Bull. Chem. Soc. Ethiop. **2014**, 28(3), 475-480.
Printed in Ethiopia
DOI: http://dx.doi.org/10.4314/bcse.v28i3.17

ISSN 1011-3924

SHORT COMMUNICATION

SYNTHESIS, CHARACTERIZATION AND RADICAL SCAVENGING ACTIVITY OF AROMATIC AMINE CONJUGATES OF 5-AMINOSALICYLIC ACID

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(Received October 8, 2013; revised August 27, 2014)

ABSTRACT. 5-Aminosalicylic acid is an anti-inflammatory drug used in the treatment of inflammatory bowel diseases including ulcerative colitis and Crohn's disease. Due to its rapid and extensive absorption in the upper gastro-intestinal tract, substantial amount of 5-aminosalicylic acid is already lost before reaching the site of action, i.e. the colon. In order to prevent this loss of drug, carrier linked prodrug approach has been used and azo prodrugs have been synthesized with a purpose of colon targeting. The present research describes the synthesis and characterization of azo prodrugs of 5-aminosalicylic acid with aromatic amines. The synthesized prodrugs were tested for antioxidant activity using DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity. All the synthesized compounds were found to possess mild to moderate radical scavenging activity.

KEY WORDS: 5-Aminosalicylic acid, Antioxidant, Inflammatory bowel disease, Prodrugs

INTRODUCTION

Inflammatory bowel diseases (IBDs) including ulcerative colitis and Crohn's disease are serious conditions of the large bowel leading to impairment of intestinal functions [1-3]. The unknown etiology of IBDs hampers the development of new therapies. Also, the inability of conventional therapies in preventing relapse and achieving remission advocates the requirement of more efficient approaches [4, 5].

The knowledge of biochemical, molecular and inflammatory events has explained the involvement of oxidative stress in IBDs [6]. Hence, the use of antioxidants is an important approach used for mucosal protection against reactive oxygen metabolites including free radicals [7]. The antioxidant activity of allupurinol, a xanthine oxidase inhibitor, showed beneficial effects in prolonging the time to relapse in ulcerative colitis. Also, the enzyme superoxide dismutase showed encouraging effects in Crohn's disease [8, 9].

Normally, most of the tissues possess significant amounts of enzymes like superoxide dismutase [10], catalase [11], GSH peroxidase [12] and non-enzymatic (thiols, ascorbates), etc [13]. Owing to their antioxidant activity, these enzymes neutralize most of the oxidative agents. But, in case of IBDs, the overproduction of reactive oxygen species overwhelms the action of natural antioxidants [14]. Thus, the use of antioxidants along with conventional regimen (anti-inflammatory, immunomodulators, immunosuppressive agents and corticosteroids) may help to prevent the remission episodes in IBDs.

5-Aminosalicylic acid (5-ASA), an aminosalicylate, used for anti-inflammatory action also possesses antioxidant activity. However, the effectiveness of this drug is limited by its extensive systemic absorption leading to gastrointestinal side effects as well. Thus, an attempt was made to synthesize new prodrugs of 5-ASA to overcome the systemic side effects, achieve colon targeting as well as maintain the antioxidant activity of the active moiety. Considering that aromatic amines can be used as inert carriers for the active drug, the present work was designed

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to synthesize the aromatic amine conjugates of 5-ASA. The synthesized conjugates were evaluated for their antioxidant activity using DPPH radical scavenging assay.

EXPERIMENTAL

General. The reactants used in the synthesis were of AR grade and were purchased from Qualikem Fine Chem. and Molychem. Pvt. Ltd., India. The solvents used were purchased from Merck Inc. The IR spectra of the synthesized compounds were recorded on Shimadzu, 530 FTIR in potassium bromide pellets. ^{1}H and ^{13}C NMR spectra of the synthesized compounds were recorded in DMSO-d_6 on Bruker Advance II 400 MHz instrument using TMS as internal standard.

