Arch. Pharm. Res. Vol.21, No.2, pp. 174-178, 1998

Synthesis and Evaluation of 5-Aminosalicyl-glycine as a Potential Colon-specific Prodrug of 5-Aminosalicylic Acid

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(Received November 9, 1997)

As a new colon-specific prodrug of 5-aminosalicylic acid (5-ASA), 5-aminosalicyl-glycine (5-ASA-Gly) was prepared by a simple synthetic route in good yield. Apparent partition coefficients of 5-ASA-Gly were lower than those of 5-ASA, which determined in $CHCl_3$/pH 6.8 buffer or n-octanol/pH 6.8 buffer system. Stability of 5-ASA-Gly by peptidases was investigated by incubation of 5-ASA-Gly with the homogenates of tissue and contents of stomach, proximal small intestine or distal small intestine of rats at 37°C. 5-ASA was not detected, indicating that the prodrug was stable in the upper intestine. The amount of 5-ASA liberated from incubation of the prodrug in cecal or colonic contents of rats was about 65% or 27% in 8 hrs, respectively, which indicated that the prodrug activation took place more readily in the rat cecum whose bacterial counts are high like human colon. Results from *in vitro* experiments suggested 5-ASA-Gly as a promising candidate of a colon-specific prodrug of 5-ASA.

Key words : 5-Aminosalicyl-glycine, Colon-specific prodrug of 5-aminosalicylic acid, Ulcerative colitis, Crohn's disease, Inflammatory bowel disease

INTRODUCTION

Delivery of orally administered drugs specifically to the colon is desirable for the treatment of diseases which developed at colonic site. It can also be utilized when colonic absorption is prefered and more beneficial (Mcleod *et al.*, 1992; Saffran, 1992). The cases of inflammatory bowel disease (IBD), such as ulcerative colitis and Crohn's disease, which have been popular among Europeans and Americans, are now getting increased in Asian countries such as Korea since the end of the 1980's. The exact etiology of the IBD is not yet clearly understood, and a allopathic therapy employing corticosteroids and salicylates is most common to relieve the symptoms of such ailments (Danel *et al.*, 1991). 5-ASA is an active ingredient of agents which used for the long-term maintenance therapy to prevent relapses of Crohn's disease and ulcerative colitis, and yet it is not suitable to be used as such because it is absorbed rapidly and extensively through the upper intestine before it reached to the colonic site (Crotty *et al.*, 1992). In addition, systematically absorbed 5-ASA is reported to induce nephrotic syndrome (Novis *et al.*, 1988). Several prodrugs aiming at the delivery of 5-ASA to the colonic site are available (Brown *et al.*, 1983; Istran *et al.*, 1991; Ryde 1991; Yamaguchi *et al.*, 1994).

Amide bond of aromatic carboxylic acid is reported to be biochemically stable in the upper instestine (Nakamura *et al.*, 1992). Glycine or taurine conjugates of bile acids are known to be undergoing enterohepatic circulation (Martin *et al.*, 1988) and p-aminohippuric acid is reported to be readily converted to p-aminobenzoic acd when it is incubated with rat cecal contents (Scheline, 1973). Based on the above facts, we choosed glycine as a colon-specific carrier of 5-ASA and synthesized 5-aminosalicyl-glycine (5-ASA-Gly) as a potential colon-specific prodrug of 5-ASA. Being a glycyl amide of 5-ASA, 5-ASA-Gly is expected to be biochemically stable in the environment of the upper intestine and nonabsorbable due to the hydrophilicity of the compound. Thus, orally administered 5-ASA-Gly might safely be delivered to the colon, where it is activated to liberate 5-ASA microbially. In the present study, simple synthetic processes were exploited to prepare 5-ASA-Gly in good yield, and biological characteristics of 5-ASA-Gly as a colon-specific prodrug were investigated.

