates with cholylglycine hydrolase was carried out according to the method reported by Nair.[24] Solutions of 5-ASA-UDCA and 5-ASA-UDCA-3G with 5 different concentrations, or 5 mL of reaction mixture containing 0.2 mM, 0.1 mM, 0.05 mM, 0.025 mM, and 0.0125 mM were used. The sodium salt of 5-ASA-UDCA and 5-ASA-UDCA-3G were dissolved in 2.5 mL of pH 5.6 acetate buffer solution containing 0.75% mercaptoethanol (1.0 mL), 1.86% ethylenediaminetetraacetic acid (1.0 mL) and 0.5 mL of cholylglycine hydrolase solution (2 mg/mL in water) were added. In the case of 5-ASA-UDCA-3S and 5-ASA-UDCA-DS, higher concentrations (2 mM, 1 mM, 0.5 mM, and 0.25 mM) were employed because of the lower hydrolysis. The incubation mixture was shaken at 37°C for 0, 3, 5, 10, 20, 30, 120, 180, and 240 min, and 5-ASA released was determined by HPLC.

Membrane Transports of 5-ASA-UDCA Derivatives in Rats' Everted Gut Sac

Rats weighing 200 to 250 g were fasted for 24 hours before experiments after feeding for several days. Under overdose pentobarbital anesthesia, laparotomy was performed, and the whole intestine was rinsed with ice-cold 0.9% NaCl solution. The small intestine, cecum, and colon were removed and everted gut sacs were prepared over a glass rod according to the method reported: 10 cm long 4 sacs from the small intestine, 1 sac from the cecum, and 2 sacs from the colon, respectively.[25-27]

The sacs were filled with a modified Krebs'-Ringer's-bicarbonate solution (pH 7.4) of the following composition: 128 mM NaCl, 1.2 mM $CaCl_2$, 0.7 mM $MgSO_4$, 5.1 mM KCl, 1.3 mM KH_2PO_4, 25 mM $NaHCO_3$, and 9 mM glucose. The initial serosal and mucosal concentrations of 5-ASA-UDCA-derivatives were set at 0.2 mM, and the sacs were incubated at 37℃ under continuous shaking and gassing with 95 % O_2-5 % CO_2 for 60 min. At the end of the incubation period, the concentrations of 5-ASA in the serosal and mucosal side medium were determined by HPLC after hydrolysis of 5-ASA-UDCA-derivatives with 6 N HCl in a boiling water bath for 60 min.

Distribution Study of 5-ASA into the Intestinal Membrane after Oral Administration of Salazosulfapyridine and 5-ASA-UDCA Derivatives in Fed Rats

Male SD rats weighing 200 to 250 g were used without fasting in experiments. 5-ASA-relating compounds such as salazosulfapyridine (78 mg/kg), 5-ASA-UDCA (103 mg/kg), 5-ASA-UDCA-3G (138 mg/kg), 5-ASA-UDCA-3S (119 mg/kg), and 5-ASA-UDCA-DS (143 mg/kg) were administered orally by stomach intubation at a dose of 30 mg/kg as 5-ASA. After 8 h of oral administration, laparotomy was performed under overdose pentobarbital anesthesia, and the intestinal tract, cecum, and colon were opened and cleaned with ice-cold 0.9% NaCl solution, immediately frozen in liquid nitrogen, and stored at -80℃ until analysis. The small intestine was divided into 4 segments with an equal length, the cecum was 1 segment, and the colon was 3 segments, and 10% (w/w) homogenates in ice-cold 0.9% NaCl solution were prepared by homogenizer (POLYTRON® PT1200E, KINEMATICA, Switzerland). The membrane concentrations of 5-ASA and AC-5-ASA in each segment were determined by HPLC.

Analysis of 5-ASA and AC-5ASA by HPLC

The 5-ASA and AC-5-ASA concentrations in the samples were determined using HPLC and 4-ASA as the internal standard (I.S.) in the same manner as reported previously.[4, 28] An L-column 2 ODS (150 mm × 4.6 mm, particle size 5 µm, CERI Co., Ltd., Saitama, Japan) was used, and the mobile phase was a mixture of 0.1 M acetic acid, acetonitrile, and triethylamine (920:100:2, v/v/v). The mobile phase flow rate was 1 mL/min. Detection was achieved using a fluorescence detector at 315 and 430 nm for the excitation and emission wavelengths, respectively. Each 5-ASA-containing sample (100 µL) was mixed with 900 µL of methanol for deproteinization. The mixture was vigorously mixed by a vortex mixer for 30 s and centrifuged at 15,000× g for 5 min at 4 °C, and all the supernatant was collected and evaporated. Then, 100 µL of I.S. solution (5 µg of 4-ASA/mL), 900 µL of 0.05 M phosphate buffer (pH 7.4), and 100 µL of propionic anhydride were added, and the reaction solutions were incubated at 37 °C for 1 h. The compounds of interest were extracted with 4 mL of acetonitrile after adding 1 mL of 10% sodium chloride solution. Liquid phases were separated by centrifugation at 3000× g for 5 min after cooling at 4 °C. The organic layer was removed and evaporated to dryness under the air. The residue was dissolved in 250 µL of mobile phase, and 10 µL aliquots were injected into the HPLC column. The HPLC system (Shimadzu, Kyoto, Japan) comprised a model LC20AD pump, a 20 µL fixed injection loop, and a fluorescence spectrophotometer (Shimadzu, RF10AXL). Data acquisition was performed

with the Sept 3000 processor (Hangzhou, China). The retention times for the AC-5-ASA, Prop-5-ASA, and Prop-4-ASA (I.S.) were 4.8, 8.7, and 10.6 min, respectively. The correlation coefficient (r) values of the calibration curves for 5-ASA and AC-5-ASA were more than 0.9997 and 0.9999, respectively, with a low Y-intercept value and variation within 5% in a concentration range from 1 to 10 µg/mL for both. The 5-ASA and AC-5-ASA detection limits were 100 and 50 ng/mL (or ng/g), respectively.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the unpaired Student's *t*-test, in which p-values less than 0.05 were regarded as statistically significant.

RESULTS

5-ASA-UDCA

5-ASA-UDCA was newly synthesized in the present study. The synthesized yield of 5-ASA-UDCA was 71 %. The purity of 5-ASA-UDCA was high since the ^{1}H-NMR spectral data displayed only specific signals of 5-ASA-UDCA. The purity of 5-ASA-UDCA was also evaluated by TLC using a mixture of benzene: dioxane: acetic acid (15: 5: 2, v/v/v) as a developing solvent. The spot detected using shortwave ultraviolet light (254 nm) was one spot, and the Rf values of UDCA and 5-ASA-UDCA were 0.88 and 0.56, respectively. Data: ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 0.59 (s, 3H), 0.84 (s, 3H), 0.90 (d, J = 5.9 Hz, 3H), 1.00-1.93 (m, 23H), 2.10-2.28 (m, 2H), 6.76 (d, J = 8.7Hz, 1H), 7.52 (dd, J = 8.9, 2.5 Hz, 1H), 7.97 (d, J = 2.7 Hz, 1H), 9.74 (s, 1H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ: 12.8, 19.2, 21.7, 24.0, 27.4, 29.0, 30.5, 32.4, 34.0, 34.4, 35.4, 35.8, 37.4, 38.0, 40.6, 42.8, 43.6, 43.9, 55.4, 56.5, 70.5, 70.7, 115.8, 117.5, 122.1, 127.6, 130.8, 157.8, 172.5, 173.2 ESI-HRMS *m/z*: calcd for $C_{31}H_{44}NO_6^-$ $[M-H]^-$ 526.3174, found 526.5559.
Chemical formula: $C_{31}H_{45}NO_6$. Molecular weight: 527.7020.

5'-ASA-UDCA-3-glucuronide

The synthesized overall yield of 5'-ASA-UDCA-3-glucuronide was 15 % from UDCA. The purity of 5'-ASA-UDCA-3-glucuronide was high since the ^{1}H-NMR spectral data displayed only specific signals of 5'-ASA-UDCA-3-glucuronide. The purity of 5'-ASA-UDCA-3-glucuronide was also evaluated by TLC using a mixture of EtOAc: MeOH: water (6:2:1, v/v/v) as a developing solvent. The spot detected using shortwave ultraviolet light (254 nm) was one spot, and the Rf values of 5'-ASA-UDCA-3-glucuronide was 0.14.

Data: ^{1}H-NMR (400 MHz, CD_3OD) 0.72 (s, 3H), 0.91-1.61 (m, 22H), 1.65-1.98 (m, 8H), 2.00-2.10 (m, 1H), 2.20-2.32 (m, 1H), 2.35-2.47 (m, 1H), 3.19 (dd, *J* = 9.1, 7.8 Hz, 1H), 3.37 (t, *J* = 9.6 Hz, 1H), 3.43-3.52 (m, 1H), 3.52 (t, *J* = 9.1 Hz, 1H), 3.59-3.71 (m, 1H), 3.78 (d, *J* = 9.6 Hz, 1H), 4.65 (d, *J* = 7.8 Hz, 1H), 6.89 (d, *J* = 9.1 Hz, 1H), 7.60 (dd, *J* = 9.1, 2.7 Hz, 1H), 8.07 (d, *J* = 2.7 Hz, 1H).

^{13}C-NMR (100 MHz, CD_3OD) 12.6, 19.1, 22.4, 23.9, 27.6, 28.0, 29.7, 33.3, 34.8, 35.3, 35.9, 36.1, 36.9, 38.6, 40.7, 41.6, 44.0, 44.5, 44.8, 56.5, 57.5, 71.9, 73.2, 74.8, 76.6, 77.5, 80.0, 102.6, 113.5, 118.3, 123.3, 129.7, 131.5, 159.8, 172.5, 173.2, 175.1.

ESI-HRMS *m/z*: calcd for $C_{37}H_{52}NO^-$ $[M-H]^-$ 702.3495, found 702.3538.
IR (ATR) 3374 (OH), 3228 (NH), 1727 (C=O), 1650 (C=O)
Chemical formula: $C_{37}H_{53}NO_{12}$. Molecular weight: 703.8260.

