Synthesis and evaluation of mutual azo prodrug of 5-aminosalicylic acid linked to 2-phenylbenzoxazole-2-yl-5-acetic acid in ulcerative colitis

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Abstract: In this study, the syntheses of 4-aminophenylbenzoxazol-2-yl-5-acetic acid, (an analogue of a known nonsteroidal anti-inflammatory drug [NSAID]) and 5-[4-(benzoxazol-2-yl-5-acetic acid)phenylazo]-2-hydroxybenzoic acid (a novel mutual azo prodrug of 5-aminosalicylic acid [5-ASA]) are reported. The structures of the synthesized compounds were confirmed using infrared (IR), hydrogen-1 nuclear magnetic resonance (1H NMR), and mass spectrometry (MS) spectroscopy. Incubation of the azo compound with rat cecal contents demonstrated the susceptibility of the prepared azo prodrug to bacterial azoreductase enzyme. The azo compound and the 4-aminophenylbenzoxazol-2-yl-5-acetic acid were evaluated for inflammatory bowel diseases, in trinitrobenzenesulfonic acid (TNB)-induced colitis in rats. The synthesized diazo compound and the 4-aminophenylbenzoxazol-2-yl-5-acetic acid were found to be as effective as 5-aminosalicylic acid for ulcerative colitis. The results of this work suggest that the 4-aminophenylbenzoxazol-2-yl-5-acetic acid may represent a new lead for treatment of ulcerative colitis.

Keywords: benzoxazole acetic acid, azo prodrug, colon drug delivery

Introduction
5-aminosalicylic acid (5-ASA) is widely used for the treatment of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease.1,2 The drug is also useful for the treatment of colorectal cancer.3–4 However, its absorption in the upper gastrointestinal tract (GIT) after oral administration leads to unwanted systemic effects and lower bioavailability at the site of action.5,6 Therefore, colon delivery systems were developed for 5-ASA, which include controlled formulations, through coating of the 5-ASA with suitable polymer.7 Another controlled delivery method is the use of prodrugs of 5-ASA. This can be achieved through utilizing an azo linkage between 5-ASA and other agents. Sulfasalazine, olsalazine and balsalzide represent examples of azo prodrugs.8–10 Although these prodrugs improve the therapeutic utility of 5-ASA, their use is associated with side effects of their own.11–13 The need for 5-ASA to be safer and more effective stimulates researchers to continue their efforts to optimize the efficacy of 5-ASA.14–16

Finding a carrier of 5-ASA that itself has anti-inflammatory or anticancer activities would represent a significant improvement in the treatment of colorectal cancer or IBD. Search of the literature reveals that a benzoxazole derivative may be a suitable target carrier for 5-ASA. Arylbenzoxazoles are reported to have a
wide range of pharmacological activities that includes anti-inflammatory\textsuperscript{17,18} and anticancer activities.\textsuperscript{19−21} Also, a recent study showed that arylbenzoxazoles have the potential to treat IBD by acting as an antagonist at the serotonin subtype 3 receptor.\textsuperscript{22} Based on the above argument, 4-aminophenylbenzoxazol-2-yl-5-acetic acid was selected for synthesis and evaluated as a carrier of 5-ASA via azo linkage. The compound represents a novel analogue of two known nonsteroidal anti-inflammatory drugs (NSAIDs) namely, benoxaprofen and flunoxaprofen.

Benoxaprofen and flunoxaprofen are well known for their anti-inflammatory properties. They belong to the class of benzoxazoleacetic acid derivatives.\textsuperscript{23,24} Benoxaprofen has been recognized to be an NSAID with a spectrum of activity that is different from other NSAIDs. It shows both lipoxigenase- and cyclooxygenase-inhibition activities.\textsuperscript{25,26} This dual inhibition has encouraged researchers to evaluate benoxaprofen activity in the treatment of UC and psoriasis.\textsuperscript{27−29} In UC, benoxaprofen showed an accelerated healing effect in a rat IBD model.\textsuperscript{30} In this study, the syntheses of 4-aminophenylbenzoxazol-2-yl-5-acetic acid (an analogue of a known NSAID) and 5-[4-(benzoxazol-2-yl-5-acetic acid)phenylazo]-2-hydroxybenzoic acid (as a novel mutual azo produg of 5-ASA) are reported. Additionally, the anti-UC activity of the compounds are investigated.