MATERIALS AND METHODS

5-Nitrosalicylic acid (5-NSA), 5-aminosalicylic acid (5-ASA), glycine methyl ester hydrochloride, *N,N'*-dicyclohexylcarbodiimide (DCC), 10% Pd/C, benzyl chloroformate and kanamycin sulfate were purchased from

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Sigma Chemical Co. (St. Louis, Mo). Solvents for NMR and HPLC were obtained from Merck Inc. All other chemicals were reagent grade, commercially available products.

IR spectra were recorded with a Bomem MB 100 FT-IR spectrophotometer. ^{1}H-NMR spectra were taken on a Brucker AC-200 spectrometer, and the chemical schifts are in ppm downfield from tetramethylsilane. A Orion 320 pH meter was used for the pH measurements. Melting points were taken on a Mel Tem II (Fisher) and were uncorrected. Pressure reactor (Parr 4562) was used for catalytic hydrogenation. A Eyela Mazela-Z tissue homogenizer was used for homogenation of tissue and contents of GI tracts of rats and a Hanil Supra K-22 centrifuge was used for centrifugation. TLC was performed on Merck Kieselgel 60 F_{254}, and RP-8 F_{254s}. Open column chromatography was performed on Merck silica gel (70~230 mesh and 230~400 mesh) and low-pressure chromatography on Merck Lichroprep RP-8 size B (230~400 mesh) columns.

Buffer solutions

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

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: phosphate buffer (5.0 mM sod. phosphate dibasic and phosphoric acid were mixed to give pH 6.0).

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 10% methanol in buffer C and 0.5 mM tetrabutylammonium chloride and filtered through 0.45 μm membrane filter before use. A Synchropac ODS (250×4.6, 5 μm) column was eluted with the mobile phase at a flow rate of 1.5 ml/min and at a pressure of about 2000 psi. The column eluent was monitored at 254 nm with a sensitivity of AUFS 0.01 and a Gilson 712 software was employed for data analysis.

Standard and sample solutions of 5-ASA, *N*-acetyl-5-ASA and 5-ASA-Gly

Standard solution of 5-ASA or N-acetyl-5-ASA was prepared in methanol (50 μg/ml). The tissue and contents of stomach, PSI or DSI and contents of cecum or colon were homogenated and diluted with buffer solution B (10 w/v%). To 100 μl of the above homogenates, 10 μl, 50 μl, 100 μl, and 200 μl of standard solutions of 5-ASA or N-acetyl-5-ASA and appropriate volumes of methanol were added to give final volume of 1 ml.

Standard solution of 5-ASA-Gly was prepared by dissolving in buffer B (50 μg/ml), and 10 μl, 50 μl, 100 μl, and 200 μl of standard solutions of 5-ASA-Gly were added to microtubes and centrifugally evaporated. To the above microtubes, 100 μl of the homogenates of the stomach, PSI, DSI, cecum or colon in buffer B (10 w/v%) and 0.9 ml of methanol were added to give final volume of 1 ml. Blank solution was prepared by following the same procedure excluding the drug.

Calibration and quantitation

Standard (0.5 μg/ml~10 μg/ml) or blank (1 ml) solution was mixed on a vortex mixer for 2 min, centrifuged at 10,000×g for 5 min, filtered through 0.45 μm filter and 20 μl of the filterate was injected on the column. A calibration curve was constructed from the peak area versus the concentration of standard solutions. Concentration of 5-ASA, 5-ASA-Gly or N-acetyl-5-ASA in the sample was calculated from the calibration curve.

Preparation of 5-[*N*-(Benzyloxycarbonyl)amino]salicylic acid (2)

Benzylchloroformate (18.5 g, 110 mmol) was added dropwise in 20 min to a suspension of 5-ASA (**1**) (15 g, 100 mmol) in saturated solution of $NaHCO_3$ (250 ml) also containing solid $NaHCO_3$ (10 g). The reaction mixture was stirred mechanically for 5 hrs at 0°C and the precipitates thus formed was filtered. The filterate was washed three times with ether (25 ml), combined with the previously obtained precipitates, acidified with 3N HCl, and extracted with ethyl acetate. The organic phase was dried over anhydrous Na_2SO_4, removed the solvent by flash evaporator. The resulting solid was dried under vaccum to give compound **2** (26 g, 90% yield). mp: 224~225°C; ^{1}H NMR (DMSO-d_6) δ: 5.17 (s, 2H, CH_2Ph), 7.00~8.05 (m, 8H, ArH), 9.80 (s, 1H, COOH).