5-ASA-UDCA-DS

5-ASA-UDCA-DS was prepared by modifying the method of Goto.[17,22] The synthesized yield of 5-ASA-UDCA-DS was 68 %. The purity of 5-ASA-UDCA-DS was high since the ^{l}H-NMR spectral data displayed only specific signals of 5-ASA-UDCA-DS. The purity of 5-ASA-UDCA-DS was also evaluated by TLC using a mixture of ethanol: ammonia solution (3: 1, v/v) as a developing solvent. The spot detected using shortwave ultraviolet light (254 nm) was one spot, and the Rf values of 5-ASA-UDCA and 5-ASA-UDCA-DS were 0.86 and 0.31, respectively. Data: ^{1}H NMR (400 MHz, D_2O) δ: 0.50 (s, 3H), 0.78 (s, 3H), 0.81 (d, J = 6.4 Hz, 3H), 0.90-1.00 (m, 2H), 1.04-1.86 (m, 22H), 2.11-2.22 (m, 2H), 4.10-4.17 (m, 2H), 6.45 (d, J = 8.7 Hz, 1H), 6.91 (dd, J = 8.7, 2.7 Hz, 1H), 7.01 (d, J = 2.7 Hz, 1H). ^{13}C-NMR (100 MHz, D2O) δ -0.0, 14.1, 20.8, 23.6, 25.1, 28.7, 29.8, 30.8, 34.7, 35.9, 36.1, 36.7, 37.5, 41.7, 42.1, 43.8, 44.7, 46.1, 57.1, 60.1, 83.5, 84.9, 122.4, 126.0, 126.9, 129.5, 164.2,179.5, 180.5.

ESI-HRMS *m/z*: calcd for $C_{31}H_{43}NNaO_{12}S_2^-$ $[M+Na-2H]^-$ 708.2130, found 708.1800. ESI-HRMS *m/z*: calcd for $C_{31}H_{42}NNa_2O_{12}S_2^-$ $[M+2Na-3H]^-$ 730.1949, found 730.1567.
Chemical formula: $C_{31}H_{45}NO_{12}S_2$ Molecular weight: 687.8160.

5-ASA-UDCA-3S

5-ASA-UDCA-3S was prepared by modifying the method of Goto.[17,22] The yield of 5-ASA-UDCA-3S was 59 %. The purity of 5-ASA-UDCA-3S was high since the ^{l} H-NMR spectral data displayed only specific signals of 5-ASA-UDCA-DS. The purity of 5-ASA-UDCA-3S was also evaluated by TLC using a mixture of ethanol: ammonia solution (3: 1, v/v) as a developing solvent. The spot detected using shortwave ultraviolet light (254 nm) was one spot, and the Rf value of 5-ASA-UDCA-3S was 0.48. Data: ^{1}H-NMR (400 MHz, CD_3OD) δ: 0.67-0.75 (m, 3H), 0.85-2.11 (m, 30H), 2.15-2.31 (m, 1H), 2.32-2.46 (m, 1H), 3.40-3.54 (m, 1H), 4.13-4.35 (m, 1H), 6.79-6.73 (m, 1H), 7.42-7.55 (m, 1H), 7.82-7.94 (m, 1H)

ESI-HRMS *m/z*: calcd for $C_{31}H_{44}NO_9S^-$ $[M-H]^-$ 606.2742, found 606.2740. ESI-HRMS *m/z*: calcd for $C_{31}H_{43}NO_9S^{2-}$ $[M-2H]^{2-}$ 302.6335, found 302.6425.
Chemical formula: $C_{31}H_{45}NO_9S$ Molecular weight: 607.7590.
When 5-ASA-UDCA-3S was analyzed by LC-MS (Acquity UPLC - Xevo G2-XS Q TOF/TOF system, Waters Corporation, Milford, USA), it was found to be a mixture of 5'-ASA-UDCA-3-sulfate (sulfation of 3-OH group of UDCA) and 5'-ASA-UDCA-7-sulfate (3 : 1).

Spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B), and ESI-MS (C) of 5-ASA-UDCA; spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B) and ESI-MS (C) of 5-ASA-UDCA-3G; The ^{1}H and ^{13}C-NMR spectral data of compound **S3-S5, S7-S8** see Supplementary Materials. Spectrum of ^{1}H-NMR (A), ESI-MS (B), and LC-MS (C) of 5-ASA-UDCA-3S; spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B), and ESI-MS (C) of 5-ASA-UDCA-DS are shown in Figures S1–S4, respectively as Supplementary Materials.

Partition Coefficient of 5-ASA-UDCA Derivatives

To evaluate the lipophilicity of 5-ASA-UDCA derivatives, their partition coefficients (PCs) were determined using a partition system of chloroform and (pH 4.0 and pH 6.5) PBS buffer (Table 1). At pH 4.0 and pH 6.5, the apparent PC of 5-ASA-UDCA was 38-75-fold and 2.5-4.3-fold higher than 5-ASA-UDCA-conjugates, respectively. All three 5-ASA-UDCA-conjugates were hydrophilic compared to 5-ASA-UDCA.

Table 1: Partition Coefficients of 5-ASA-UDCA Derivatives.

Compound	P.C. at pH 4.0	P.C. at pH 6.5
5-ASA-UDCA	1.50	0.30
5-ASA-UDCA-3G	0.02	0.07
5-ASA-UDCA-3S	0.04	0.07
5-ASA-UDCA-DS	0.04	0.12

PC: Partition coefficient was determined in a chloroform/0.1 M phosphate buffered saline (pH 4.0 and pH 6.5) partition system at 25 ◦C.

Chemical and Enzymatic Stability of 5-ASA-UDCA Derivatives and Salazosulfapyridine

The stability of 5-ASA-UDCA derivatives and salazosulfapyridine in the small intestine, intestinal membrane, plasma, and liver was evaluated in vitro. 5-ASA-UDCA derivatives were fairly in pH 4.0 and pH 8.0 Tris-HCl buffer, 10 % plasma, 5 % intestinal mucosa homogenate, 5 % liver homogenate, and 5 % feces' homogenate, for 6 h, compared to salazosulfapyridine (Table 2).

Table 2: Stability of 5-ASA-UDCA derivatives and salazosulfapyridine in buffer solution, and enzymatic solution at 37℃.

Time (h)	Hydrolysis (%)					
	0.2 M Tris-HCl (pH4)	**0.2 M Tris-HCl (pH8)**	**5% Mucosal homogenate**	**5% Liver homogenate**	**10% plasma**	**5% feces**
5-ASA-UDCA						
1 h	0	0	0	0	0	0.1±0.1
3 h	0	0.3±0.1	0.1±0.1	0.3±0.1	0	1.2±0.5
6 h	0.7±0.2	2.9±0.5	0.4±0.1	0.8±0.2	0	1.8±0.3
5-ASA-UDCA-3G						
1 h	0	0	0	0	0	0
3 h	0	0	0	0	0	0
6 h	0	1.6±0.9	0	0	0	1.0±0.2
5-ASA-UDCA-3S						
1 h	0	0	0	0	0	0
3 h	0	0	0	0	0	0
6 h	0	0	0	0	0	0.7±0.7
5-ASA-UDCA-DS						
1 h	0	0	0	0	0	0
3 h	0	0	0	0	0	0
6 h	0.3±0.1	0.2±0.1	0.3±0.1	0.1±0.1	0	0.2±0.1
Salazosulfapyridine						
1 h	3.1±1.4	2.7±0.9	19.0±7.1	6.8±4.1	9.4±7.8	23.4±7.4
3 h	3.7±0.6	3.7±0.6	24.4±4.6	17.3±2.3	20.0±7.7	44.7±8.1
6 h	6.6±2.1	5.7±1.6	35.6±6.7	18.3±2.7	24.0±7.5	65.3±13.2

The initial concentration of each 5-ASA-related compound was 0.1 mM. All biological samples were taken from SD rats. Each value represents the mean±S.D. of 3 trials.

Hydrolysis of 5-ASA-UDCA Derivatives by Cholylglycine Hydrolase

The time courses of the enzymatic hydrolysis of 5-ASA-UDCA derivatives for 60 min are shown in Figure 4. 5-ASA-UDCA and 5-ASA-UDCA-3G were relatively rapidly hydrolyzed and released 5-ASA by cholylglycine hydrolase compared to 5-ASA-UDCA-3S and 5-ASA-UDCA-DS. In the case of glycine- and/or taurine-conjugated bile acids, cholylglycine hydrolase deconjugates and releases free bile acids. The Km values estimated by the Lineweaver-Burk

plot of these data were 0.053 mM for 5-ASA-UDCA, 0.078 mM for 5-ASA-UDCA-3G, 2.91 mM for 5-ASA-UDCA-3S, and 0.93 mM for 5-ASA-UDCA-DS, respectively (Figure 5).

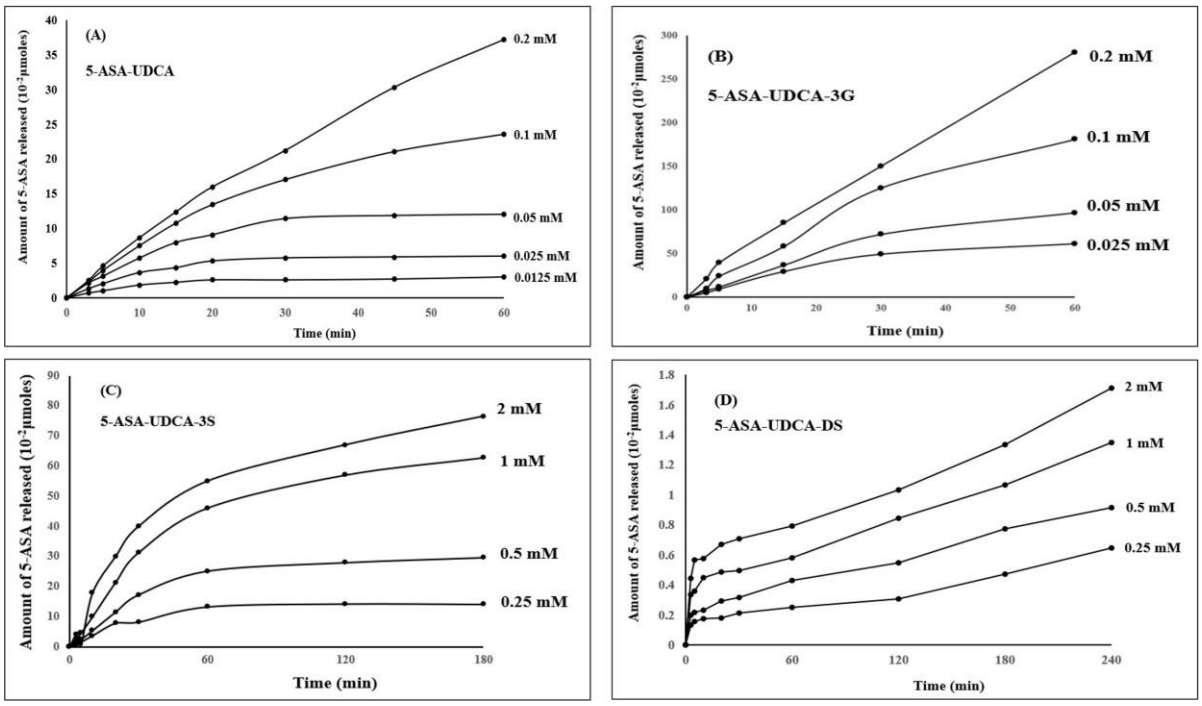

Figure 4: Time course of enzymatic hydrolysis of 5-ASA-UDCA (A), 5-ASA-UDCA-3G (B), 5-ASA-UDCA-3S (C), and 5-ASA-UDCA-DS (D) by cholylglycine hydrolase.