Synthetic procedures. Aromatic amine (**1a-i**) (0.01 mol) was dissolved in 1:1 ratio of conc. HCl and water. Sodium nitrite solution (0.02 mol/mL) was added to the reaction mixture dropwise with continuous stirring. The reaction mixture was stirred for 45 min. at a temperature of 0-5 ^{o}C. The diazotization step was followed by coupling of the diazotized amines by drop wise addition to the solution of sodium salicylate (0.01 mole of salicylic acid dissolved in 0.02 mol/mL solution of sodium hydroxide). The reaction was stirred at 0-5 ^{o}C for 2 hours under alkaline conditions. The conjugates of salicylic acid and aromatic amines (**3a-i**) were obtained as orange to red powders after the pH was neutralized to 6-7.

*2-Hydroxy-5-phenylazo-benzoic acid (**3a**).* Yield 56%, orange to brownish crystals, m.p. 218-220 ^{o}C. R_f 0.31 ($CHCl_3$/CH_3OH/CH_3COOH 8:1:1). IR (KBr): ν_{max} 3425 (phenolic -OH str), 3063 (aromatic CH str), 1585 (C=O str), 1483 (N=N str), 1286 (CH bend aromatic ring), 1251 (C–O str), 1174 cm^{-1} (C–N str). ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 16.6 (s, 1H), 8.42 (d, J = 2.4 Hz, 1H), 7.8 (m, 3H), 7.46 (t, J = 7.6 Hz, 2H), 7.38 (t, J = 7.2 Hz, 1H), 6.78 (d, J = 8.80 Hz, 1H), 3.82 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.8, 166.9, 152.17, 143.14, 129.85, 129, 126.6, 126, 121.9, 119, 117.2, 78.8, 39.4.

*2-Hydroxy-5-o-tolyl benzoic acid (**3b**).* Yield 59%, red crystals, m.p. 202-204 ^{o}C. R_f 0.38 ($CHCl_3$/CH_3OH/CH_3COOH 8:1:1). IR (KBr): ν_{max} 3628 (phenolic -OH str), 3064 (aromatic CH str), 1670 (C=O str), 1485 (N=N str), 1286 (C–O str), 1193 cm^{-1} (C–N str). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 11.3 (s, 1H), 8.38 (d, J = 2.40 Hz, 1H), 8.04 (dd, J = 8.84, 2.48 Hz, 1H), 7.57 (d, J = 7.60 Hz, 1H), 7.36 (d, J = 4.04 Hz, 2H), 7.27 (m, 1H), 7.09 (d, J = 8.80 Hz, 1H), 2.68 (s, 3H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 164.3, 149.8, 144.3, 137, 131.1, 130.5, 129.1, 128.1, 127.6, 126.4, 122.2, 118, 115, 78.8, 39.5, 17.1.

*2-Hydroxy-5-m-tolyl benzoic acid (**3c**).* Yield 61%, orange amorphous solid, m.p. 199-201 ^{o}C. R_f 0.42 ($CHCl_3$/CH_3OH/CH_3COOH 8:1:1). IR (KBr): ν_{max} 3333 (phenolic O-H str), 1599 (C=O str), 1483 (N=N str), 1257 cm^{-1} (C–O str). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 8.40 (d, J = 2.56 Hz, 1H), 7.83 (dd, J = 8.76, 2.56 Hz, 1H), 7.6 (d, J = 8.00 Hz, 2H), 7.38 (t, J = 8.00 Hz, 1H), 7.24 (d, J = 8.00 Hz, 1H), 6.82 (d, J = 8.72 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.9, 166.2, 152.2, 143.4, 138.3, 130.5, 128.7, 126.6, 126, 122.1, 119.4, 118.9, 117.1, 78.6, 39.3, 20.9.

*2-Hydroxy-5-p-tolyl benzoic acid (**3d**).* Yield 58%, red crystals, m.p. 195-197 ^{o}C. R_f 0.40 ($CHCl_3$/CH_3OH/CH_3COOH 8:1:1). IR (KBr): ν_{max} 3383 (phenolic -OH str), 1585 (C=O str), 1485 (N=N str), 1288 (C–O str), 831 cm^{-1} (aromatic out of plane bend). ^{1}H NMR (400 MHz, DMSO-d_6): 17.0 (s, 1H), 8.32 (d, J = 2.52 Hz, 1H), 7.78 (dd, J = 4.72, 2.56 Hz, 2H), 7.69 (d, J = 8.16 Hz, 2H), 7.29 (d, J = 8.16 Hz, 1H), 6.78 (d, J = 8.76 Hz, 1H), 2.50 (t, J = 1.60 Hz, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.3, 167.5, 150.3, 142.7, 139.5, 129.5, 125.9, 121.8, 119.4, 117, 78.8, 39.5, 20.9.