Materials and methods

The 5-ASA was obtained from the Jordanian Pharmaceutical Manufacturing Co, PLC (JPM), Naour, Jordan. Trinitrobenzenesulfonic acid (TNB) was purchased from Sigma-Aldrich Corp, St Louis, MO, USA. All other reagents were obtained from commercially available sources. The melting points were determined using a Gallenkamp capillary melting point apparatus (model MPD 350 BM 2.5; SANYO Gallenkamp PLC, Loughborough, UK).\textsuperscript{1} H NMR spectra were obtained using a Varian Unity 300 Spectrometer (Varian Medical Systems, Inc, Palo Alto, CA, USA), and chemical shifts (δ) were reported as parts per million (ppm) relative to the internal standard, tetramethylsilane. IR spectra were obtained with a Nicolet Impact 410 (Nicolet Instrument Corp, Fitchburg, WI, USA). MS data were obtained by VG7070 mass spectrometer (M-Scan Inc, West Chester, PA, USA). The ultraviolet (UV)-visible spectra were recorded using a Shimadzu UV-1800 UV-VIS Spectrophotometer (Shimadzu Corp, Kyoto, Japan). Thin layer chromatography (TLC) was conducted using Silica gel 60 GF254 precoated sheets (E Merck KG, Darmstadt, Germany) and was visualized by UV-lamp at wavelength 254 nm.

Chemistry

Methyl-4-nitrophenylbenzoxazol-2-yl-5-acetate (5)

A solution of methyl-3-amino-4-hydroxyphenylacetate (20.0 g, 0.109 mol) and 4-nitrobenzaldehyde (17.0 g, 0.113 mol) in absolute ethanol was heated under reflux for 4 hours. Evaporation of ethanol gave a thick product, which was dissolved in hot glacial acetic acid (250 mL). To the formed solution, lead tetraacetate (14.25 g) was added, and the formed mixture was allowed to cool to room temperature, yielding 28 g (82%) of compound 5, a pure solid precipitate, which was collected by filtration. Melting point (mp) 195°C−196°C. IR: \( \text{KBr} 1731 \text{ cm}^{-1} \), 1516 \text{ cm}^{-1} ; \text{1} \text{H NMR} \) (CDCl3), δ 3.5 (s, 3H, OCH3), 3.6 (s, 2H, CH2), 7.3 (d, 1H, ArH, J = 8.43 Hz), 7.6 (d, 1H, ArH, J = 8.4 Hz), 7.75 (s, 1H, ArH), 8.4 (m, 4H, ArH). MS m/z 313 (MH+).

Methyl-4-aminophenylbenzoxazol-2-yl-5-acetate (6)

The amount 4.5 g (0.014 mol) of methyl-4-nitrophenylbenzoxazol-2-yl-5-acetate and 7.0 g of tin were suspended in 300 mL methanol. Hydrochloric acid (HCl) gas was introduced to the mixture until all of the tin was consumed, and the color changed. The formed solution was concentrated to 50 mL, yielding 3.5 g of the HCl salt upon cooling, which was collected as an orange precipitate. The formed salt was dissolved in 50 mL deionized water, forming a solution that was adjusted to pH 8.0 by adding sodium hydroxide (NaOH) solution. Stirring for 10 minutes, followed by filtration, gave a solid precipitate, which was washed with water. The solid was collected and recrystallized from ethyl acetate, yielding 28 g (82%) of compound 6, a pure solid precipitate, which was washed with water. The solid was dissolved in hot glacial acetic acid (250 mL). Hydrochloric acid (HCl) gas was introduced to the mixture until all of the tin was consumed, and the color changed. The formed solution was concentrated to 50 mL, yielding 3.5 g of the HCl salt upon cooling, which was collected as an orange precipitate. The formed salt was dissolved in 50 mL deionized water, forming a solution that was adjusted to pH 8.0 by adding sodium hydroxide (NaOH) solution. Stirring for 10 minutes, followed by filtration, gave a solid precipitate, which was washed with water. The solid was collected and recrystallized from ethyl acetate, yielding 2.75 g (70%) of 6, mp 185°C−186°C. \text{1} \text{H NMR} \) (CDCl3), δ 3.65 (s, 3H, OCH3); 3.75 (s, 2H, CH2); 4.0 (s, 2H, NH2); 6.75 (d, 2H, ArH, J = 8.5 Hz), 7.15 (dd, 1H, ArH, J = 1.2, 8.3 Hz), 7.45 (d, 1H, ArH, J = 8.3 Hz); 7.6 (d, 1H, J = 1.2 Hz, ArH), 8.05 (d, 2H, ArH, J = 8.5 Hz); MS m/z 283 (MH+).