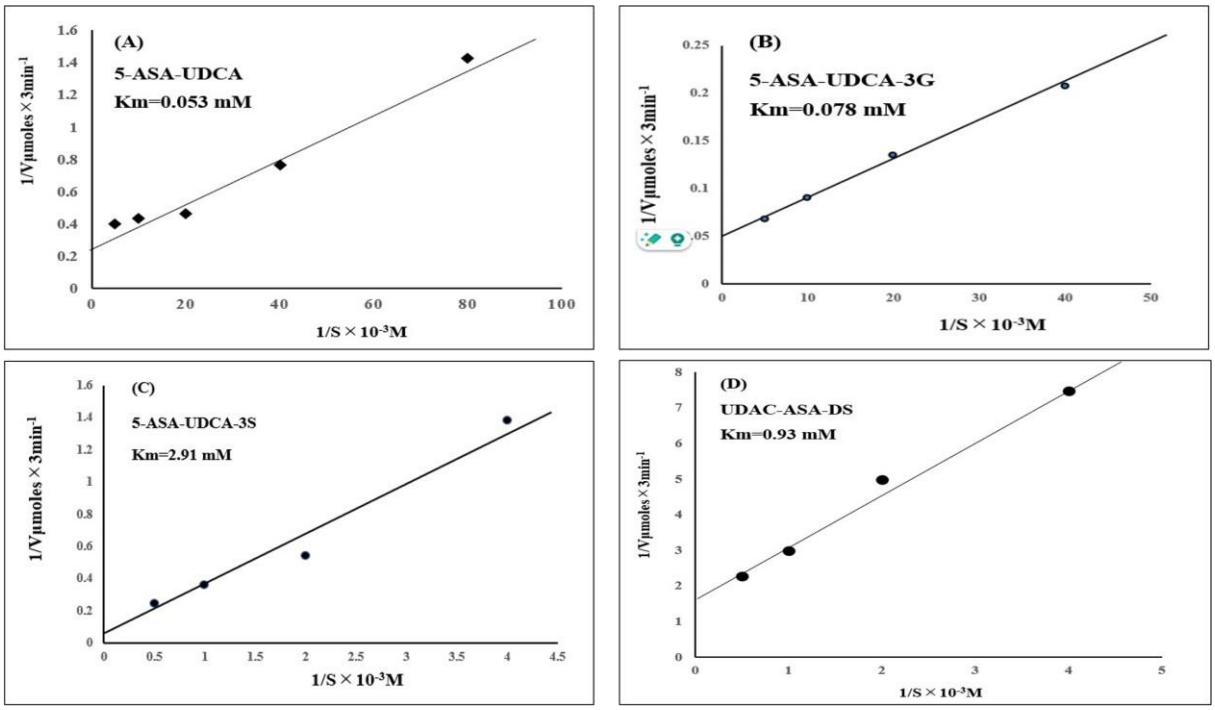

Figure 5: Lineweaver-Burk Plot of the Hydrolysis of 5-ASA-UDCA derivatives by cholylglycine hydrolase. The estimated Km values were 0.053 mM for 5-ASA-UDCA, 0.078 mM for 5-ASA-UDCA-3G, 2.91 mM for 5-ASA-UDCA-3S, and 0.93 mM for 5-ASA-UDCA-DS.

Transport Characteristics of 5-ASA-UDCA Derivatives in Everted Sac of Rat's Intestine

The contribution of active transport in the intestinal membrane permeability of 5-ASA-UDCA derivatives was examined using a rat's everted sac of the segmental intestine. 5-ASA-UDCA alone showed an active transport in the ileum, whereas other UDCA conjugated derivatives didn't show such active transport (Figure 6).

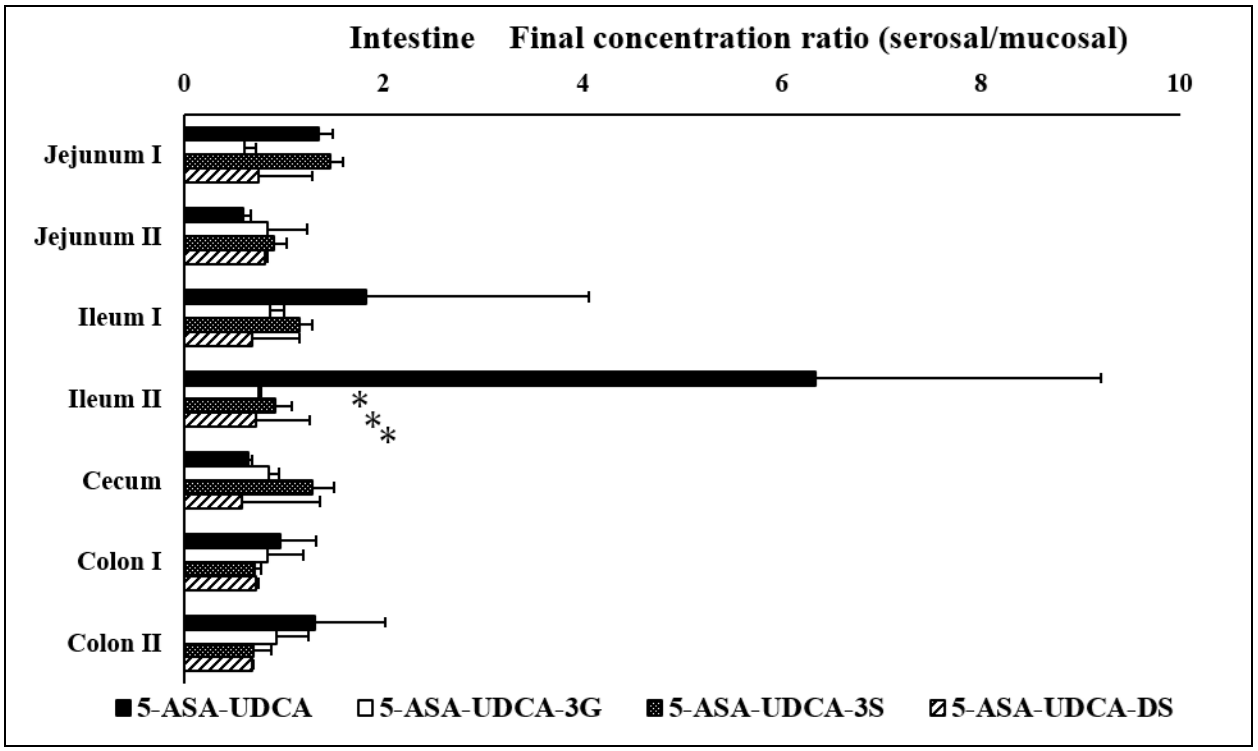

Figure 6: Transport of 5-ASA-UDCA derivatives across the everted gut sacs prepared from various segments of the rat intestine. The initial concentration of each 5-ASA-UDCA derivative was 0.2 mM in both the serosal and mucosal compartments, and incubation was made for 60 min at 37°C. Concentrations of each derivative in both compartments were measured as 5-ASA after hydrolysis. Each bar represents the mean ±S.D. of three trials. *; $p<0.05$.

Concentrations of 5-ASA and AC-5-ASA in Each Intestinal Segment after Oral Administration of salazosulfapyridine and 5-ASA-UDCA Derivatives

The oral dose of these compounds was 30 mg/kg as 5-ASA, and the concentrations of 5-ASA and AC-5-ASA were determined 8 h after oral administration. Salazosulfapyridine, 5-ASA-UDCA and 5-ASA-UDCA-3G showed higher total concentrations of 5-ASA and AC-5-ASA compared to those after 5-ASA-UDCA-DS and 5-ASA-UDCA-3S. The rank order of the total concentrations of 5-ASA and AC-5-ASA in the colonic membranes was as follows: 5-ASA-UDCA-3G $\geq$ 5-ASA-UDCA $\geq$ salazosulfapyridine >> 5-ASA-UDCA-DS = 5-ASA-UDCA-3S, although there was no significant difference among 5-ASA-UDCA-3G, salazosulfapyridine, and 5-ASA-UDCA. (Figure 7, Table 3)

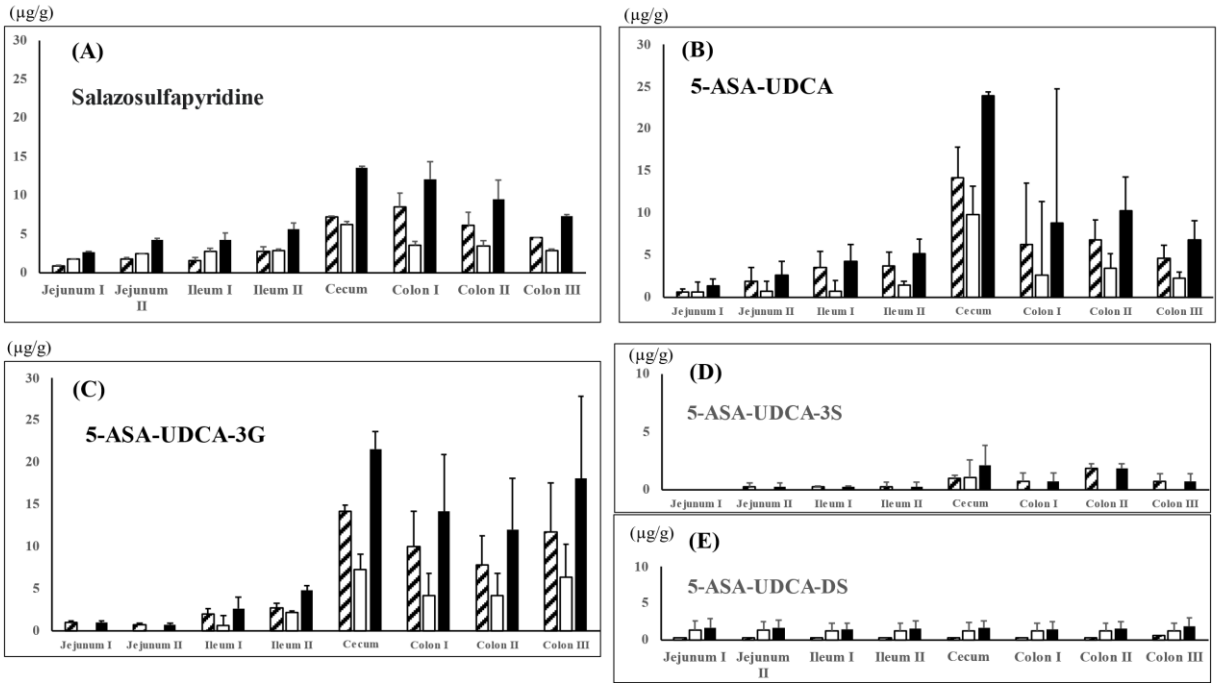

Figure 7: Concentrations of 5-ASA and AC-5-ASA in each intestinal segment 8 h after oral administration. Salazosulfapyridine (A), 5-ASA-UDCA (B), 5-ASA-UDCA-3G (C), 5-ASA-UDCA-3S (D), and 5-ASA-UDCA-DS (E) to fed rats. The dose was 30 mg/kg as AC-5-ASA concn. ▨; 5-ASA. 5-ASA concn., □; total (5-ASA + AC-5-ASA) concn., ■. Each value represents the mean ± S.D. of three trials.

