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Original article

Design, synthesis and anti-ulcerogenic effect of some of furo-salicylic acid derivatives on acetic acid-induced ulcerative colitis

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ABSTRACT

Ulcerative colitis is a chronically recurrent inflammatory bowel disease of unknown origin. The present work describes design and synthesis of 3-aminofurosalicylic acid **4**, azo-conjugates with aniline **2a**, 4-ASA **2b** or sulphapyridine **2c** as well as N-arylsulphonamido **5**, chlorosulphonyl **6**, aminosulphonyl **7** and N-arylaminosulphonyl derivatives **8** (positional isosters of **5**). All the synthesized compounds were evaluated for their anti-ulcerogenic effect on acetic acid-induced ulcerative colitis in rats. It was noticed that oral treatment with sulphasalazine (a reference drug) and the tested compounds **2a**, **2c**, **4** and **5c** in equimolar doses significantly reduced the intensity of lesion score, ulcer area, ulcer index and wet weight/length ratio compared to the control group. On the other hand, compounds **2b**, **5a**, **5b** and **7** had a lower anti-ulcerogenic efficacy. Also, the antimicrobial activity of the synthesized compounds was screened *in vitro* using the agar diffusion assay technique. In addition, docking of the tested compounds into cycloxygenase II using molecular operating environment (MOE) was performed in order to rationalize the obtained biological results and their mechanism of action.

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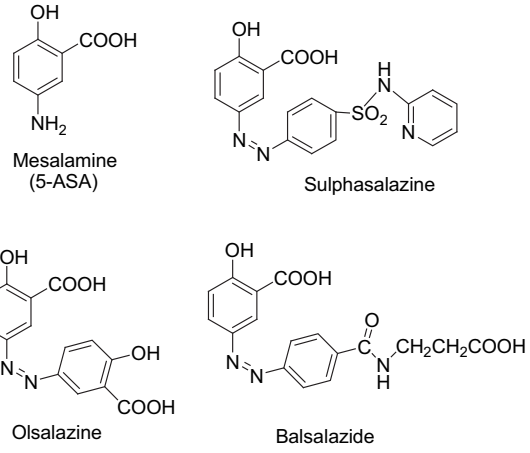
1. Introduction

Inflammatory bowel disease (IBD) is a chronic relapsing inflammation afflicting any part of the entire bowel wall which can take place anywhere in the gastrointestinal tract (GIT) between the mouth and anus. IBD is the result of a deregulated, aberrant and even inappropriate over activation of mucosal response in the intestinal wall due to the defects in the barrier function of the intestinal epithelium and mucosal immune system [1,2]. Two specific IBD subtypes based on the site and extent of inflammation are Crohn's disease and ulcerative colitis. Crohn's disease was first described as a disease in the distal ileum, however, it may be found anywhere in the GIT. Ulcerative colitis (UC) invariably affects the rectum and may extend proximally in a confluent pattern to involve a part of or the entire colon [3,4]. There is no complete cure for IBD (other than colectomy in ulcerative colitis) and the major goal is to decrease the relapse episodes and to increase the patient quality of life [5]. Aminosalicylates [6] and glucocorticoids [7] are the drugs of choice for treatment of the active phase of IBD. Immunosuppressants (such as methotrexate), folate antagonists [8], and infliximab, an anti-tumor necrosis factor (anti-TNF) alpha agent [9], are usually used to establish, and importantly, maintain remission of IBD. Azathiopurine or 6-mercaptopurine and 5-aminosalicylates are co-prescribed in UC [10]. Recently, mesalamine and the non-pathogenic probiotic *Escherichia coli* Nissle 1917 are co-prescribed and effective for treatment of UC [11].



Mesalamine (5-aminosalicylic acid, 5-ASA) is a drug used for treating UC. The mechanism of action of mesalamine is believed to be by reducing inflammation in the colon. UC and other

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inflammatory diseases cause excessive production of chemicals, for example, prostaglandins that produce inflammation in the colon. Prostaglandins are produced by the enzymes, cyclo-oxygenase and lipoxygenase. These enzymes are over-active in individuals with ulcerative colitis. Mesalamine may work by blocking the activity of cyclooxygenase and lipoxygenase, thereby, reducing the production of prostaglandins. Reduced production of prostaglandins decreases inflammation in the colon and the symptoms associated with UC. Available forms of mesalamine differ in their route of administration and how often they are administered, generally available as rectal enema [12]. Sulphasalazine, an azo conjugate of 5-ASA with sulphapyridine, is a drug that is used primarily for treating UC. It is a colon-specific prodrug that is broken down by bacteria in the colon into 5-aminosalicylic acid (5-ASA), and sulphapyridine. More importantly, 5-ASA was shown to be the active therapeutic moiety while sulphpyridine serves simply as a carrier [13]. Olsalazine [14] and Balsalazide [15] also release 5-ASA by the bacteria in the large intestine. Thus they are believed to act as delivery systems for the active agent (5-ASA) to the large intestine, the active site of UC. Very recently the focus of IBD research seems to have shifted from 5-ASA to 4-ASA (4-aminosalicylic acid, a second line anti-tuberculosis agent) with few of its prodrugs. Research studies showed that 4-ASA provides a stable, inexpensive alternative to 5-ASA for topical treatment of UC [16]. So, induction and maintenance of remission, mucosal healing, the avoidance of surgical intervention, and decreasing the likelihood of cancer developing are the primary therapeutic goals in UC [17–19].

In the light of the above consideration, the aim of this study is designing and synthesis of furo-salicylic acid derivatives, where 4 and 5-positions of salicylic acid nucleus are incorporated in furan ring (benzofurans) and their evaluation for treatment of UC. Benzofuran derivatives exhibit a promising gastro-protective activity [20,21] and show dual cycloxygenase (COX-2) and 5-lipoxygenase (5-LOX) inhibition, so they are potential candidates as anti-inflammatory agents [22,23]. In addition, it is known that bacterial infections often produce pain and inflammation, therefore, the antimicrobial activities of benzofurans have been extensively investigated [24,25].

The present work describes design and synthesis of 3-aminofurosalicylic acid **4**, azo-conjugates with aniline **2a**, 4-ASA **2b** or sulphapyridine **2c** as well as N-arylsulphonamido **5**, chlorosulphonyl **6**, aminosulphonyl **7** and N-arylaminosulphonyl derivatives **8** (positional isosters of **5**). The effects of the new synthesized compounds on acetic acid-induced UC in rats' model and their antimicrobial activities were evaluated.