4-aminophenylbenzoxazol-2-yl-5-acetic acid (1)

A solution of 6 (0.5 g, 0.002 mol) and NaOH (1.4 g, 0.035 mol) in 90% ethanol (EtOH) (30 mL) was stirred at room temperature for 4 hours. A solid precipitate was formed, washed with Acetone, and dissolved in H$_2$O (70 mL). The aqueous solution was acidified with HCl (2 mL), giving a solid precipitate, which was collected and purified by recrystallization from HOAc (acetic acid) to give 0.3 g of 1, mp 220°C−224°C. IR: \( \text{KBr} \) cm$^{-1}$ 1705, \text{1} \text{H NMR} \) (DMSO-d$_6$), δ 3.8 (s, 2H, CH2), 3.95 (s, 2H, NH2); 6.75 (d, 2H, ArH, J = 8.5 Hz); 7.2 (d, 1H, ArH, J = 8.5 Hz); 7.55 (s, 1H, ArH), 7.6 (d, 1H, ArH, J = 8.5 Hz); 7.95 (d, 2H, ArH, J = 8.5 Hz), MS m/z 269 (MH+).
2-hydroxy-5-[4-(benzoazol-2-yl-5-acetic acid) phenylazo]benzoic acid (4)
The amount of 16.0 g (0.053 mol) of HCl salt of 4-amino-phenylbenzoxazole-5-acetic acid 1 was suspended in 250 mL 6 N HCl and cooled to 0°C. To this suspension, a pre-cooled (0°C) solution of sodium nitrite (5.0 g in 50 mL H2O) was added dropwise within 15–20 minutes. The temperature of the mixture was kept between 0°C–5°C. In another flask, 8.0 g (0.057 mol) of salicylic acid was dissolved in 200 mL of 25% NaOH solution, forming a solution, to which the diazonium salt solution was added dropwise within 40–60 minutes, while keeping the temperature at 0°C–5°C and the pH above 8.0 (by adding NaOH solution). The formed mixture was stirred at 0°C–5°C for 30 minutes, and the pH was adjusted to about 7.0, then filtered. A red-to-orange color powder was collected, washed with water, and dissolved in 200 mL boiling 5% NaOH solution. The formed solution was filtered; and the filtrate was adjusted to a pH of 2.0. Upon cooling, a precipitate was formed, which was collected, yielding 8.0 g (38%) of 4 as a pure, colored solid. Mp: decomposition at 160°C. IR: (KBr) 3280, 1720 cm−1 (C=O) stretching. 1H NMR: (DMSO-d6) δ 3.73 (s, CH2), 6.79 (d, 1H, ArH, J = 8.82 Hz), 7.34 (dd, 1H, ArH, J = 1.37, 8.37 Hz), 7.75 (d, 2H, ArH, J = 8.7 Hz), 7.84 (dd, 1H, ArH, J = 2.63, 8.8), 7.98 (d, 2H, ArH, J = 8.56 Hz), 8.25 (m, 2H, ArH). MS m/z 418 (MH+).

Animals
Female Wistar rats (200–250 g) were utilized in this study. The animals were housed in metallic cages (maximum five rats per cage) under hygienic conditions and maintained at room temperature, with free access to food and water. Animals were housed on a 12-hour light/dark cycle (light on 7 am), at 25°C. All experimental procedures were performed during the light cycle and were in accordance with international laboratory animals care and use procedures.

In vitro study
The stability study of the azo prodrug under acidic conditions was performed by dissolving a sufficient quantity of the prodrug in HCl buffer (pH 1.2). The resulting solution was incubated at 37°C, with stirring. The stability of the prodrug was monitored by measuring the absorbance at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 hours.

On the other hand, the susceptibility of the azo bond to reduction by the bacterial azo reductase enzyme was evaluated according to a previously reported method. In a group of test tubes flushed with N2 gas, 1 g of rat fecal matter was placed. To each tube, 5 mL of 8 µg/mL solution of the prodrug in phosphate buffer (pH 7.4) was added. The formed suspensions were incubated in a shaking water bath at 37°C, under anaerobic conditions. At predetermined time intervals (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 hours), aliquots were taken, filtered and monitored using the UV spectrophotometer.