Preparation of 5-[*N*-(Benzyloxycarbonyl)amino]-2-acetoxysalicylic acid (3)

To the suspension of compound **2** (10 g, 35 mmol)) in acetic acid (52 ml), acetic anhydride (6.7 g, 60 mmol) and pyridine (0.3 ml) were added, and the mixture was stirred for 24 hrs. The resulting precipitates were filtered and dried under vaccum to give compound **3** (9.5 g, 83% yield). mp: 202~204°C; ^{1}H NMR (MeOH-d_4) δ: 2.15 (s, 3H, $COCH_3$), 5.10 (s, 2H, CH_2Ph), 6.90~8.00 (m, 8H, ArH).

Preparation of 5-[*N*-(benzyloxycarbonyl)amino]-2-acetoxysalicylchloride (4)

To a suspension of compound **3** (10 g, 30 mmol) in

anhydrous benzene (40 ml), $SOCl_2$ (7.5 g, 60 mmol) and pyridine (0.3 ml) were added and refluxed for 3 hrs under nitrogen. After cooling the reaction mixture, the precipitates were collected and extracted with hot benzene, and removed the solvent *in vacuo* to give compound **4** (4.2 g, 41% yield).

Preparation of 5-aminosalicyl-glycine (10, 5-ASA-Gly) from compound 4

Glycine methyl ester hydrochloride (4.2 g, 47.4 mmol) in methanol (10 ml) was neutralized by adding triethylamine (11 g, 108.6 mmol) dropwise at 0°C. It was stirred for 2 hrs, filtered, and removed solvent by flash evaporator. Compound **4** (14.3 g, 47.4 mmol) in CCl_4 (150 ml) was added to the oily glycine dimethyl ester, and refluxed for 12 hrs under nitrogen. After cooling, the precipitates were collected and washed with small amount of ether until it shows one spot on TLC. 5-Benzyloxycarbonylamino-2-acetoxysalicyl-glycine methyl ester (**5**), thus obtained, was catalytically hydrogenated at 50 psi with 10% Pd/C in a Parr apparatus to yield 5-amino-2-acetoxysalicyl-glycine methyl ester (**6**). Compound **6** was hydrolyzed with 1 N NaOH (10 molar excess) for 5 hrs under nitrogen, neutralized to pH 3~4 with 3 N HCl. The precipitates were collected, and dried in vacuo to yield 5-ASA-Gly (10). mp: (~297°C, decomp.); IR (nujol) ν_{max} (C=O): 1620 cm^{-1}, 1648 cm^{-1}; ^1H NMR (DMSO-d_6) δ: 3.9 (d, 2H, $NHCH_2CO$), 6.6~7.0 (m, 3H, ArH).

Preparation of 5-nitrosalicyl-glycine methyl ester (8)

5-Nitrosalicylic acid (**7**) (5 g, 27.3 mmol) was dissolved in anhydrous ethyl acetate (170 ml), added DCC (6.2 g, 30.0 mmol) in portions at 0°C, and stirred for 1 hr. To the reaction mixture, glycine methyl ester (2.4 g, 27.3 mmol) was added and stirred for 3 hrs at 0°C and 72 hrs at room temperature. It was filtered and removed the solvent in vacuo. The oily residue, thus obtained, was extracted with saturated solution of $NaHCO_3$. The combined extract was acidified with 3N HCl, extracted with ethyl acetate, dried with anhydrous Na_2SO_4, and removed the solvent *in vacuo*. The residue was loaded on a silica gel open column and eluted with $CHCl_3$/MeOH (100/1.5), from which compound **8** (4.4 g 65% yield) was obtained. mp: 140~142°C; IR (nujol) ν_{max} (C=O): 1635 cm^{-1}, 1747 cm^{-1}; ^1H NMR ($CDCl_3$) δ: 3.8 (s, 3H, $COOCH_3$), 4.9 (d, 2H, $NHCH_2CO$), 7.0~8.8 (m, 3H, ArH).