*2-Hydroxy-5-(o-nitro-phenylazo)-benzoic acid (**3e**)*. Yield 66%, orange colored amorphous solid, m.p. 219-221 ^{o}C., R_f 0.40 ($CHCl_3/CH_3OH/CH_3COOH$ 8:1:1). IR (KBr): ν_{max} 3479 (phenolic -OH str), 3101 (aromatic CH str), 1589 (C=O str), 1494 (N=N str), 1303 (CH bend aromatic ring), 1249 (C–O str), 1130 (C–N str). ^{1}H NMR (400 Hz, DMSO-d_6) δ (ppm) 17.6 (s, 1H), 8.35 (d, *J* = 2.64 Hz, 1H), 7.93 (m, 1H), 7.58 (m, 1H), 6.79 (t, *J* = 4.40 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 170.6, 146.8, 144.5, 142.4, 132.8, 129.4, 128, 126.5, 123.5, 118.2, 78.7, 39.5.

*2-Hydroxy-5-(p-nitro-phenylazo)-benzoic acid (**3f**)*. Yield 60%, orange crystals, m.p. 216-218 ^{o}C. R_f 0.37 ($CHCl_3/CH_3OH/CH_3COOH$ 8:1:1). IR (KBr): ν_{max} 3425 (phenolic -OH str), 3063 (aromatic CH str), 1585 (C=O str), 1483 (N=N str), 1251 (C–O str), 1174 (C–N str), 831 cm^{-1} (aromatic out of plane bend). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 17.5 (s, 1H), 8.41 (d, *J* = 2.36 Hz, 1H), 8.33 (d, *J* = 8.92 Hz, 2H), 7.94 (d, *J* = 8.88 Hz, 2H), 7.85 (dd, *J* = 9.16, 2.48 Hz, 1H), 6.78 (d, *J* = 8.84 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 170.6, 155.9, 147, 142.6, 127.9, 126.8, 124.6, 122.5, 118.9, 118.1, 78.7, 39.5.

*2-Hydroxy-5-(p-methoxy-phenylazo)-benzoic acid (**3g**)*. Yield 70%, yellowish green amorphous solid, m.p. 222 ^{o}C (decomp.). R_f 0.35 ($CHCl_3/CH_3OH/CH_3COOH$ 8:1:1). IR (KBr): ν_{max} 3502 (phenolic -OH str), 3333 (aromatic CH str), 1641 and 1484 (aromatic C=C) 1583 (C=O str), 1494 (N=N str), 1251 (C–O str), 1176 (C–N str), 833 cm^{-1} (aromatic out of plane bend). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 16.6 (s, 1H), 8.33 (d, *J* = 2.56 Hz, 1H), 7.76 (m, 3H), 7 (m, 2H), 6.79 (d, *J* = 8.68 Hz, 1H), 3.82 (s, 3H), ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.5, 166.7,160.7, 146.3, 142.8, 125.7, 123.5, 119.4, 116.9, 114.1, 78.8, 55.3, 39.5.

*5-(p-Chloro-phenylazo)-2-hydroxy-benzoic acid (**3h**)*. Yield 64%, orange crystals, m.p. 192-194 ^{o}C. R_f 0.42 ($CHCl_3/CH_3OH/CH_3COOH$ 8:1:1). IR (KBr): ν_{max} 3446 (phenolic -OH str), 3063 (aromatic CH str), 1587 (C=O str), 1483 (N=N str), 1288 (CH bend aromatic ring), 1172 cm^{-1} (C–N str). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 17.0 (s, 1H), 8.41 (d, *J* = 2.52 Hz, 1H), 7.81 (m, 3H), 7.49 (dd, *J* = 15.48, 8.64 Hz, 2H), 6.82 (d, *J* = 8.76 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.0, 168.8, 151, 142.5, 134.1, 128.9, 126.3, 119.4, 117.4, 78.8, 39.5