Since, UC is an inflammation disease of the lining of the colon, or large intestine and since the clinically used drugs for UC treatment e.g. mesalamine may work by blocking the activity of cyclo-oxygenase, molecular docking studies using molecular operating environment (MOE) and murine COX enzymes were performed in order to rationalize the obtained biological results and their mechanism of action.

2. Results and discussion

2.1. Chemistry

6-Hydroxy-4-methoxybenzofuran-5-carboxylic acid **1** as a starting material was prepared *via* oxidative alkaline cleavage of γ-pyranone ring of the naturally isolated 4-methoxy-7-methyl-5*H*-furo[3,2-*g*][1]benzopyran-5-one "visnagin" according to the literature procedure [26]. It was convenient to use the highest electron density on position 7 provided by the adjacent OH group to prepare the azo compounds **2a**–**c** by coupling compound **1** with the appropriate benzene diazonium chloride in presence of sodium hydroxide. Mono 7-nitro derivative **3** was prepared by controlled mild nitration of compound **1** using nitric acid/acetone and the temperature did not exceed 5 °C to avoid the formation of 3,7-dinitro derivative [27]. The 7-amino furo-salicylic acid derivative **4** was prepared by two methods. The first method was reductive cleavage of the azo compounds **2** using sodium dithionite. The second was reduction of 7-nitro derivative **3** using sodium dithionite. The NH_2 group was elucidated through IR spectrum, which offered two absorption bands at ν 3450 and 3350 cm^{-1} and a broad signal at δ 3.26–3.43 ppm in H NMR spectrum. The sulphonamides **5a**–**c** were prepared by reacting compound **4** with aryl sulphonyl chlorides in acetone and pyridine as an acid acceptor. IR spectra of **5a**–**c** exhibit absorption bands at ν 3368–3399 cm^{-1} (NH) and 1378–1416 and 1156–1189 (SO_2) and ^{1}HNMR spectra showed exchangeable signals at δ 5 ppm (NH). The chlorosulphonation of furo-salicylic acid derivative **1** with excess chlorosulphonic acid afforded 7-sulphonyl chloride **6**. IR spectrum of the latter compound was characterized by two strong absorption bands at ν 1399 and 1138 cm^{-1} arising from the SO_2 group. Subsequent conversion of arylsulphonyl chloride **6** to the respective arylsulphonamide **7** (by treatment with concentrated ammonia solution or with ammonium carbonate) or the substituted arylsulphonamides **8a**–**e** (by reaction with the appropriate amino compounds) was done. The OH group at position-6 was clearly shown free and was proved by IR, ^{1}H NMR spectra and a positive ferric chloride test.

2.2. Pharmacology

All the new synthesized compounds and sulphasalazine as a reference were initially screened in acetic acid-induced colitis in rats, a well-established and a reproducible experimental model for acute ulcerative colitis [28–32]. Data and parameters of anti-ulcerogenic effect are listed in Table 1.

2.2.1. Statistical analysis

Statistical analysis was performed using SPSS 14.0 statistical software. Differences among groups were examined using parametric one-way analysis of variance (ANOVA). Results are expressed as the mean ± SEM. The minimal level of significance was identified at $P<0.05$ and $P<0.01$.

Table 1
Effects of sulphasalazine and the new synthesized compounds on the macroscopic parameters of ulcerative colitis induced by acetic acid in rats. ($n = 5$).

Groups	Lesion score (0–4)	Ulcer area (cm^2)	Ulcer index	Wet W/L Ratio
Sham	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	66.4 ± 3.17
Control (DMSO)	3.8 ± 0.20	5.3 ± 0.27	9.1 ± 0.34	123.7 ± 8.01
Sulphasalazine	1.6 ± 0.24**	2.1 ± 0.10**	3.7 ± 0.30**	79.0 ± 6.32**
2a	2.2 ± 0.20**	2.6 ± 0.24**	4.8 ± 0.20**	84.5 ± 3.95**
2b	3.0 ± 0.31*	4.4 ± 0.23*	7.4 ± 0.39**	99.4 ± 6.86*
2c	2.8 ± 0.20**	3.9 ± 0.33**	6.7 ± 0.20**	95.2 ± 6.14**
4	2.2 ± 0.20**	3.3 ± 0.20**	5.5 ± 0.38**	90.0 ± 7.24**
5a	3.0 ± 0.44*	4.4 ± 0.24*	7.4 ± 0.50**	99.1 ± 6.48*
5b	3.0 ± 0.31*	4.3 ± 0.18*	7.6 ± 0.52**	98.6 ± 7.66**
5c	2.0 ± 0.31**	2.6 ± 0.24**	4.6 ± 0.40**	86.4 ± 5.90**
6	3.4 ± 0.24	4.7 ± 0.40	8.1 ± 0.56	112.0 ± 7.40
7	3.0 ± 0.15*	4.4 ± 0.24*	7.4 ± 0.18**	103.4 ± 7.18*
8a	3.4 ± 0.24	5.1 ± 0.33	8.5 ± 0.31	119.9 ± 6.90
8b	3.4 ± 0.24	4.7 ± 0.20	8.1 ± 0.40	111.0 ± 6.20
8c	3.4 ± 0.24	4.8 ± 0.37	8.2 ± 0.37	112.0 ± 7.23
8d	3.4 ± 0.24	4.8 ± 0.43	8.2 ± 0.52	110.0 ± 6.89
8e	3.6 ± 0.24	5.1 ± 0.38	8.7 ± 0.60	120.1 ± 7.30

W/L = Weight/Length, *denote significant difference vs. control groups at $P<0.05$ and **$P<0.01$.

2.2.2. LD_{50}

LD_{50} figures are frequently used as a general indicator of a substance's acute toxicity.

Oral administration of the tested compounds in doses less than 220 mg kg^{-1} body weight (b.wt) failed to kill mice within 24 h. LD_{50} was 410 mg kg^{-1} b.wt for compounds **2a**, **2c**, **8c** and **8d** and 400 mg kg^{-1} b.wt for compounds **5a**, **5b** and **7**. On the other hand, LD_{50} for compounds **4**, **8a**, **8b** and **8e** was 396 mg kg^{-1} b.wt. Finally, LD_{50} of compounds **2b**, **5c** and **6** was 390 mg kg^{-1} b.wt. Thus the tested compounds are considered safe, since substances possessing LD_{50} equal to 50 mg or less per kilogram of body weight when administered orally to albino rats, are contemplated as toxic [33].