Table 3: Total concentrations of 5-ASA and AC-5-ASA in the colonic regions 8 h after oral administration of 5-ASA-relative compounds at 30 mg/kg as 5-ASA in fed rats.

	Concentration (μg/g)	
Compounds / Tissue	**Colon I + II + III**	**Colon III**
Salazosulfapyridine	28.9±4.5	7.3±0.2
5-ASA-UDCA	42.6±21.4	8.2±2.3
5-ASA-UDCA-3G	44.2±18.2	18.1±9.7
5-ASA-UDCA-3S	2.9±1.5*	0.7±0.5
5-ASA-UDCA-DS	1.9±0.8*	1.3±1.2

Each value represents the mean±S.D. of three trials. *:Significantly different from others values (Salazosulfapyridine, 5-ASA-UDCA, and 5-ASA-UDCA-3G).

DISCUSSION

The targeted delivery of 5-ASA, the first-line therapy for UC, to the colonic region, is important because UC is the colonic inflammation. In the present study, several 5-ASA-UDCA derivatives were synthesized as carriers of 5-ASA and their targetability to the colonic region was evaluated by comparing them with salazosulfapyridine in fed rats. Salazosulfapyridine is known to be split into 5-ASA and sulfapyridine, a sulfonamide, by bacterial azo-reductase. In Caco-2 cells, 5-ASA permeates the membrane via the paracellular route[29], and via the transcellular route by the influx transporters such as organic anion transporting polypeptide 2B1 (OATP2B1) and sodium-coupled monocarboxylate transporter 1 (SMCT1), with a greater contribution of OATP2B1 than SMCT1.[30] In the mucosal membrane, a part of 5-ASA absorbed is metabolized to AC-5-ASA by N-acetyltransferase (NAT), and AC-5-ASA is also a useful biomarker of 5-ASA efficacy.[31]

AC-5-ASA is a substrate for ATP-dependent efflux transporter, multidrug resistance-associated protein 2 (MRP2)[32-34], suggesting that the intestinal absorption of AC-5-ASA itself is low, if any. Salazosulfapyridine is poorly absorbed (3 - 12% of dose) and its elimination half-life is about 5 to 10 hours. It is reported that salazosulfapyridine is split into 5-ASA and sulphapyridine by bacterial diazo reductase, at least 25% of the released sulphapyridine in the colon is absorbed and acetylated in the membrane and excreted into the urine, and 50% of sulphapyridine is eliminated in the feces.

Common adverse reactions of salazosulfapyridine at high serum levels are hepatitis, nausea, headache, anorexia, and hemolysis. Adverse reactions of 5-ASA reported are pancreatitis, hepatitis, and lung fibrosis, and the common adverse effects of sulfapyridin include headache, nausea, anorexia, malaise, and other allergic effects including fever, rash, hemolytic anemia, hepatitis, pancreatitis, paradoxical worsening of colitis, and reversible sperm abnormalities. These reports would suggest that the common side effects of salazosulfapyridine come mostly from sulfapyridine.[12,35-38]

Compared to sulfapyridine, UDCA may be safer with fewer side effects. As side effects of UDCA, diarrhea in a small portion of patients and drug interactions with cholestyramine, colestimide, colestipol, aluminum hydroxide and smectite in the absorption process are reported.[39] UDCA exhibiting hepatoprotective effects in liver diseases can improve functional dyspepsia and reduce methane values on lactulose breath tests in patients with small intestinal bacterial overgrowth.[40,41] In rats, hamsters and humans, the main metabolites of UDCA are glycoursodeoxycholic acid (GUDCA) and tauroursodeoxycholic acid (TUDCA)[42-44], and these UDCA and its amino acid-conjugates can reduce colitogenic dysbiosis and suppress experimental colitis (dextran sodium sulfate-induced colitis) in mice.[45] Separately, it was also reported that sulfation of the 3-hydroxy group is assumed to be a major metabolic route of UDCA.[46] The safety and tolerability of UDCA are reported under various disease states, for example, in patients with intrahepatic cholestasis of pregnancy[47], with Parkinson's disease[48], with amyotrophic lateral sclerosis[44], and so on.

UDCA, an enterohepatic drug as well as other bile acids, is absorbed by the apical sodium-dependent bile acid transporter (ASBT) preferentially in the ileum and enters into the portal circulation by heteromeric organic solute transporter OSTalpha-OSTbeta. Conjugated UDCA is taken up into hepatocytes from plasma by human Na(+) taurocholate cotransporting polypeptide (NTCP), organic anion transporting polypeptide (OATP)1B1, and OATP1B3 expressed on the sinusoidal membrane, whereas UDCA is transported significantly by NTCP. These bile acids and conjugates are secreted into the intestinal lumen by the bile salt export pump (BSEP) via the bile canalicular membrane.[49,50]

Bile acids including their conjugates excreted into the lumen are re-absorbed in the ileum by the apical sodium-dependent bile acid transporter.[50] In the present study, 5-ASA-UDCA, 5-ASA-UDCA-3G, 5-ASA-UDCA-3S and 5-ASA-UDCA-DS were synthesized (Figure 1). Then, to evaluate their efficacy as a colon-targeted prodrug, in vitro studies such as their chemical stabilities, enzymatic stabilities in biological samples, hydrolytic stabilities against cholylglycine hydrolase (Table 2 and Figures 4 and 5, in vitro membrane permeability study across the everted sac (Figure 6), and in vivo studies to compare the intestinal membrane distribution of 5-ASA between 5-ASA-UDCA derivatives and salazosulfapyridine after oral administration in fed rats were conducted (Figure 7). In these studies, 5-ASA-UDCA was found to be hydrolyzed chemically and by biological samples slowly in addition to cholylglycine hydrolase. In addition, 5-ASA-UDCA alone was actively absorbed in the ileum possibly as it is by ASBT (Figure 6). The 5-ASA-UDCA would act as an ASBT-targeted prodrug of 5-ASA, as well as many other solute carrier (SLC)

transporter-targeted prodrugs.[47] Thus, although 5-ASA-UDCA showed higher distribution to the colonic region as well as the case of salazosulfapyridine in in-vivo studies, it was suspected that the colonic distribution of 5-ASA from 5-ASA-UDCA could be varied among individuals due to the chemically and enzymatically instability of 5-ASA-UDCA in the small intestinal lumen and loss of 5-ASA by the active absorption in the ileum. In addition, 5-ASA-UDCA was found to be relatively stable against intestinal and hepatic first-pass metabolism in the body. These properties may indicate that 5-ASA-UDCA is not an ideal prodrug of 5-ASA. In contrast, no active transport of 5-ASA-UDCA-3G was observed in the intestine including the ileum (Figure 6) and hydrolyzed by bacteria-mediated cholylglycine hydrolase (Figures 4 and 5). Thus, 5-ASA-UDCA-3G was thought to have good properties as a colon-targeted prodrug. In fact, in vivo studies, 5-ASA-UDCA-3G showed higher colonic distribution of 5-ASA, although a significant difference was not observed compared to salazosulfapyridine (Figure 7). Both 5-ASA-UDCA-DS and 5-ASA-UDCA-3S were fairly stable even against cholylglycine hydrolase (Figures 4 and 5), which suggests that 5-ASA is not fully released from 5-ASA-UDCA-DS and 5-ASA-UDCA-3S. The concentrations of 5-ASA and AC-5-ASA in the intestinal membrane after oral administration of these UDCA sulfate-conjugates were significantly lower than those after 5-ASA-UDCA, 5-ASA-UDCA-3G and salazosulfapyridin (Figure 7 and Table 3). Taken together, 5-ASA-UDCA-3G was thought to be a feasible prodrug of 5-ASA in the targeted delivery to the colonic region, although further study is necessary

CONCLUSION

In the present study, we evaluated the efficacy of 5-ASA-UDCA prodrugs in the colonic delivery of 5-ASA. The rank order of 5-ASA colonic delivery was as follows; 5-ASA-UDCA-3G $\geq$ 5-ASA-UDCA $\geq$ salazosulfapyridine >> 5-ASA-UDCA-DS = 5-ASA-UDCA-3S. Thus, 5-ASA-UDCA-3G, as well as salazosulfapyridine, was found to possess feasible properties as a colon-target prodrug and show a higher distribution of 5-ASA to the colonic membranes. Although further detailed studies are needed regarding the safety of 5-ASA-UDCA-derivatives, the use of UDCA-3G as a carrier of 5-ASA may be safer and more effective than salazosulfapyridine in the colon-targeted delivery of 5-ASA.

ACKNOWLEDGEMENT

We thank Satoshi Kawano, Shintaro Natsuyama, Fumiya Ohnishi, Syoutarou Koga, and Yuta Goto, for their technical assistance in experimenting at the Laboratory of Drug Information Analytics, Faculty of Pharmacy & Pharmaceutical Sciences, Fukuyama University. We would like to thank Terumasa Ohune for supporting this research with donations.

Funding: This research received no external funding.