*2-Hydroxy-5-(1-naphthaylazo)-benzoic acid (**3i**)*. Yield 66%, orange crystals, m.p. 188-190 ^{o}C (decomp.). R_f 0.40 ($CHCl_3/CH_3OH/CH_3COOH$ 8:1:1). IR (KBr): ν_{max} 3470 (phenolic -OH str), 3047 (aromatic CH str), 1585 (C=O str), 1491 (N=N str), 1288 (CH bend aromatic ring), 763 cm^{-1} (aromatic out of plane bend). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 17.2 (s, 1H), 8.87 (d, *J* = 8.28 Hz, 1H), 8.52 (d, *J* = 1.96 Hz, 1H), 7.97 (m, 3H), 7.74 (d, *J* = 7.36 Hz, 1H), 7.65 (m, 3H), 6.87 (d, *J* = 8.72 Hz, 1H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 171.5, 167.8, 147, 143.7, 133.8, 130.3, 129.7, 127.8, 126.5, 125.7, 122.9, 117.4, 111.1, 78.8, 39.4.

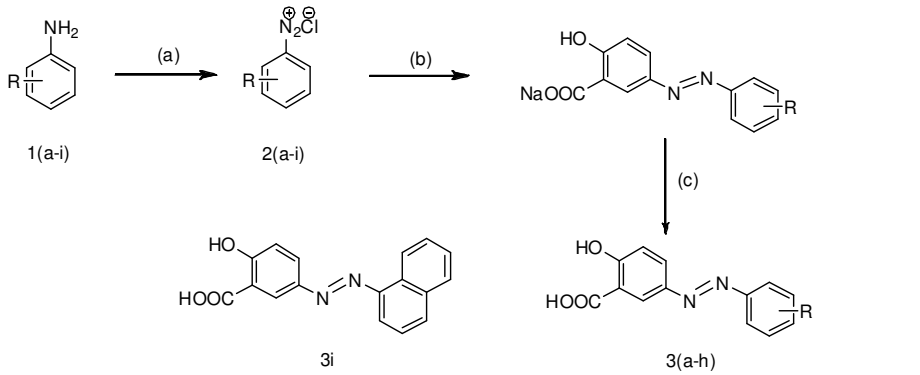
Antioxidant activity. The 0.1 mM solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol was prepared by dissolving 3.94 mg of DPPH in methanol and making the volume up to 100 mL. This solution (1 mL) was added to 3 mL of synthesized compound solution in methanol at different concentrations (10-100 µg/mL). The solution was then incubated at room temperature for 45 min. in dark and the absorbance was measured at 517 nm against blank solution [15, 16]. Percentage inhibition of DPPH free radical was calculated based on the control reading, which contains DPPH solution without any compound using the following equation

$$[\text{DPPH scavenging activity} = (A_{control} - A_{test}/A_{control}) \times 100]$$

where, $A_{control}$ is absorbance of DPPH solution, A_{test} is absorbance of DPPH solution in presence of synthesized compounds.

RESULTS AND DISCUSSION

Commercially available aniline (**1a**) was treated with sodium nitrite in an acidic medium (35% HCl) to give the diazonium salt **2a** (Scheme 1). Since the diazonium salts are unstable moieties and readily convert back to amines or to corresponding phenols, **2a** was generated *in situ* and reaction was continued for coupling with salicylic acid in alkaline medium. The product thus obtained was a sodium salt that was acidified to pH 6-7 to yield **3a**. The other derivatives **3b-3i** were synthesized following the same procedure (Scheme 1).



Scheme 1. Reagents and conditions: (a) $NaNO_2$, 35% HCl, 0-5 ^oC, (b) aq. NaOH (0.02 M), 0-5 ^oC, salicylic acid, and (c) H^+, pH 6-7.