2.2.3. Anti-ulcerogenic effect (Table 1)

Intra-rectal administration of 4% acetic acid in rats (control group) significantly increased the colon weight and markedly decreased the colon length, leading to elevation in wet weight/length (W/L) ratio. Also, macroscopic examination of the colon of control rats revealed hyperemia, edema, erosion, and ulceration in their mucosa. No pathological changes were observed in sham group suggesting that handling procedure had no interference with the experimental outputs. It was noticed that oral treatment with sulphasalazine (5 mg kg^{-1} b.wt = 12.5 mmol kg^{-1} b.wt) as a reference drug and the tested compounds **2a**, **2c**, **4** and **5c** in equimolar doses significantly reduced the intensity of lesion score, ulcer area, ulcer index and wet weight/length ratio ($P<0.01$) compared to the control group (Table 1). On the other hand, compounds **2b**, **5a**, **5b** and **7** had a lower anti-ulcerogenic efficacy ($P<0.05$). Other compounds **6**, **8a**, **8b**, **8c**, **8d** and **8e** failed to decrease the intensity of lesion score, ulcer area, ulcer index and wet weight/length ratio.

From the previous results, it is clear that 3-aminofurosalicylic acid **4** has a significant effect in correcting ulcerative colitis. Its azo-conjugates with aniline **2a** or with sulphapyridine **2c** also have a significant anti-ulcerogenic effect and their advantage over compound **4** is believed to be the capacity to deliver the active agent **4** past the small intestine and into the large intestine, the active site of UC. Also, the azo conjugate with PAS **2b** exhibited a mild anti-ulcerogenic.

It is also clear that compound **5c** proved to be the most pharmacologically active among the newly synthesized compounds. It could be easily broken in the body by hydrolysis – decomposition, a process which proceeds more quickly in presence of acids [34], with the liberation of the parent pharmacologically active compound **4**. It is also interesting to observe that compound **5c** (the sulphonamido derivative of **4**) proves to be more active than **4** (3-aminofurosalicylic acid). Hydrolysis of N-arylsulphonamido derivatives **5** is attributed to the electronic contribution of the substituents: electron withdrawing groups, for example chloride of **5c** or electron donating groups, for example methyl of **5b**. N-arylsulphonamido derivatives **5a** and **5b** exhibited moderate anti-ulcerogenic effect for UC as well as aminosulphonyl derivative **7**. On the other hand, both of chlorosulphonyl derivative **6** and N-arylaminosulphonyl derivatives **8** (positional isosters of **5**) exhibited no pharmacologic effect. This may be attributed to the fact that the intact compounds **8a**–**e** and their hydrolytic products (furo-salicylic acid nucleus substituted at 3-position with sulphonic acid rather than amino group) were pharmacologically inactive.

2.3. Antimicrobial screening (Table 2)

All the newly synthesized compounds **2**–**8**, sulphsalazine and amphotercin B (as standards) were tested *in vitro* using the agar diffusion assay technique [35]. The antimicrobial activity was screened against two Gram-positive bacteria namely *Staphylococcus aureus* (MTCC 3160) and *Bacillus subtilis* (MTCC 1456), two Gram-positive bacteria namely *E. coli* (ATCC 8739) and *Pseudomonas aeruginosa* (MTCC 3541) and the fungus *Candida albicans* (ATCC 10231). The antimicrobial activity is reflected as zone of growth inhibition of the tested microorganisms (measured in mm). Azo derivatives **2a**, **2b** and **2c** exhibited antibacterial activity against both Gram-positive and Gram-negative bacteria. Compounds **5a**, **5b**, **5c**, **8a** and **8d** showed antibacterial activity against Gram-positive bacteria *B. subtilis* while, compounds **4**, **8a**, **8b** and **8c** showed antibacterial activity against Gram-negative bacteria *E. coli.* All the tested compounds displayed inhibitory activity against the fungus *C. albicans* except **1**, **3**, **4**, **8d** and **8e**. Azo derivatives **2a**–**2c** and chlorosulphonyl compound **6** showed a promising antifungal activity closely related to that of the standard, amphotercin B. The antifungal activity of compound **2b** (azo derivative of PAS) was better than that of amphotercin B.

Table 2
In vitro antibacterial activity using agar diffusion assay technique.

Compound	Diameter of zone of growth inhibition (mm)			
	Staphylococcus aureus	*Bacillus subtilis*	*Escherichia coli*	*Candida albicans*
Control (DMF)	6	6	6	6
Sulphasalazine	30	25	8	7
Amphotercin B	–	–	–	20
1	–	–	–	8
2a	8	10	10	18
2b	10	12	10	25
2c	10	12	12	22
3	–	–	–	–
4	8	8	10	8
5a	–	10	–	10
5b	–	10	–	15
5c	–	12	–	8
6	8	8	8	20
7	8	8	8	14
8a	–	12	–	15
8b	8	–	12	10
8c	–	–	12	12
8d	–	–	–	8
8e	–	10	–	8

2.4. Molecular docking study (Table 3)

Molecular docking studies of compounds **4**–**8** using MOE and murine COX enzymes were performed to rationalize the obtained biological results and their mechanism of action (Fig. 1). Besides, molecular docking studies helped in understanding the various interactions between the ligands and enzyme active site in detail. Since it is highly probable that the investigated compounds **2a**–**c** undergo *in vivo* enzymatic cleavage of azo moiety to afford the corresponding amino analogue, molecular docking study for the amino derivative **4** was performed.

Docking studies of the inhibitors were performed using Molecular Operating Environment (MOE 2008.10; Chemical Computing Group, Canada) [36] using murine COX-2 co-crystallized with celecoxib (PDB: 6COX) as a template (Fig. 2). We performed 100 docking iterations for each ligand and the top-scoring configuration of each of the ligand–enzyme complexes was selected on energetic grounds. Docking of compounds **2a**–**c**, **4**, **5a**–**c**, **8a**, **8c** and **d** showed that the ligand was oriented so that the carboxylate moiety was in the vicinity of Arg120 residue forming a hydrogen bond interaction (side chain acceptor) the NH_2 group of guanidine side chain (distance 2.22–2.84 Å, score 11–31%), Table 3. A hydrogen bond interaction between Arg120 and OH of 5-ASA or carboxylate of salicylic acid (SA) was observed, while in case of

Table 3
Molecular Docking data using MOE.