Preparation of 5-aminosalicyl-glycine (10, 5-ASA-Gly) from compound 8

Compound **8** (1 g, 3.95 mmol) in methanol (20 ml) was catalytically hydrogenated at 50 psi with 200 mg of 10% Pd/C in a Parr reactor for 1 hr. After removal of methanol, 1N NaOH (30 ml) was added and reacted for 5 hrs under nitrogen. Upon adjustment of the pH to 3~4, 5-ASA-Gly (**10**) (0.69 g, 83% yield) was obtained as white precipitates.

Preparation of *N*-acetyl-5-aminosalicylic acid (*N*-acetyl-5-ASA)

5-ASA (**1**) (3 g, 20 mmol) in acetic anhydride (5 g, 49 mmol) and acetic acid (10 ml) was refluxed for 10 min and added cold distilled water (30 ml). The precipitates (1 g), thus formed, were filtered and reacted with 1 N NaOH (20 ml) at room temperature for 1 hr, acidified with 3 N HCl, and extracted with ethyl acetate, from which N-acetyl-5-ASA was obtained. mp: 222~225°C; IR (nujol) ν_{max} (C=O): 1630 cm^{-1}.

Determination of apparent partition coefficient

The solution (10 ml) of 5-ASA (155 μg/ml) or 5-ASA-Gly (155 μg equivalent of 5-ASA/ml) in buffer B was added to 10 ml of chloroform or *n*-octanol which was previously saturated with buffer B. The mixture was shaken for 24 hrs at 37°C and analyzed the amount of 5-ASA or 5-ASA-Gly in the aqueous phase by HPLC. 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.

Release of 5-ASA after incubation of 5-ASA-Gly with the homogenates of tissue and contents of stomach, PSI or DSI of rats

A male Sprague-Dawley rat was anesthetized by ether and a midline incision was made. Tissue and contents of stomach was diluted with buffer A and that of PSI or DSI with buffer B to half concentration and homogenated. 5-ASA-Gly in buffer B (140 μg equivalent of 5-ASA/0.8 ml), 0.8 ml, was added to the above homogenate (0.2 g) in a microtube and the mixture was incubated for 6 hrs at 37°C. At appropriate time interval, a portion of the sample was centrifuged at 5,000 rpm for 3 min. Methanol (0.9 ml) was added to the supernatant (0.1 ml). It was vortexed for 2 min, centrifuged for 5 min at 10,000×g and analyzed the amount of 5-ASA in 20 μl of the supernatant by HPLC.

Determination of 5-ASA and *N*-acetyl-5-ASA after incubation of 5-ASA-Gly with the cecal and colonic contents of rats

The cecal and colonic segments of the intestine were cut open and their contents were collected separately in a glover box which was previously displaced by nitrogen. 5-ASA-Gly (140 μg equivalent of 5-ASA/0.9 ml) in buffer B (0.9 ml) and the gut con-

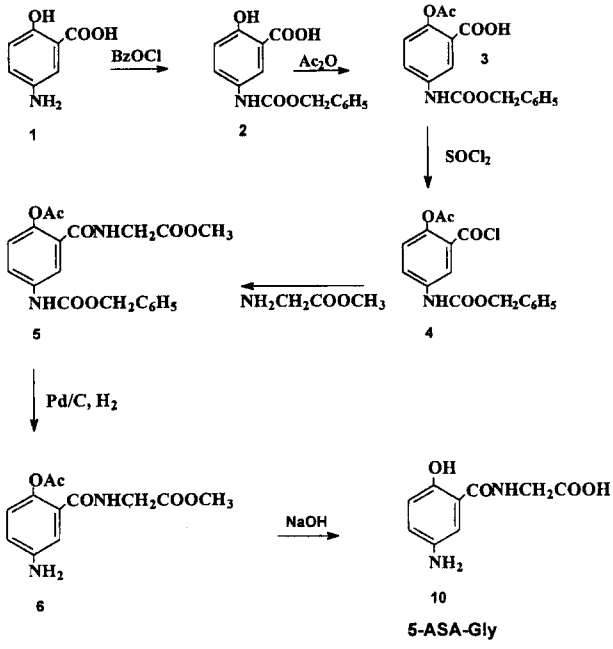
tents (0.1 g) were placed in a microtube and incubated for 6 hrs at 37°C. At appropriate time interval, a portion of the sample was centrifuged at 5,000 rpm for 3 min. Methanol (0.9 ml) was added to the supernatant (0.1 ml). It was vortexed for 2 min, centrifuged for 5 min at 10,000×g and analyzed the amount of 5-ASA and *N*-acetyl-5-ASA in 20 μl of the supernatant by HPLC.