The electrophilic aromatic substitution takes place at *para* position since phenols direct *ortho* and *para* position for the substituent to the hydroxyl group. However, as *ortho* position is substituted here with -COOH group and also the *ortho*- substituted product is unstable, thus this electrophilic substitution of aromatic diazonium salt occurs at *para* position of salicylic acid yielding the required conjugates (**3a**-**3i**). Different aromatic amines were used as reactants (**1a**-**1i**) to get corresponding conjugates with same reaction mechanism.

Biological activity. The synthesized compounds were screened for *in vitro* antioxidant activity using DPPH (1,1 diphenyl-2-picrylhydrazyl) radical. Ascorbic acid was used as the positive control in the assay.

Antioxidants react with the stable DPPH free radical reducing it to the DPPH-H and as consequence the absorbance decreases. The degree of discoloration indicates the scavenging potential of the antioxidant compounds in terms of hydrogen donating ability. The scavenging reaction between (DPPH) and an antioxidant (H-A) can be written as:

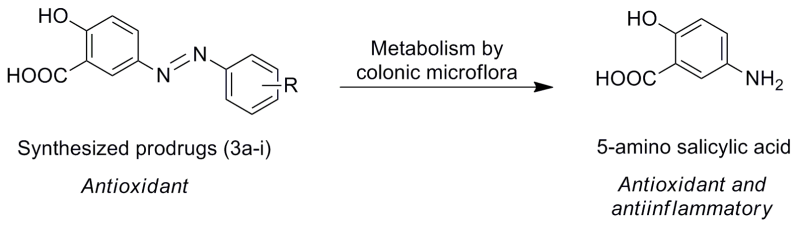
$$\underset{\text{(Yellow)}}{\text{(DPPH)} + \text{(H-A)}} \longrightarrow \underset{\text{(Purple)}}{\text{DPPH-H} + \text{(A)}}$$

The hydrogen donating activity measured at 540 nm showed a significant relationship between concentration of novel molecules and percentage inhibition. Table 1 presents the results of DPPH free radical scavenging activity bioassay.

Table 1. Results of the DPPH radical scavenging activity.

Entry	Mean % inhibition DPPH	Entry	Mean % inhibition DPPH
3a	47.44	**3g**	52.14
3b	44.84	**3h**	51.38
3c	46.66	**3i**	41.96
3d	47.60	Ascorbic acid (positive control)	67.20
3e	48.50	5-ASA (standard drug)	53.76
3f	49.66		

The antioxidant activity of the compounds **3g** and **3h** is comparable to that of the standard drug 5-ASA. The activity of all the synthesized prodrugs is expected to increase *in vivo* because of the release of the active moiety, i.e. 5-ASA into the system. The synthesized prodrugs bear structural resemblance with prontosil, a prodrug of antibacterial sulphanilamide. Prontosil is metabolized in the body to give sulphanilamide [17]. Therefore, it is expected that 5-ASA will be liberated from the prodrugs in an analogous manner (Scheme 2).



Scheme 2. Metabolic pathway of the synthesized prodrugs.

This metabolism is brought about by the enzyme azo-reductases secreted by colonic microflora [18]. Therefore, aromatic amines may serve as carriers in the synthesis of colon-targeted azo-prodrugs of 5-ASA. Further, 5-ASA has anti-inflammatory as well as antioxidant activities. Hence, it protects the cells from the inflammatory damage and the oxidative stress in the IBDs. The synthesized prodrugs may further be tested for their anti-inflammatory potential.

CONCLUSION

Nine novel conjugates of salicylic acid with aromatic amines were synthesized. Aromatic amines (such as aniline, *o*-toluidine, *m*-toluidine, *p*-toluidine, *o*-nitroaniline, *p*-nitroaniline, *p*-chloroaniline, *p*-anisidine and naphthylamine) were conjugated as inert carriers to the active moiety through azo linkage making it colon specific. All the synthesized conjugates showed moderate inhibition of DPPH radical. Compound **3g** was found to have most potent antioxidant activity amongst the synthesized conjugates.

ACKNOWLEDGEMENTS

Authors are grateful to Prof. Monica Gulati and Dr. Amit Mittal, School of Pharmaceutical Sciences, Lovely Professional University for support.

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