Institutional Review Board Statement: The animal study was performed in compliance with the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Fukuyama University. This study was approved on October 2023, by the Ethics Committee of the Faculty of Pharmacy & Pharmaceutical Science of Fukuyama University. The license number is 2023-A-10.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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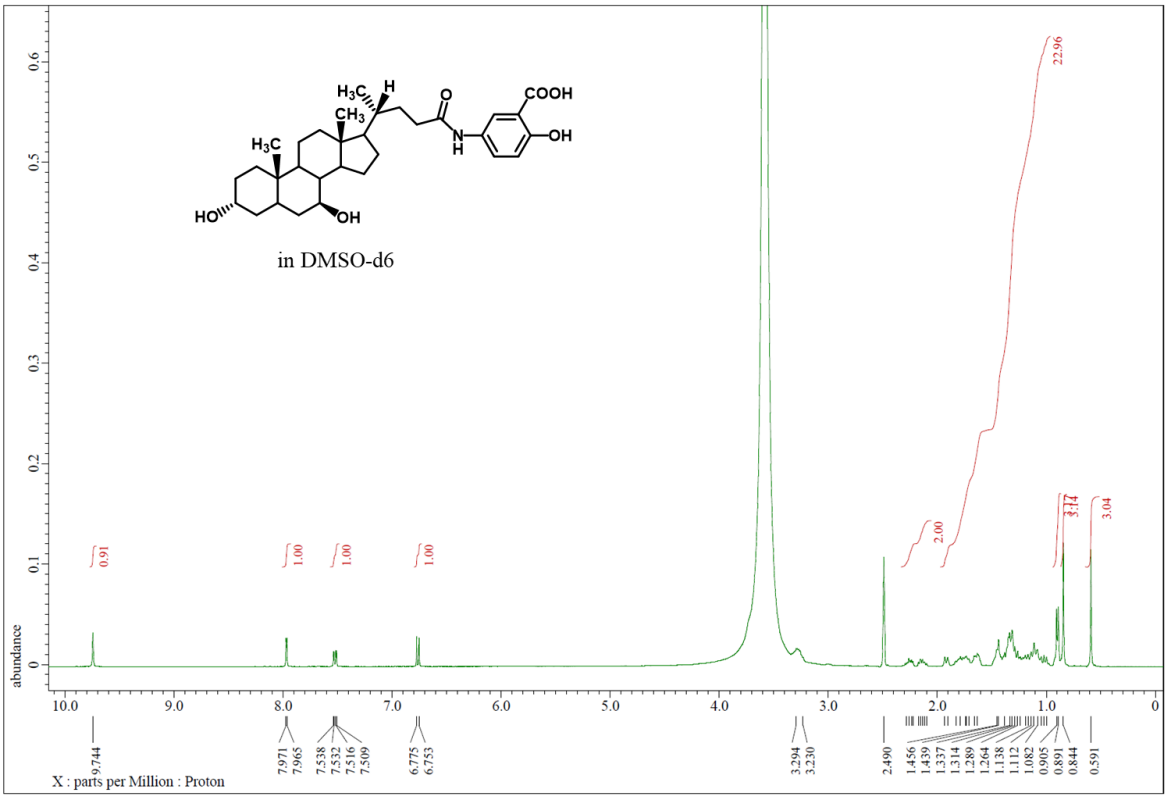
Supplementary Materials



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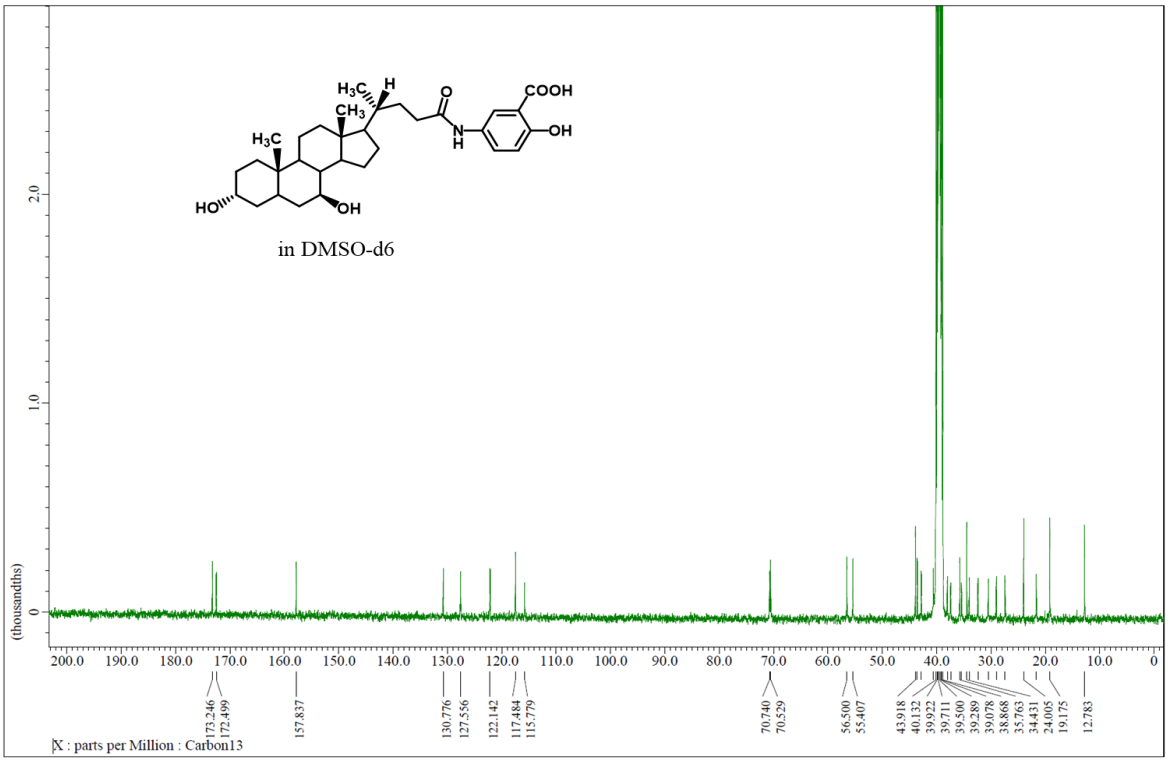

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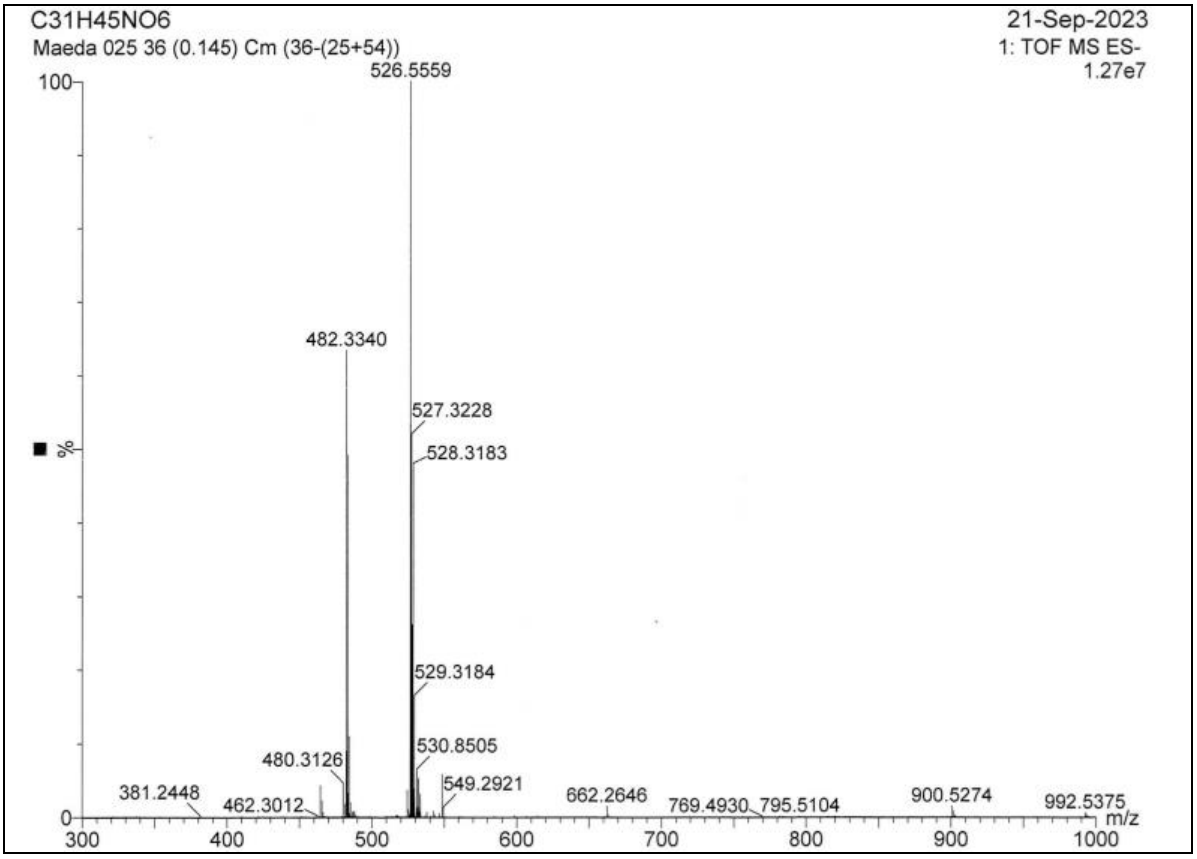
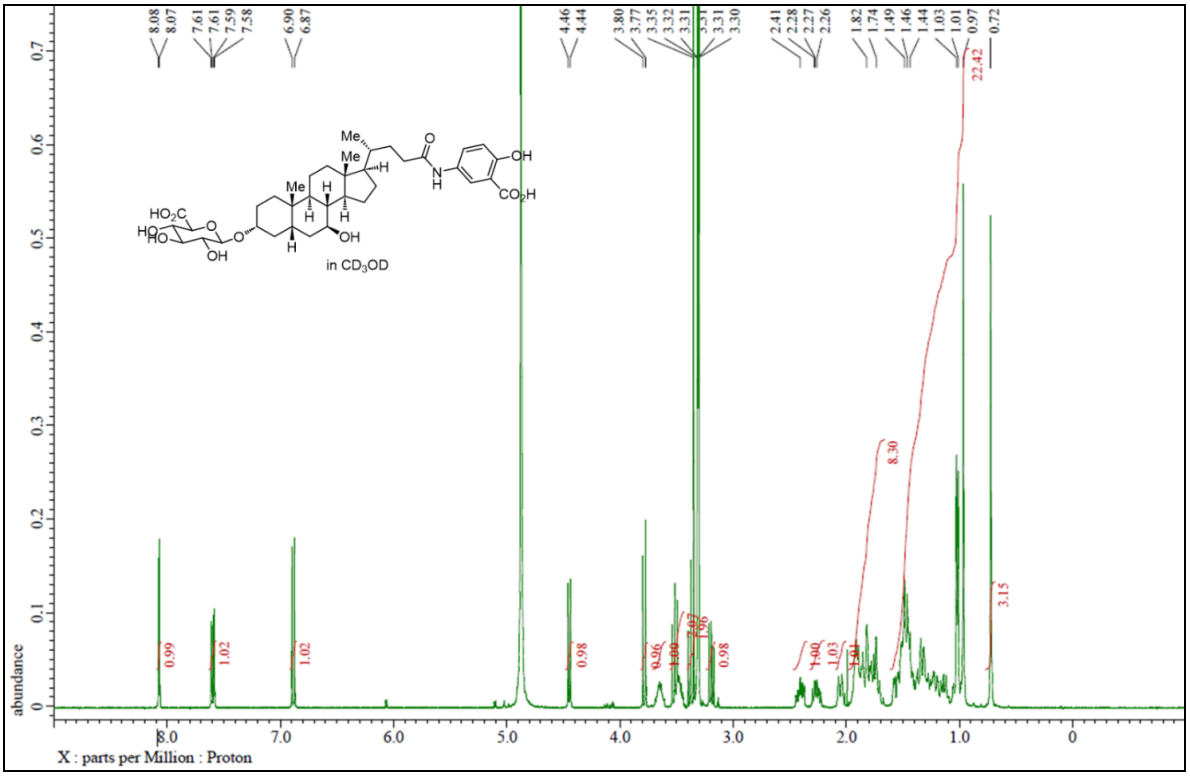


(C)

Figure S1. Spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B), and ESI-MS (C) of 5-ASA-UDCA.