Kanamycin sulfate (5×200 mg/rat) was administered to rats twice a day for 2 days and 4 hrs prior to the experiment via an oral zonde (Yamaguchi *et al.*, 1994) and executed the experiments by following the same procedure.

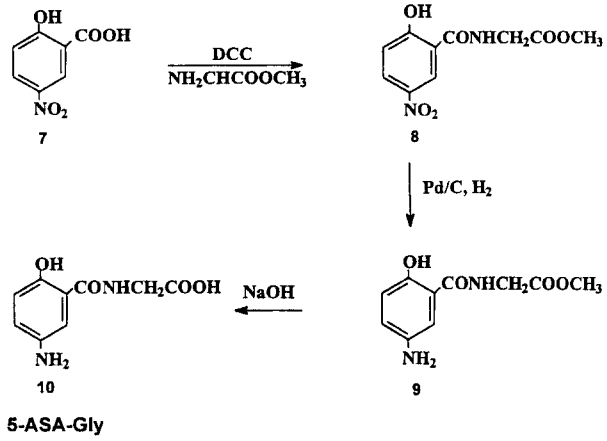
RESULTS AND DISCUSSION

Preparation of 5-aminosalicyl-glycine (10, 5-ASA-Gly)

One route which we prepared 5-ASA-Gly was started from 5-ASA as shown in Scheme 1. In this method, compound **3** was prepared from 5-ASA by protecting amino and hydroxy groups with benzyloxycarbonyl and acetyl, respectively. Conversion of compound **3** to acid chloride and subsequent reaction with glycine methyl ester produced compound **5**, from which 5-ASA-Gly was obtained after removal of benzyloxycarbonyl by hydrogenolysis with 10% Pd/C, and hydrolysis of methyl ester. High tendency of by-product formation in the processes of acid chloride formation and catalytic reduction and low overall yield urged us to develop a alternative synthetic route which would produce 5-ASA-Gly in good overall yield. As shown



Scheme 1. Synthesis of 5-aminosalicyl-glycine from 5-aminosalicylic acid.



Scheme 2. Synthesis of 5-aminosalicyl-glycine from 5-nitrosalicylic acid.

in Scheme 2, 5-NSA (**7**) was directly reacted with glycine methyl ester in the presence of DCC. Compound **8**, thus produced, was reduced with 10% Pd/C to give compound **9**, from which 5-ASA-Gly was obtained after hydrolysis of ester. The reaction processes were relatively simple and the overall yield was highly improved.

Determination of apparent partition coefficient

Apparent partition coefficients of 5-ASA or 5-ASA-Gly were determined at 37°C employing $CHCl_3$/buffer B or *n*-octanol/buffer B as solvent system. Apparent partition coefficients of 5-ASA or 5-ASA-Gly were 1.85 or 0.20 in $CHCl_3$/buffer B solvent system, and 1.30 or 1.23 in *n*-octanol/buffer B solvent system, respectively. HPLC retention times of 5-ASA, *N*-acetyl-5-ASA and 5-ASA-Gly were determined on Synchropak ODS column (4.5×250, 5 m) employing methanol/buffer C (1/9) and 0.5 mM tetrabutylammonium chloride as mobile phase at a flow rate of 1.5 ml/min. HPLC retention times of 5-ASA, *N*-acetyl-5-ASA and 5-ASA-Gly were 280 sec, 820 sec and 370 sec, respectively. Unexpectedly, apparent partition coefficients of 5-ASA-Gly varied greatly on the above two solvent systems, which might be due to the specific interaction of n-octanol with glycyl group. If $CHCl_3$/buffer system represents the absorption phenomena of 5-ASA-Gly through GI tract better than n-octanol/buffer system, absorption through GI tract should be lower with 5-ASA-Gly than 5-ASA. *In vivo* studies are in progress.