In vivo study
The synthesized compounds were evaluated for IBD activity using a TNB-induced UC rat model, as previously described. In brief, inflammation of the colon was induced in a pre-anesthetized rat by a single intracolonic administration of TNB solution. Rats (n = 24) were randomly grouped in groups A, B, C, and D. Group A was a positive control, and each of the other three groups received predetermined equimolar doses of the compounds under testing. Briefly, animals were fasted for 12 hours. Thereafter, all animals received a solution (0.25 mL) of methylcellulose solution (50% methylcellulose in absolute ethanol) containing 20 mg of TNB. The TNB solution was administered by intracolonic route, using a syringe and rubber cannula. The cannula was inserted into the lumen of the colon via the anus. The cannula was advanced so that its tip was located at 8 cm from the anus. Six hours after administering the TNB solution, the compound and placebo suspensions were given orally twice daily to each animal as follows: group A was administered 0.5 mL of the suspension containing 2.5 mg of methylcellulose solution, twice daily; group B was administered 0.5 mL of the methylcellulose suspension containing 5 mg of 5-ASA, twice daily; group C was administered 0.5 mL of the methylcellulose suspension containing 18 mg of compound 4, twice daily; and group D was administered 0.5 mL of the methylcellulose suspension containing 12 mg of compound 1, twice daily. The treatments continued for 8 days, and on the ninth day, the animals were sacrificed and opened along their abdomen for evaluation. The morphological and histological changes in the rat colons were assessed. A gross inspection of the intestine, before opening, was carried out. For the histological evaluation, the distal colons were excised, and each was examined under a stereomicroscope (SMZ 445, Nikon, Tokyo, Japan) by an observer who was unaware of the treatment. Then, samples of inflamed colons were excised and fixed in 10% formalin and processed by routine technique that included sectioning at 5 µm thickness, mounting on glass slides, and staining with hematoxylin and eosin. The sections were examined under the light microscope, and the extent of inflammation and damage was assessed with respect to three
parameters: the presence of mucosal ulcers, the extent of wall thickness, and the transmural inflammation. Each parameter was graded (grade 1 = normal; grade 2 = very minimal; grade 3 = mild; grade 4 = moderate; and grade 5 = severe). Then, the overall score of damage was considered as the sum of the grades of each parameter and divided as the following (score 1 = normal, sum of the grades 3; score 2 = mild damage, sum of the grades 4–7; score 3 = moderate damage, sum of the grades 8–11; and score 4 = severe damage, sum of the grades 12–15).

Results
The preparation of 4-aminophenylbenzoxazol-2-yl-5-acetic acid 1 (Figure 1) was done by coupling 4-nitrobenzaldehyde with methyl-3-amino-4-hydroxyphenyl acetate in the presence of lead tetraacetate. Once the nitro group of the resulted compound was reduced, the ester function then hydrolyzed to give the target compound 1, as shown in Figure 2. On the other hand, the diazo compound 4 (Figure 3) was synthesized by diazotization of the aminophenylbenzoxazole, followed by the attack of salicylic acid (Figure 4). The structures of the compounds were proven by 1H NMR, IR, and MS.

The stability study of the prepared prodrug 4 in acidic conditions — similar to those of the upper GIT — showed that the compound is stable during the time of the study, which was 5 hours. The absorbencies at 650 nm were 0.041, 0.042, 0.041, 0.042, 0.039, 0.040, 0.043, 0.041, and 0.040 at zero time, and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 4.0, at 5.0 hours, respectively. The release of 5-ASA from the prodrug in rat fecal matter showed a continuous increase in 5-ASA concentration, as reflected by the increase in UV absorbance, which reached a plateau phase at the third hour and continued to the end of the study (at 5.0 hours). In the in vivo studies, the administration of TNB solution resulted in severe inflammation. In group A, the intestines were swollen from the cecum to rectum, the colon was rigid and full of stool in contrast to normal, and the intestine was almost completely damaged. On the other hand, the changes in the treated groups (B, C, and D) were much less evident, swelling was mild, only a small amount of stool was present, and the damage was minimal. Histopathological examination of the intestines of group A showed inflammation and ulcerations in the distal colon. The inflammation was severe, with multiple superficial ulcers present, and there was a marked increase in wall thickness due to fibrosis and transmural extension of the inflammation, which was prominent in most of the animals (Figure 5A). The overall score of the damage was 3–4. In groups B, C, and D, section of the colons showed only occasional superficial ulcerations, and there was no significant increase in wall thickness or transmural inflammation (Figure 5B–D). There were no significant differences in changes among the treated groups, whereas the differences between the three treated groups and the positive control (group A) were remarkable (Table 1).