Compound	Bond number	Amino acid (bond length Å, score %)	Docking energy (kcal./mole)
Celecoxib	3	Arg120 ↔ Arg513 → (3.15, 17), His90 → (2.69, 20).	−111.0077
Indomethacin	2	Tyr355 → (2.57, 11), Glu524 ← (1.30, 34).	−110.9509
Salicylic acid	2	Arg120 → (2.91, 13), Tyr355 → (2.74, 35).	−39.7747
5-ASA	3	Arg120 → (2.84, 14), Arg513 → (2.32, 23), Leu352 ← (1.94, 30).	−55.6055
2a	3	Arg120 → (2.85, 25), Arg120 → (2.23, 30), Tyr355 → (2.34, 56).	−76.4459
2b	7	Arg120 ↔ Gly526 ↔ Arg513 → (2.82, 24), Arg513 → (2.59, 15), Tyr355 → (3.13, 20), Val523 → (1.87, 36), Glu524 → (1.30, 65).	−62.9665
2c	3	Arg120 ↔ Arg120 ↔ Arg120 → (3.39, 13).	−76.8431
4	3	Arg120 → (3.08, 13), Arg120 → (2.29, 31), Tyr355 → (2.35, 68).	−57.9414
5a	3	Arg120 → (2.33, 17), Arg513 → (2.80, 60), Tyr355 → (2.72, 41).	−64.3142
5b	3	Arg120 → (2.71, 27), Tyr355 → (2.36, 60), Tyr355 → (2.81, 38).	−88.2576
5c	3	Arg120 ↔ Ser530 → (2.73, 31), Ser530 → (2.93, 27).	−60.1337
6	1	Tyr355 → (2.52, 47).	−82.0727
7	2	Tyr355 → (2.83, 15), Tyr355 ← (3.24, 19).	−65.1178
8a	3	Arg120 → (2.22, 31), Arg120 → (3.13, 12), Tyr355 → (2.45, 90).	−104.8033
8b	1	Arg513 → (2.39, 53)	−86.5446
8c	3	Arg120 → (2.73, 28), Tyr355 → (2.73, 57), Tyr355 → (2.86, 34).	−97.1818
8d	3	Arg120 → (2.41, 15), Arg120 → (2.66, 29), Tyr355 → (2.35, 65).	−107.2287
8e	2	Arg120 → (3.22, 11), Val523 ← (2.03, 32).	−81.3963

N.B. → means the bond is a side chain acceptor.
← is a side chain donor.
↔ is a hydrophobic.

celecoxib the interaction with Arg120 was a hydrophobic bond. Furthermore, a hydrogen bond interaction between the OH group of Tyr355 and the ligand carboxylate and/or methoxy group was observed (distance 2.35–3.24 Å, score 15–90%) through docking studies of all the new synthesized compounds – except **5c** and **8b** – and the standard compounds: indomethacin and SA. The amino group of guanidine residue of Arg513 interacts with OCH_3 group of compounds **5a** and **8b** (distance 2.72, 2.39 Å, score 60, 53% respectively). The ligand **5c** *p*-chlorophenyl moiety formed a hydrophobic bond with Arg120 and two hydrogen bonds between of the NH group of Ser530 and carboxylate and OCH_3 (distance 2.73, 2.93 Å, score 31, 27% respectively). Docking energy (kcal/mole) of the new synthesized compounds showed that their interaction is more stable than that of SA or 5-ASA and some of them are closely related to that of celecoxib and indomethacin (**8a** and **8d**).

From the abovementioned data, the molecular docking studies of the examined compounds **4**–**8** showed that they bind to the COX-2 active site with position and orientation very close to that resulting from the crystal structure of celecoxib complex with COX-2 and docking studies of the standard compounds SA, 5-ASA and indomethacin. Consequently, these observations provided a good explanation for the assumed inhibitory activity of COX enzyme with compounds **2b**, **2c**, **4** and **5c**.

2.5. Evaluation of lipophilicity

Partition coefficients (Log *P*) of sulphasalazine and the synthesized compounds were calculated using ChemDraw Ultra V 8.0. In this study, the pharmacological activity is not attributed to the value of Log *P*. Although, the pharmacologically active compounds **2a**, **2c** and **5c** and sulphasalazine are considered to be lipophilic (Log *P* are 3.12, 2.91, 1.65 and 3.42, respectively), the active parent compound **4** is hydrophilic (Log *P* is 0.11).

3. Conclusion

The data generated as an outcome of this work demonstrates that four new synthesized compounds: 3-aminofurosalicylic acid **4**, its azo conjugate with aniline **2a**, or with sulphapyridine **2c** and N-(*p*-chloro)sulphonamido derivative **5c** have a remarkable anti-ulcerogenic effect on acetic acid-induced ulcerative colitis in rats.

SAR:The presence of aminosalicylic acid structure is essential for biological activity. Substitution of salicylic acid with amino group either at position 5, 4, or 3 are biologically equivalents. For activity; the amino group should be unsubstituted or have a substituent that is removed readily *in vivo*.

4. Experimental

4.1. Chemistry

Melting points are uncorrected and determined in one end open capillary tubes using Gallen Kamp melting point apparatus MFB-595-010M (GallenKamp, London, England). Microanalysis was carried out at the microanalytical unit, Faculty of Science, Cairo University. IR spectra were determined using KBr discs (cm^{-1}) on Shimadzu Infrared Spectrometer IR-435 (Shimadzu, Kyoto, Japan), Perkin–Elmer FT-IR 1650 (Perkin–Elmer, Waltham, Massachusetts 02451, USA) and Mattson Genesis II FTIR™ Spectrometer (Mattson, Madison, WI, USA). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm spectra were determined using Joel NMR Varian Gemini 200 MHz Spectrometer (Joel, Tokyo, Japan) and Varian Mercury VX-300 MHz NMR Spectrometer (Varian, Oxford, England). Mass spectra were recorded using Hewlett Packard Varian (Varian, Polo, USA) and Shimadzu Gas Chromatograph Mass spectrometer-QP 1000 EX (Shimadzu, Kyoto, Japan). TLC were carried out using Art. DC-Plastikfolien, Kieselgel 60 F_{254} sheets (Merck, Darm-stadt, Germany), the developing solvents were $CCl_4/CH_3COOC_2H_5$ (9:1) or (4:1) and the spots were visualized at 366, 254 nm by UV Vilber Lourmat 77 202 (Vilber, Marne La Vallee, France).

4.1.1. Synthesis of 6-hydroxy-4-methoxybenzofuran-5-carboxylic acid **1** (Scheme 1)

A compound **1** was prepared according to the literature procedure [26].