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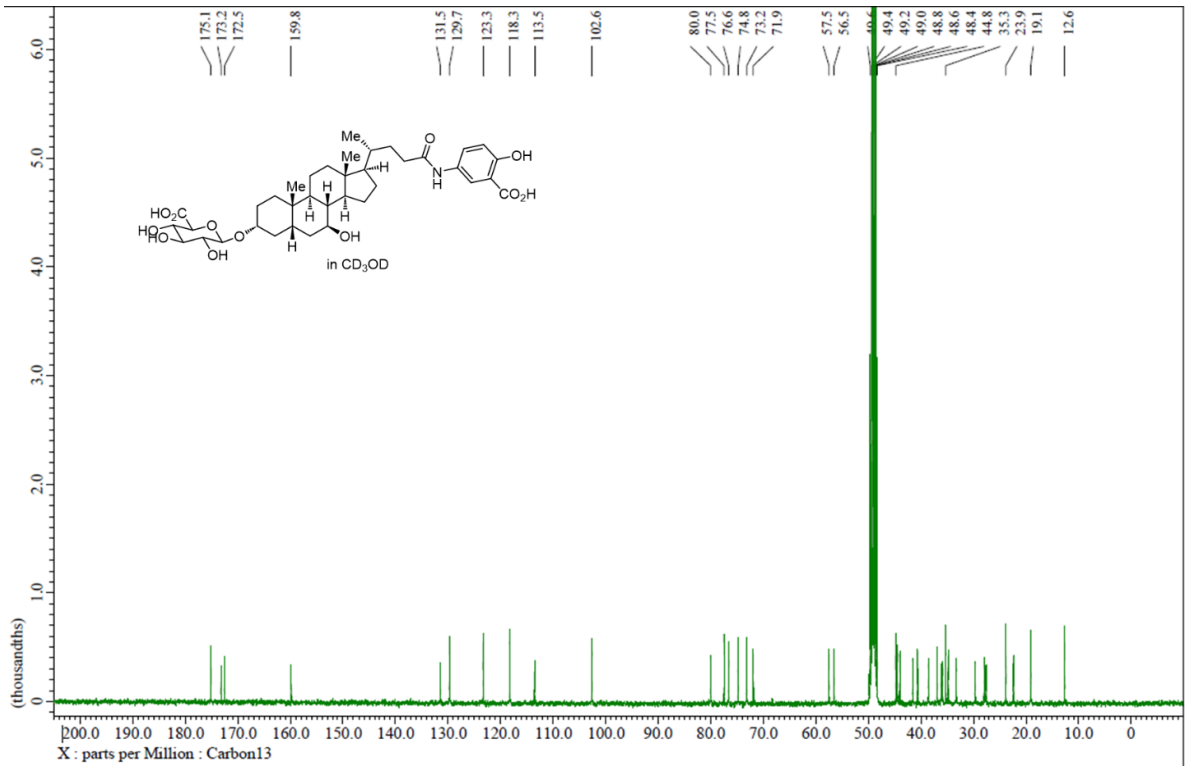

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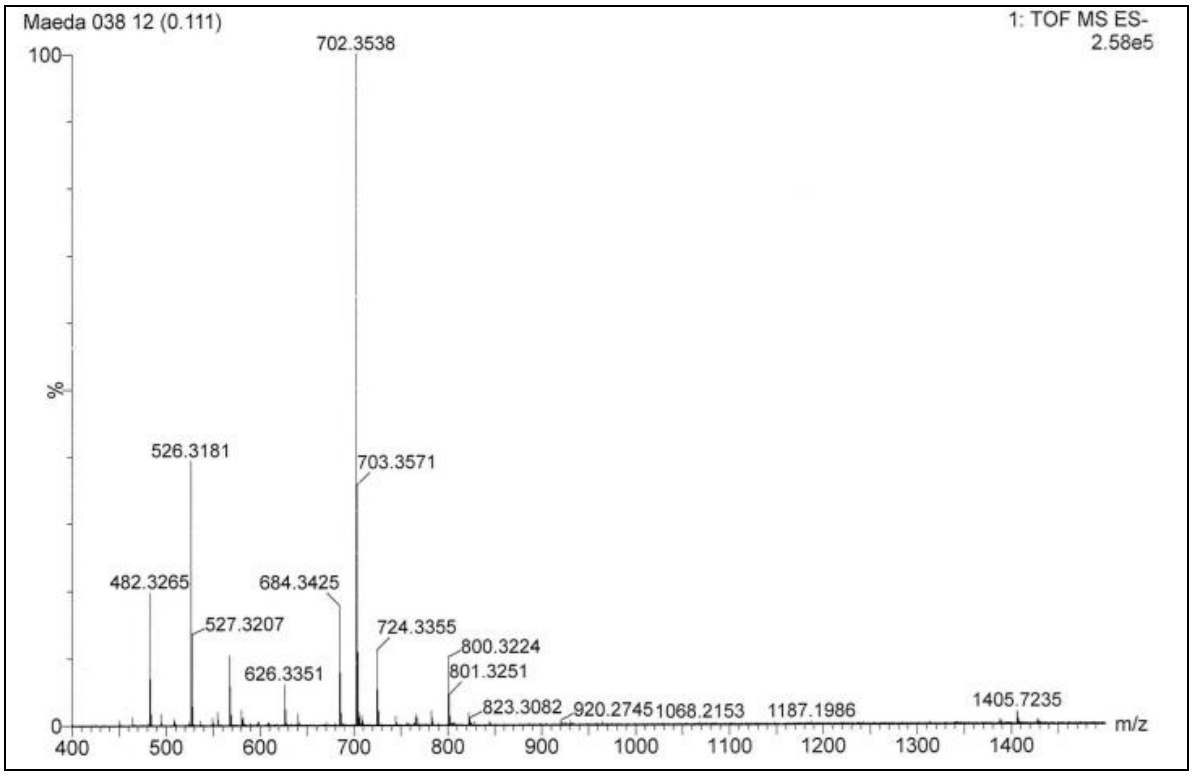


(C)

Figure S2: Spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B), and ESI-MS (C) of 5'-ASA-UDCA-3-glucuronide.

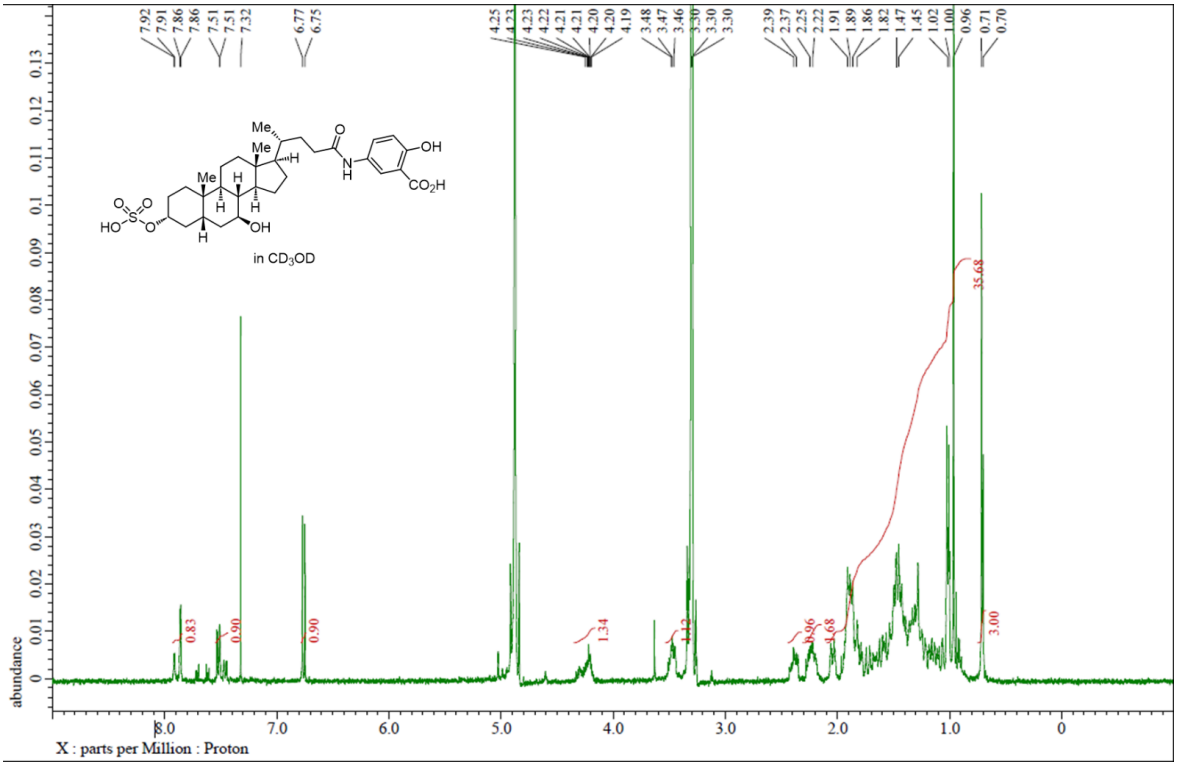
Benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate (S3)
^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.65 (s, 3H), 0.87-0.93 (m, 3H), 0.96 (s, 3H), 0.97-1.08 (m, 2H), 1.08-1.59 (m, 13H), 1.59-1.88 (m, 9H), 1.93-2.02 (m, 4H), 2.21-2.33 (m, 1H), 2.34-2.46 (m, 1H), 3.52-3.64 (m, 1H), 4.71-4.82 (m, 1H), 5.04-5.17 (m, 2H), 7.28-7.42 (m, 5H)
^{13}C-NMR (100 MHz, $CDCl_3$) δ 12.2, 18.5, 21.3, 22.0, 23.4, 25.8, 28.5, 30.3, 31.0, 31.4, 33.1, 34.1, 34.9, 35.3, 37.2, 39.5, 40.0, 40.1, 42.4, 43.7, 55.1, 55.3, 66.2, 71.5, 73.9, 128.3, 128.4 (2C), 128.7 (2C), 136.2, 170.8, 174.2