Discussion
UC is a major gastrointestinal disease for which current treatments are still lacking. This study reports the synthesis of a novel mutual azo produg of 5-ASA prodrug with pharmacological activity both in vitro and in vivo. This prodrug could have the potential to reduce 5-ASA adverse effects in human, especially those related to its absorption in the upper GIT.

The activity of 4-aminophenylbenzoxazole acetic acid was shown to be similar to that of 5-ASA, in the treatment of TNB-induced colitis. Similarly, previous studies have shown that arylbenzoxazoles possess anti-inflammatory, and anticancer activities. In fact, it has been recently shown that arylbenzoxazoles have potential anti-UC properties. In addition, the presence of the amino group, together with the carboxylate in compound 1, possibly decreases the absorption due to zwitterion formation. Thus, this study suggests that 4-aminobenzoxazole 1 may represent a new lead for the treatment of IBD.

Concerning the diazo compound 4, current results showed that it has similar activity to the 4-aminophenylbenzoxazole acetic acid compound 1 and 5-ASA. The similar potency between the diazo compound 4 and both 5-ASA and 1 could be explained based on poor solubility and/or incomplete diazo reduction in the large intestine. However, since the absorption of this compound is expected to be poor, it could represent a promising colon delivery system for both 5-ASA and the aminobenzoxazole 1. This matter requires further future studies.

The diazo compound reported in the current study represents a novel mutual azo produg that should deliver 5-ASA as well as the aminobenzoxazole 1.
is an analogue of benoxaprofen and flunoxaprofen, which are known NSAIDs with anti-inflammatory properties.\textsuperscript{23,24} In fact, benoxaprofen has been shown to promote accelerated healing in a rat UC model of inflammatory bowel disease, which could be explained by its action in leukotriene B4 inhibition.\textsuperscript{30} The reported toxicities of benoxaprofen and flunoxaprofen, which should be taken into consideration in this study, are due to their systemic effect after absorption. The prodrug under investigation is expected to exhibit very minimal absorption, as it is a highly charged compound with low solubility. In a previous study, olsalazine, which is an azo dimer of 5-ASA, showed an oral bioavailability of only 3\%.\textsuperscript{36} Yet, studying the pharmacokinetic and pharmacological profile of the currently reported prodrug and related compound could be a recommended future study.

Results of this study showed that the prepared azo prodrug is stable under acidic conditions similar to those of the upper GIT, which ensures its ability to reach the lower intestine intact. Also, the in vitro study showed that the azo prodrug was susceptible to bacterial azo reductase. When this is correlated with the anti-UC capacity of the synthesized compound, it indicates that bacteria within the large intestine will very likely digest the azo component of the synthesized compound, leading to a release of the 5-ASA and aminobenzoxazole from the moieties. Both of these possess anti-inflammatory and anti-UC activities,
Figure 4 Synthesis of compound 2.

Figure 5 Representative histopathology pictures from rat colons treated with synthesized prodrugs: (A) a representative picture of group A (negative control) showing severe mucosal and transmural inflammation, in addition to increased wall thickness; (B) a representative picture of group B (treated with 5-ASA), shows that mucosal inflammation and ulceration were minimal and no increase in wall thickness was present; (C) a representative picture of group C (treated with compound 4) showing mucosal ulceration with no transmural inflammation or increase of wall thickness; and (D) a representative picture of group D (treated with compound 1) shows mucosal ulceration with no transmural inflammation and that wall thickness was minimally increased.

Notes: All pictures show staining with hematoxylin and eosin. Pictures A and C are at 40 x, pictures B and D are at 10 x.
yet, we do not exactly know which one of these compounds primarily manifest the UC-related activity. The exact determination of the mechanism of action of our newly synthesized compound requires more investigations. Once this is known, further work could result in modified diazo derivatives with augmented anti-UC activities compared with 5-ASA and the currently reported diazo model prodrug.

In conclusion, the current results describe the synthesis of a novel diazo prodrug derivative 4-aminophenylbenzoxazol-2-yl-5-acetic acid and 5-[4-(benzoxazol-2-yl-5-acetic acid) phenylazo]-2-hydroxybenzoic acid. These two compounds were shown to be effective and promising for the treatment of UC.

Disclosure
The authors report no conflicts of interest in this work.

References