4.1.2. Synthesis of 7-arylazo-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid **2a**–**c** (Scheme 1)

The appropriate aniline derivative (54 mmol) "aniline, *p*-aminosalicylic acid or sulphapyridine" was dissolved in a mixture of

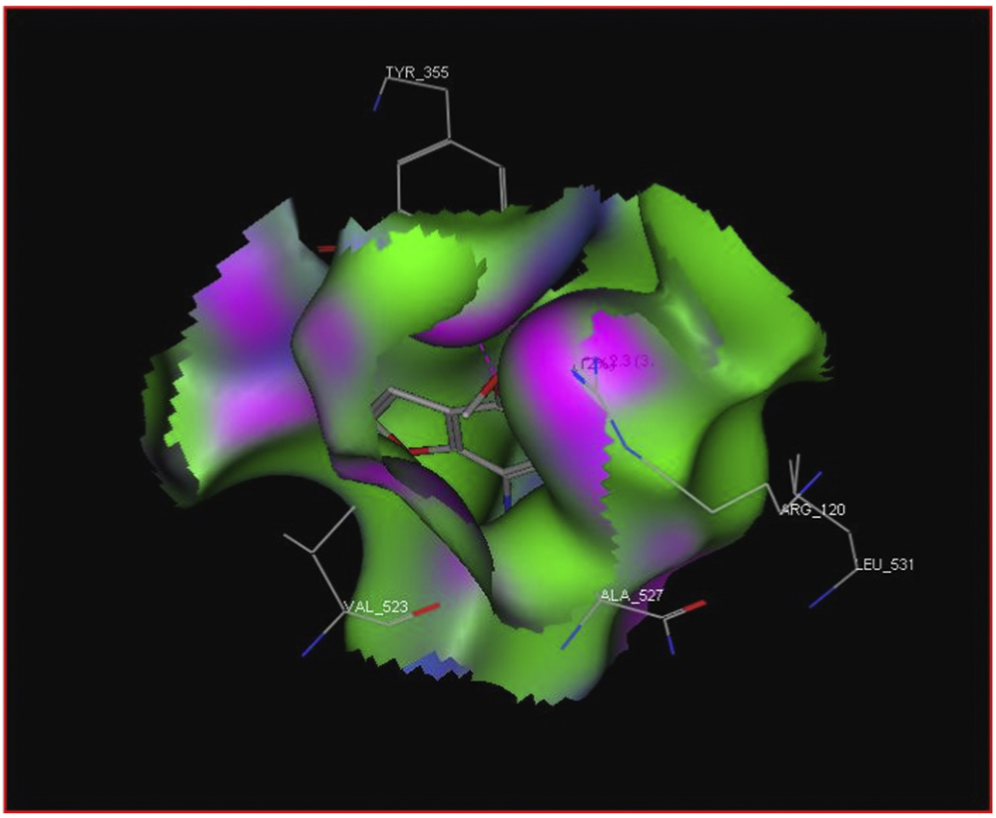


Fig. 1. Docking of compound **4** in the active site of murine COX-2 (3D Contact Style).

concentrated hydrochloric acid (16 ml) and water (16 ml) and cooled to 0–5 °C. Sodium nitrite (4.0 g, 58 mmol) was dissolved in water (16 ml) and cooled. The cold sodium nitrite solution was added in small volumes to the cold aniline hydrochloride solution, and the mixture was kept well shaken (solution A). The temperature was not allowed to rise above 10 °C. A solution of the salicylic acid derivative **1** (11.2 g, 54 mmol) in 10% NaOH (45 ml) was cooled to 5 °C (solution B). Solution A was added very slowly to solution B; a dark-red solution was produced. When all the diazonium salt solution had been added, the mixture was allowed to stand in an ice bath for 30 min with occasional stirring. The dark-red precipitate was filtered off, washed well with water, dried and crystallized from acetic acid/water 1:1.

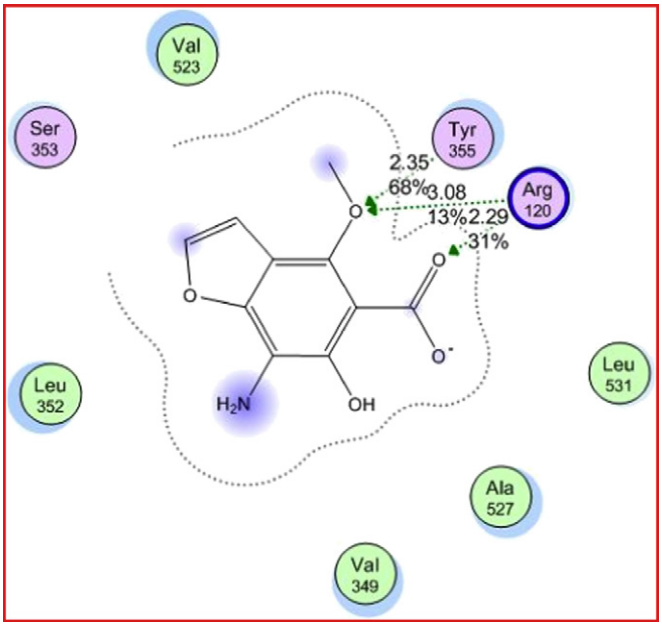
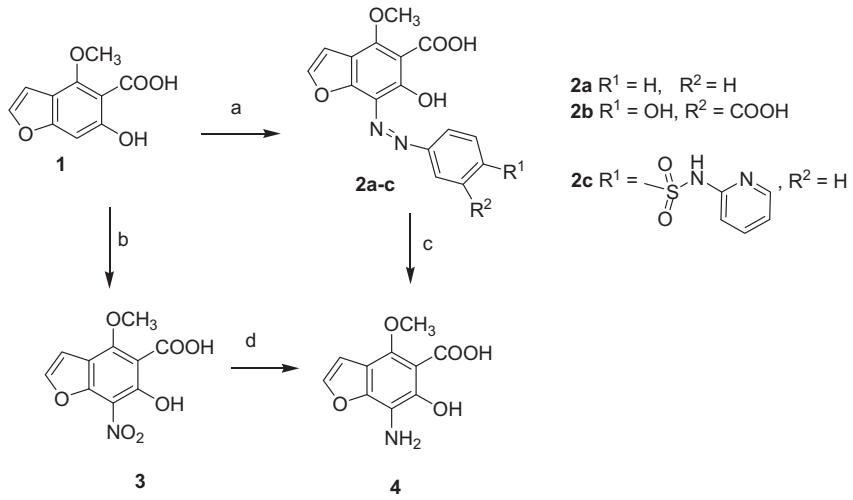


Fig. 2. Docking of compound **4** in the active site of murine COX-2 (2D Contact Style).