Benzyl 7β-acetoxy-3α-hydroxy-5β-cholanoate 3-1'β-glucuronide methyl ester-triacetate (S4)
^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.64 (s, 3H), 0.85-1.88 (m, 30H), 1.97 (s, 3H), 2.02 (s, 6H), 2.04 (s, 3H), 2.22-2.33 (m, 1H), 2.34-2.46 (m, 1H), 3.48-3.62 (m, 1H), 3.77 (s, 3H), 3.97-4.06 (m, 1H), 4.61-4.68 (m, 1H), 4.69-4.81 (m, 1H), 4.90-5.00 (m, 1H), 5.06-5.16 (m, 2H), 5.20-5.28 (m, 2H), 7.29-7.40 (m, 5H);
^{13}C-NMR (100 MHz, $CDCl_3$) δ 12.2, 18.5, 20.7, 20.8, 20.9, 21.3, 21.9, 23.4, 25.7, 26.9, 28.5, 31.1, 31.4, 33.0, 34.1, 34.7 (2C), 35.3, 39.3, 40.0, 40.1, 42.4, 43.7, 53.1, 55.0, 55.1, 66.2, 69.6, 71.7, 72.3, 72.8, 73.7, 79.9, 99.6, 128.3, 128.4 (2C), 128.7 (2C), 136.2, 167.4, 169.4, 169.5, 170.4, 170.5, 174.2

7β-acetoxy-3α-hydroxy-5β-cholanoic acid 3-1'β-glucuronide methyl ester-triacetate (S5)
^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.67 (s, 3H), 0.88-1.91 (m, 30H), 1.97 (s, 3H), 2.02 (s, 6H), 2.05 (s, 3H), 2.19-2.32 (m, 1H), 2.33-2.45 (m, 1H), 3.50-3.62 (m, 1H), 3.77 (s, 3H), 3.97-4.06 (m, 1H), 4.61-4.68 (m, 1H), 4.70-4.81 (m, 1H), 4.90-5.01 (m, 1H), 5.19-5.29 (m, 2H)
^{13}C-NMR (100 MHz, $CDCl_3$) δ 12.2, 18.5, 20.7, 20.8, 20.9, 21.3, 21.9, 23.4, 25.7, 26.9, 28.5, 30.8, 31.0, 33.0, 34.1, 34.7 (2C), 35.3, 39.3, 40.0, 40.1, 42.4, 43.7, 53.1, 55.0, 55.1, 69.6, 71.7, 72.3, 72.8, 73.7, 79.9, 99.6, 167.4, 169.4, 169.5, 170.4, 170.6, 179.5

***N*-(4''-(*tert*-butyldimethylsilyl)oxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholano-24-amide 3-1'β-glucuronide methyl ester-triacetate (S7)**
^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.19 (s, 6H), 0.68 (s, 3H), 0.88-1.93 (m, 37H), 1.94-2.08 (m, 14H), 2.16-2.29 (m, 1H), 2.33-2.46 (m, 1H), 3.49-3.62 (m, 1H), 3.77 (s, 3H), 3.85 (s, 3H), 3.97-4.07 (m, 1H), 4.61-4.68 (m, 1H), 4.70-4.81 (m, 1H), 4.90-5.01 (m, 1H), 5.19-5.29 (m, 2H), 6.80-6.88 (m, 1H), 7.05 (brs, 1H), 7.66-7.76 (m, 2H)
^{13}C-NMR (100 MHz, $CDCl_3$) δ -4.3 (2C), 12.2, 18.4, 18.7, 20.7, 20.8, 20.9, 21.3, 21.9, 23.3, 25.7, 25.8 (3C), 27.0, 28.6, 31.7, 33.0, 34.1, 34.5, 34.7, 35.4, 39.3, 40.0, 40.1, 42.4, 43.7, 52.1, 53.1, 55.1 (2C), 69.6, 71.7, 72.3, 72.8, 73.7, 80.0, 99.7, 121.9, 122.7, 123.0, 125.5, 131.4, 151.8, 166.8, 167.4, 169.4, 169.5, 170.4, 170.5, 171.8

***N*-(4''-hydroxy-3''-methoxycarbonylphenyl)-7β-acetoxy-3α-hydroxy-5β-cholano-24-amide 3-1'β-glucuronide methyl ester-triacetate (S8)**
^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.68 (s, 3H), 0.88-1.93 (m, 29H), 1.94-2.08 (m, 13H), 2.16-2.30 (m, 1H), 2.32-2.46 (m, 1H), 3.49-3.62 (m, 1H), 3.78 (s, 3H), 3.94 (s, 3H), 3.97-4.06 (m, 1H), 4.60-4.68 (m, 1H), 4.70-4.81 (m, 1H), 4.91-5.02 (m, 1H), 5.19-5.29 (m, 2H), 6.95 (d, J = 8.7 Hz, 1H), 7.08 (brs, 1H), 7.50 (dd, J = 8.7, 2.7 Hz, 1H), 8.08 (d, J = 2.7 Hz, 1H), 10.80 (s, 1H)
^{13}C-NMR (100 MHz, $CDCl_3$) δ 12.2, 18.7, 20.7, 20.8, 20.9, 21.3, 21.9, 23.3, 25.7, 27.0, 28.6, 31.7, 33.0, 34.1, 34.2, 34.7 (2C), 35.3, 39.3, 40.0, 40.1, 42.4, 43.7, 52.5, 53.1, 55.0, 55.1, 69.6, 71.7, 72.3, 72.8, 73.7, 80.0, 99.7, 111.2, 118.1, 121.6, 128.7, 129.8, 158.5, 167.1, 167.4, 169.4, 169.5, 170.4, 170.6, 171.9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X : parts per Million : Proton

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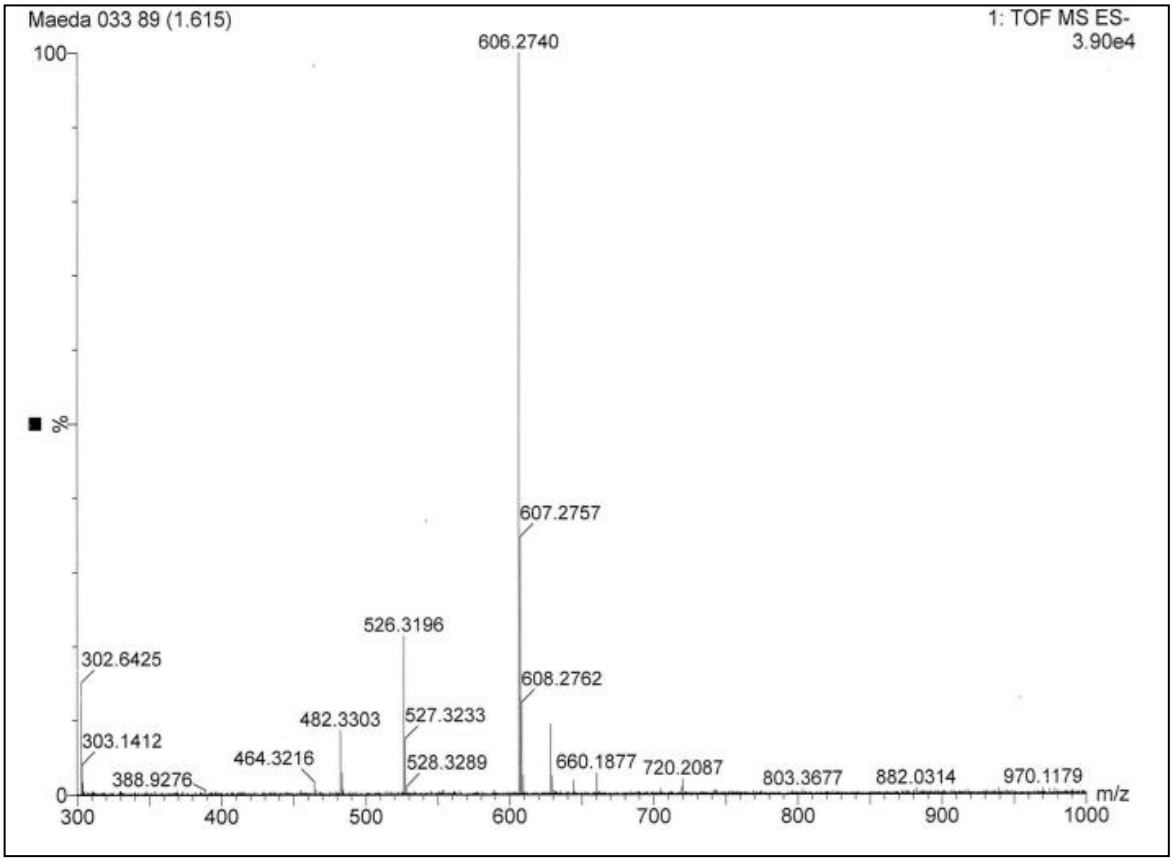
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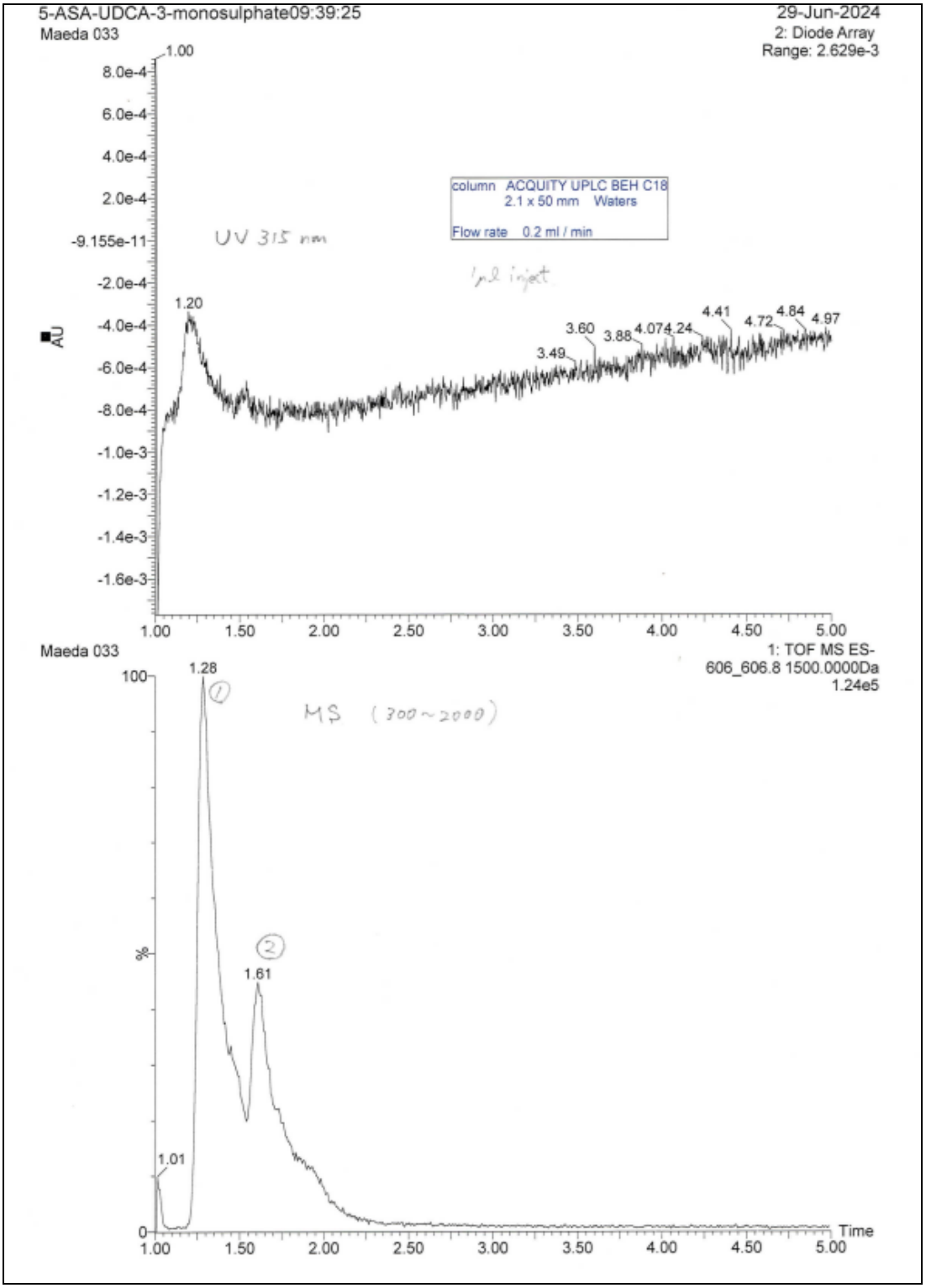
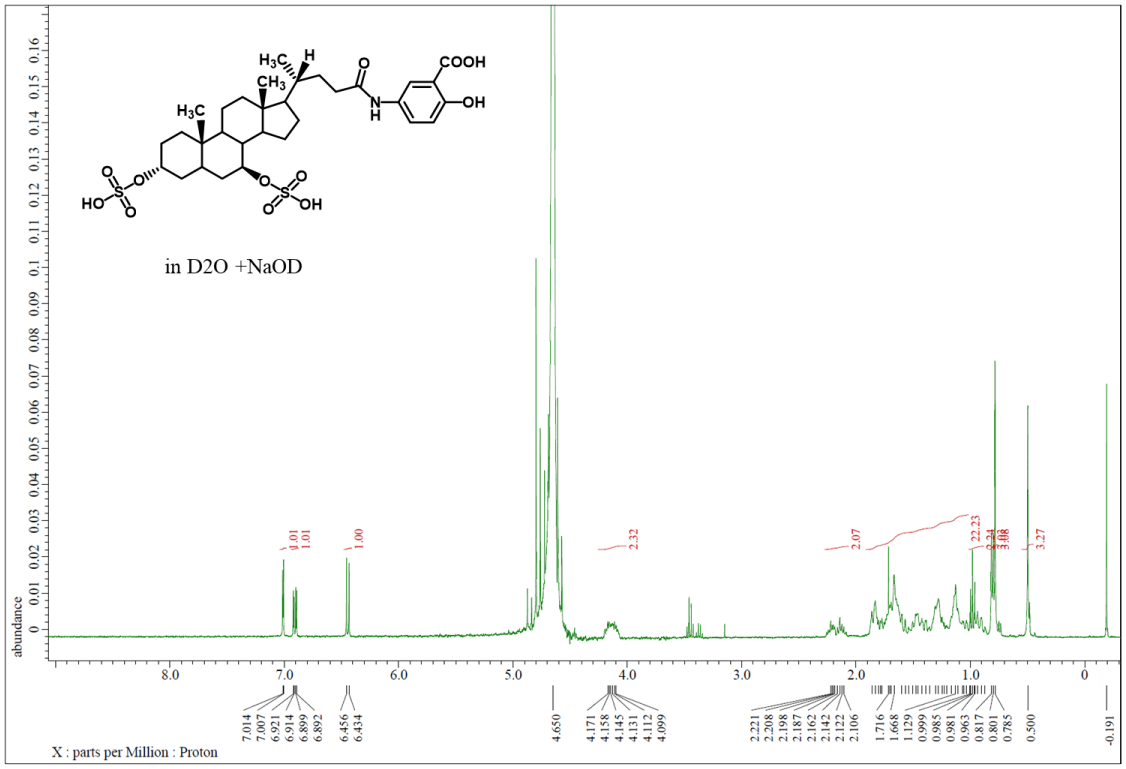