Release of 5-ASA after incubation of 5-ASA-Gly with homogenates of various segments of GI tract of rats

Release of 5-ASA versus time after incubation of 5-ASA-Gly at 37°C with homogenates of various segments of GI tract of rats is shown in Fig. 1.

To investigate the hydrolysis of 5-ASA-Gly by the peptidases which are known to be highly distributed

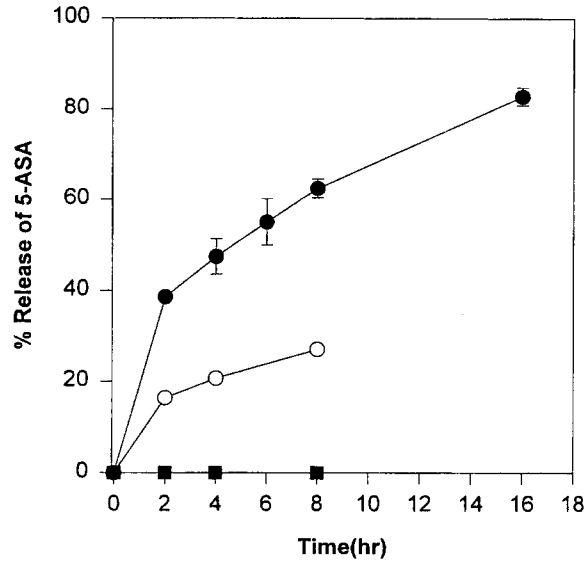


Fig. 1. Release of 5-ASA during incubation of 5-ASA-Gly (equiv. to 140 μg of 5-ASA) in 1.0 ml of ten-fold dilution of GI tract segments in isotonic phosphate buffer (pH6.8). Data are mean±S.E. (n=5). ●: 5-ASA 0.14 mg eqv./100 mg cecal contents, ○: 5-ASA 0.14 mg eqv./100 mg colonic contents, ■: 5-ASA 0.14 mg eqv./100 mg stomach, PSI or DSI tissue & contents.

in mucosal membranes of PSI and DSI, 5-ASA-Gly was incubated with homogenate of tissue and contents of stomach, PSI or DSI at 37°C. 5-ASA was not detected, which indicated that the prodrug was stable in the upper intestine. The amount of 5-ASA released was about 65% in 8 hrs and 80% in 24hrs after incubation with the cecal contents. In comparison, only 27% of 5-ASA was released in 8 hrs with colonic contents, which indicated that prodrug activation took place more readily in the rat cecum whose environments are more alike to human colon with high bacterial count. *N*-Acetyl-5-ASA was not detected when 5-ASA-Gly was incubated with the cecal contents of rats, verifying the commonly known fact that colonocytic, not bacterial, acetylation is responsible for the acetylation of the aromatic amino group to form *N*-acetyl-5-ASA, which is a major colonocytic metabolite of 5-ASA in this case (Scheline, 1973; Crotty *et al.*, 1992). No significant prodrug conversion (less than 0.1 μg of 5-ASA/ml) was observed in the cecal and colonic contents of rats pretreated with kanamycin sulfate, which suggested that microbial enzymes were responsible for the prodrug activation.

In summary, a simple route for the synthesis of 5-ASA-Gly in good yield was developed and results of *in vitro* experiments suggested 5-ASA-Gly as a promising candidate of a colon-specific prodrug of 5-ASA. *In vivo* studies are in progress.

ACKNOWLEDGEMENT

This work was partially supported by a grant from Korea Research Foundation ('97-Graduate Fellowship).

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