*4.1.2.1. 6-Hydroxy-4-methoxy-7-(2-phenylazo) benzofuran-5-carboxylic acid **2a**.* Yield 80%, mp 152–154 °C. IR (KBr, cm^{-1}): 3200, 2850 (2OH), 1697 (C=O), 1590 (N=N). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.01 (s, 3H, OCH_3), 6.73 (d, 1H, furan H, *J*: 2 Hz), 7.10–7.79 (m, 5H, ArH), 7.92 (d, 1H, furan H, *J*: 2.1 Hz), 12.98 (s, 1H, OH exch.), 14.85 (s, 1H, COOH exch.). ^{13}C NMR (DMSO-d_6): 60.1 (OCH_3), 92.7 (C5), 105.3 (C3), 109.3 (C3a), 119.5 (C2Ar, C6Ar), 123.3 (C7), 128.6 (C3Ar, C5Ar), 139.2 (C4Ar), 143.7 (C7a), 150.8 (C6), 154.6 (C1Ar), 156.9 (C4), 164.7 (C=O). Anal. Calcd. For $C_{16}H_{12}N_2O_5$ (312.28): C, 61.54; H, 3.87; N, 8.97. Found: C, 61.36; H, 4.05; N, 8.67. Log *P*: 3.12.

*4.1.2.2. 7-[2-(4-Carboxy-3-hydroxy phenyl) azo]-6-hydroxy-4-methoxy benzofuran-5-carboxylic acid **2b**.* Yield 90%, mp 170–172 °C. IR (KBr, cm^{-1}): 3156 (2OH), 2964 (2COOH), 1691, 1675 (2C=O), 1608 (N=N). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.14 (s, 3H, OCH_3), 6.71 (d, 1H, furan H, *J*: 2.1 Hz), 7.05–7.40 (m, 3H, ArH), 7.73 (d, 1H, furan H, *J*: 2.4 Hz), 12.0 (s, 1H, OH exch.), 14.0 (s, 1H, COOH exch.). Anal. Calcd. For $C_{17}H_{12}N_2O_8$ (372.29): C, 54.85; H, 3.25; N, 7.52. Found: C, 54.90; H, 3.23; N, 7.72. Log *P*: 2.29.

*4.1.2.3. 6-Hydroxy-4-methoxy-7-[2-(2-pyridinylsulphamoyl) phenylazo] benzofuran-5-carboxylic acid **2c**.* Yield 90%, mp 148–150 °C. IR (KBr, cm^{-1}): 3300 (NH), 3128 (OH), 2925 (COOH), 1693 (C=O), 1674 (NH bending), 1633 (N=N), 1373, 1145 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.01 (s, 3H, OCH_3), 6.6–6.9 (m, 3H, furan H and 2ArH), 7.1–7.3 (m, 3H, 2ArH and py. C^4–H), 7.6–7.9 (m, 2H, py. C^3–H and py. C^5–H), 8.1–8.3 (m, 2H, furan H and py. C^6–H), 9.5 (s, 1H, NH



Scheme 1. Reagents and solvents: a: Diazonium salt, 10% NaOH, 5 °C. b: HNO_3, acetone, 0 °C. c: Sodium dithionite, ethanol, reflux. d: Sodium dithionite, 5% NaOH, heat.

exch.), 11.5 (s, 1H, OH exch.), 14.0 (s, 1H, COOH exch.). ^{13}C NMR (DMSO-d_6): 60.1 (OCH_3), 92.7 (C5), 105.2 (C3), 109.8 (C3a, C3py), 113.9 (C5py), 120.1 (C2Ar, C6Ar), 128.8 (C3Ar, C5Ar), 140.2 (C4py), 143.2 (C4Ar), 146.6 (C2), 148.0 (C6py), 151.4 (C6, C7a), 153.1 (C2py), 154.9 (C1Ar), 157.1 (C4), 168.7 (C=O). Anal. Calcd. For $C_{21}H_{16}N_4O_7S$ (468.44): C, 53.84; H, 3.44; N, 11.96. Found: C, 53.87; H, 3.78; N, 11.95. Log *P*: 2.91.

4.1.3. Synthesis of 6-hydroxy-4-methoxy-7-nitrobenzofuran-5-carboxylic acid ***3*** *(Scheme 1)*

A solution of the salicylic acid derivative **1** (1 g) in acetone (20 ml) was treated at 0–5 °C with nitric acid (10 ml, obtained by mixing 50 g nitric acid "d 1.43" with 35 ml water). The reaction mixture was allowed to stand for 30 min. The yellow precipitate was filtered off and crystallized from ethanol.

Yield 75%, mp 110–112 °C. IR (KBr, cm^{-1}): 3207 (OH), 2961 (COOH), 1688 (C=O), 1522, 1334 (NO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 3.85 (s, 3H, OCH_3), 7.40 (d, 1H, furan H, *J*: 1.9 Hz), 8.03 (d, 1H, furan H, *J*: 1.9 Hz), 8.95 (s, 1H, OH exch.), 11.00 (s, 1H, COOH exch.). Anal. Calcd. For $C_{10}H_7NO_7$ (253.17): C, 47.44; H, 2.79; N, 5.53. Found: C, 46.96; H, 3.12; N, 5.02. Log *P*: 3.12.

4.1.4. Synthesis of 7-amino-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***4*** *(Scheme 1)*

Method A: A suspension of the azo derivative **2a** (1.56 g, 5 mmol) in hot ethanol (60 ml) was treated gradually with a solution of sodium dithionite (4.2 g, 20 mmol) in water (15 ml). The mixture was refluxed for 1 h, cooled and filtered. The filtrate was diluted with water to 2–3 volumes and the solid was filtered off and crystallized from methanol.

Yield 60%, mp 164–166 °C. IR (KBr, cm^{-1}): 3456, 3327 (NH_2), 3097 (OH), 2962 (COOH), 1693 (C=O), 1675 (NH bending). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 3.26 (broad, 2H, NH_2 exch.), 4.06 (s, 3H, OCH_3), 7.12 (d, 1H, furan H, *J*: 2.0 Hz), 7.90 (d, 1H, furan H, *J*: 2.1 Hz), 10.27 (s, 1H, OH exch.), 10.35 (s, 1H, COOH exch.). Anal. Calcd. For $C_{10}H_9NO_5$ (223.18): C, 53.82; H, 4.06; N, 6.28. Found: C, 54.09; H, 3.89; N, 6.48. Log *P*: 0.11.

Method B: A solution of the nitro derivative **3** (1.26 g, 5 mmol) in NaOH (10 ml, 5%), was treated with a solution of sodium dithionite (4.2 g, 20 mmol) in water (15 ml). The mixture was refluxed for 30 min, cooled and filtered. The solid was washed several times with water, dried and crystallized from methanol.