(C)

Figure S3: Spectrum of ^{1}H-NMR (A), ESI-MS (B), and LC-MS(C) of 5'-ASA-UDCA-3-sulfate.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abundance
X : parts per Million : Proton

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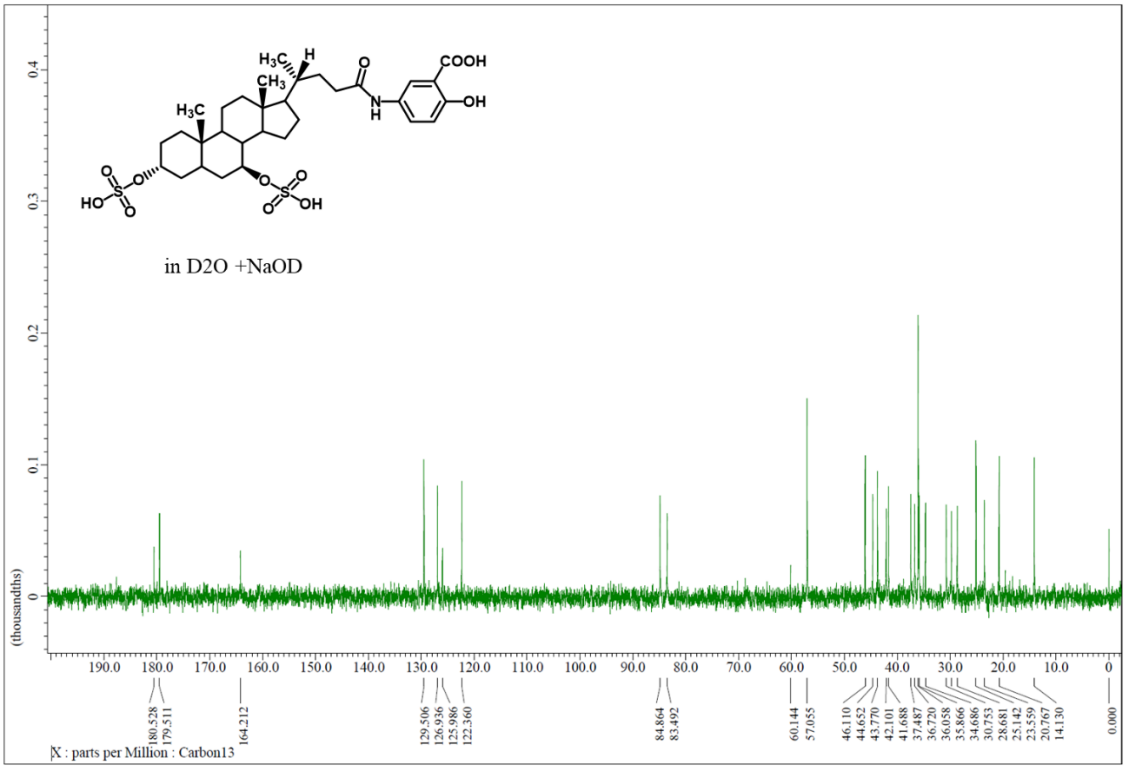
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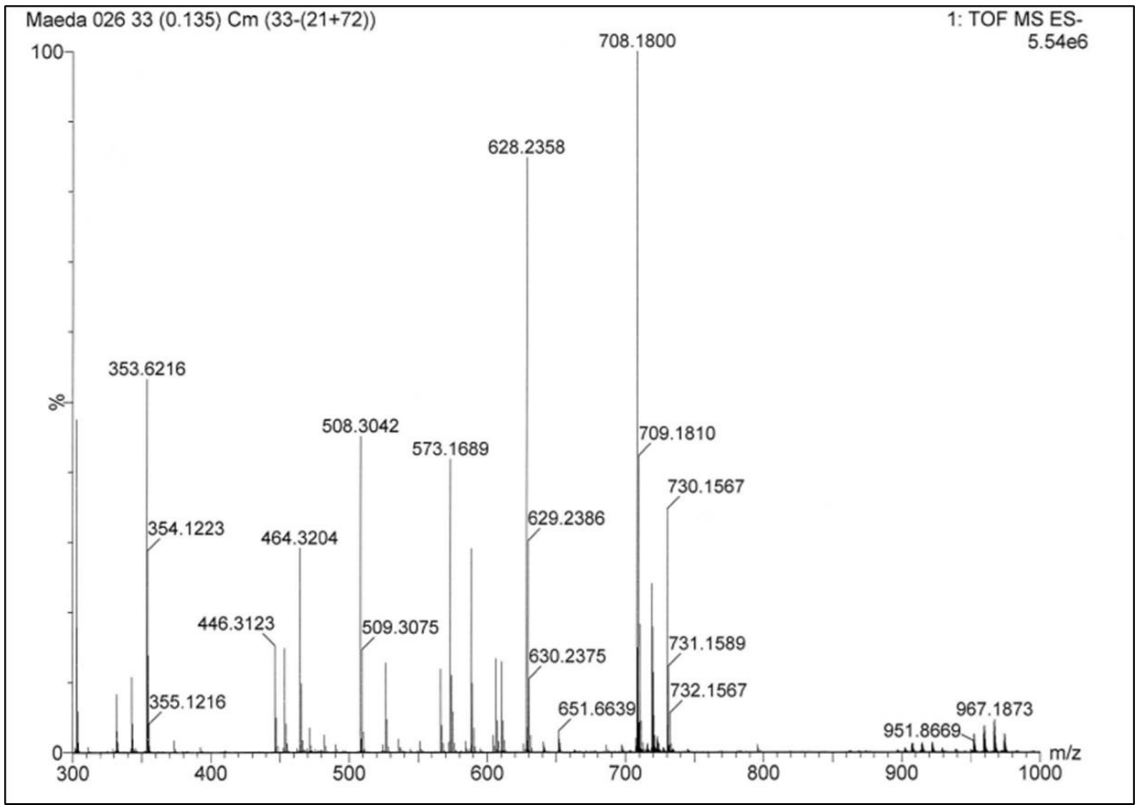

(C)

Figure S4: Spectrum of ^{1}H-NMR (A), ^{13}C-NMR (B), and ESI-MS (C) of 5'-ASA-UDCA-3,7-disulfate.