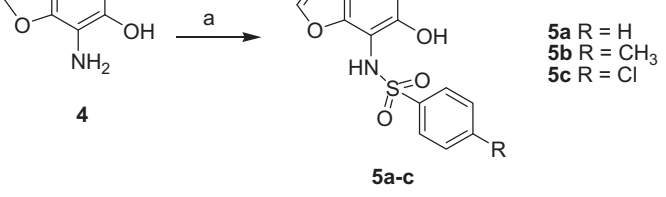
Yield 60%, mp 163–165 °C. IR (KBr, cm^{-1}): 3450, 3350 (NH_2), 3090 (OH), 2950 (COOH), 1693 (C=O), 1677 (NH bending). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 3.43 (broad, 2H, NH_2 exch.), 4.09 (s, 3H, OCH_3), 7.14 (d, 1H, furan H, *J*: 2.4 Hz), 7.84 (d, 1H, furan H, *J*: 2.4 Hz), 12.90 (s, 1H, OH exch.), 13.01 (s, 1H, COOH exch.).

4.1.5. Synthesis of 7-arylsulphonamido-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***5a–c*** *(Scheme 2)*

The amino derivative **4** (2.23 g, 10 mmol) was dissolved in a mixture of dry acetone (20 ml) and pyridine (few drops), and the appropriate benzene sulphonyl chloride derivative (10 mmol), benzene sulphonyl chloride (1.77 g), *p*-toluene sulphonyl chloride (1.91 g), or *p*-chlorobenzenesulphonyl chloride (2.11 g), was added. The reaction mixture was refluxed for 3 h and left at room temperature overnight with stirring. The separated solid was filtered off; by diluting the filtrate with double volume water, a further crop is obtained. The total product was recrystallised from acetone.

4.1.5.1. 6-Hydroxy-4-methoxy-7-(phenylsulphonamido) benzofuran-5-carboxylic acid ***5a****.* Yield 70%, mp 158–160 °C. IR (KBr, cm^{-1}): 3368 (NH), 3100 (OH), 2900 (COOH), 1697 (C=O), 1675 (NH bending), 1400, 1189 (SO_2). ^{1}H NMR ($CDCl_3$, D_2O) δ ppm: 4.37 (s, 3H, OCH_3), 4.71 (s, 1H, NH exch.), 6.86 (d, 1H, furan H, *J*: 1.8 Hz), 7.40–7.60 (m, 5H, ArH), 7.96 (d, 1H, furan H, *J*: 1.8 Hz), 11.63 (s, 1H, OH exch.), 12.20 (s, 1H, COOH exch.). ^{13}C NMR (DMSO-d_6): 56.3 (OCH_3), 92.6 (C5), 105.2 (C3), 109.2 (C7), 111.6 (C3a), 127.22 (C2Ar, C6Ar), 129.6 (C3Ar, C5Ar), 133.5 (C4Ar), 139.5 (C1Ar), 141.0 (C6), 143.4 (C2), 150.7 (C7a), 154.6 (C4), 164.8 (C=O). Mass spectrum showed *m*/*z* 365 (M^++2). Anal. Calcd. For $C_{16}H_{13}NO_7S$ (363.35): C, 52.89; H, 3.61; N, 3.85. Found: N, 3.88. Log *P*: 1.09.

4.1.5.2. 6-Hydroxy-4-methoxy-7-[(p-methylphenyl) sulphonamido] benzofuran-5-carboxylic acid ***5b****.* Yield 65%, mp 148–150 °C. IR (KBr, cm^{-1}): 3395 (NH), 3063 (OH), 2923 (COOH), 1700 (C=O), 1619 (NH



Scheme 2. Reagents and solvents. a: $ClSO_2C_6H_4R$, dry acetone, pyridine, reflux.

bending), 1378, 1156 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 2.27 (s, 3H, CH_3), 4.01 (s, 3H, OCH_3), 4.20 (s, 1H, NH exch.), 6.07 (d, 1H, furan H, *J*: 1.8 Hz), 7.40 (d, 2H, ArH, *J*: 7.4 Hz), 7.53 (d, 2H, ArH, *J*: 7.4 Hz), 8.05 (d, 1H, furan H, *J*: 1.8 Hz), 9.00 (s, 1H, OH exch.), 11.20 (s, 1H, COOH exch.). Anal. Calcd. For $C_{17}H_{15}NO_7S$ (377.37): C, 54.12; H, 4.01; N, 3.71. Found: C, 54.00; H, 4.00; N, 3.89. Log *P*: 1.58.

4.1.5.3. 7-[4-(p-Chlorophenyl) sulphonamido]-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***5c***. Yield 75%, mp 120–122 °C. IR (KBr, cm^{-1}): 3399 (NH), 3131 (OH), 2927 (COOH), 1683 (C=O), 1604 (NH bending), 1416, 1157 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.08 (s, 3H, OCH_3), 5.00 (s, 1H, NH exch.), 6.90 (d, 1H, furan H, *J*: 2.0 Hz), 7.62 (d, 2H, ArH, *J*: 7.6 Hz), 7.75 (d, 2H, ArH, *J*: 7.6 Hz), 8.03 (d, 1H, furan H, *J*: 2.0 Hz), 10.5 (s, 1H, OH exch.), 11.00 (s, 1H, COOH exch.). Anal. Calcd. For $C_{16}H_{12}ClNO_7S$ (397.79): C, 48.31; H, 3.04; N, 3.52. Found: C, 48.31; H, 3.21; N, 3.33. Log *P*: 1.65.

4.1.6. Synthesis of 7-(chlorosulphonyl)-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***6*** *(Scheme 3)*

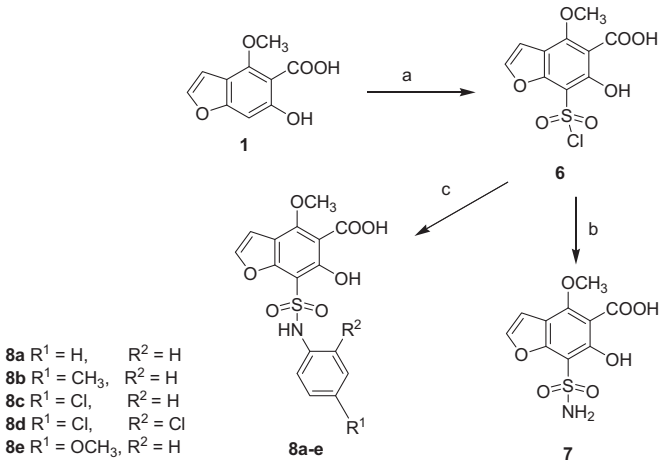
Furo-salicylic acid derivative **1** (2.08 g, 5 mmol) was added to chlorosulphonic acid (5.8 g, 3.41 ml, 50 mmol), magnetically stirred at 0 °C, over 30 min period. The stirring was continued for a further period of 60 min at room temperature and the mixture poured on to crushed ice. The obtained solid was filtered off, dried and crystallized from water/methanol.

Yield 65%, mp 318–320 °C. IR (KBr, cm^{-1}): 3200 (OH), 2925 (COOH), 1625 (C=O), 1399, 1138 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.36 (s, 3H, OCH_3), 6.89 (d, 1H, furan H, *J*: 2.0 Hz), 7.69 (d, 1H, furan H, *J*: 2.0 Hz), 10.0 (s, 1H, OH exch.), 12.00 (s, 1H, COOH exch.). Mass spectrum showed *m*/*z* 307 (M^+). Anal. Calcd. For $C_{10}H_7ClO_7S$ (306.68): C, 39.16; H, 2.30. Found: C, 39.28; H, 2.50. Log *P*: 0.42.

4.1.7. Synthesis of 6-hydroxy-4-methoxy-7-(aminosulphonyl) benzofuran-5-carboxylic acids ***7*** *(Scheme 3)*

Method A: To concentrated ammonia solution (20 ml, d 0.88), sulphonyl chloride derivative **6** (2 g) was added. The mixture was heated with occasional swirling to just below the boiling point for 15 min. The suspension was cooled in ice, and acidified with acetic acid. The product was filtered, dried and crystallized from water.

Yield 80%, mp 252–254 °C. IR (KBr, cm^{-1}): 3400–3200 (NH_2 and OH), 2901 (COOH), 1652 (C=O), 1372, 1164 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.47 (s, 3H, OCH_3), 4.83 (broad, 2H, NH_2), 6.91 (d, 1H, furan H, *J*: 2.0 Hz), 7.82 (d, 1H, furan H, *J*: 2.0 Hz), 11.25 (s, 1H, OH exch.), 13.80 (s, 1H, COOH exch.). Anal. Calcd. For $C_{10}H_9NO_7S$ (287.25): C, 41.81; H, 3.16; N, 4.88. Found: C, 42.26; H, 2.83; N, 4.83. Log *P*: 0.57.



Scheme 3. Reagents and solvents. a: $ClSO_3H$, 0–5 °C, b: Conc NH_3, reflux or anhydrous $(NH_4)_2CO_3$, dry ether, pyridine, reflux. c:$R^1R^2C_6H_3NH_2$, dry ether, pyridine, reflux.

Method B: A mixture of sulphonyl chloride derivative **6** (1.5 g, 5 mmol) and anhydrous ammonium carbonate (0.77 g, 10 mmol) in dry ether (50 ml) and pyridine (few drops) was refluxed for 3 h. The solvent was evaporated and the residue was triturated with dilute HCl, washed with small volume of water and crystallized from water. Yield 60%, mp 250–252 °C.

4.1.8. Synthesis of 7-arylaminolsulphonyl-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***8a–e*** *(Scheme 3)*

A mixture of sulphonyl chloride derivative **6** (1.5 g, 5 mmol) and the appropriate amine (5 mmol) in dry ether (50 ml) and pyridine (few drops) was refluxed for 3 h. The solvent was evaporated and the residue was triturated with dilute HCl, washed with water and crystallized from methanol/water.

4.1.8.1. 6-Hydroxy-4-methoxy-7-(phenylaminolsulphonyl) benzofuran-5-carboxylic acid ***8a***. Yield 55%, mp 179–181 °C. IR (KBr, cm^{-1}): 3365 (NH), 3123 (OH), 2939 (COOH), 1669 (C=O), 1606 (NH bending), 1307, 1154 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.08 (s, 3H, OCH_3), 4.04 (s, 1H, NH exch.), 7.08 (d, 1H, furan H, *J*: 1.8 Hz), 7.25–7.62 (m, 5H, ArH), 8.08 (d, 1H, furan H, *J*: 1.8 Hz), 11.30 (s, 1H, OH exch.), 12.65 (s, 1H, COOH exch.). Mass spectrum showed *m*/*z* 365 (M^++2). Anal. Calcd. For $C_{16}H_{13}NO_7S$ (363.35): C, 52.89; H, 3.61; N, 3.85. Found: C, 53.10; H, 3.81; N, 4.49. Log *P*: 1.09.

4.1.8.2. 6-Hydroxy-4-methoxy-7-[(p-methylphenyl) aminosulphonyl] benzofuran-5-carboxylic acid ***8b***. Yield 55%, mp 200–202 °C. IR (KBr, cm^{-1}): 3363 (NH), 3186 (OH), 2925 (COOH), 1660 (C=O), 1600 (NH bending), 1314, 1129 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 2.11 (s, 3H, CH_3), 4.08 (s, 3H, OCH_3), 5.30 (s, 1H, NH exch.), 6.88 (d, 2H, ArH, *J*: 8.2 Hz), 7.09 (d, 1H, furan H, *J*: 2.0 Hz), 7.31 (d, 2H, ArH, *J*:8.0 Hz), 8.09 (d, 1H, furan H, *J*: 1.8 Hz), 11.40 (s, 1H, OH exch.), 12.50 (s, 1H, COOH exch.). Anal. Calcd. For $C_{17}H_{15}NO_7S$ (377.37): C, 54.12; H, 4.01; N, 3.71. Found: C, 54.22; H, 3.90; N, 4.11. Log *P*: 1.58.

4.1.8.3. 7-[4-(p-Chlorophenyl) aminosulphonyl]-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***8c***. Yield 60%, mp 144–146 °C. IR (KBr, cm^{-1}): 3366 (NH), 3150 (OH), 2923 (COOH), 1660 (C=O), 1600 (NH bending), 1319, 1151 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.03 (s, 3H, OCH_3), 5.40 (s, 1H, NH exch.), 6.81 (d, 2H, ArH, *J*: 8.7 Hz), 7.10 (d, 1H, furan H, *J*: 2.0 Hz), 7.38 (d, 2H, ArH, *J*: 8.7 Hz), 7.96 (d, 1H, furan H, *J*: 2.0 Hz), 11.5 (s, 1H, OH exch.), 12.50 (s, 1H, COOH exch.). Anal. Calcd. For $C_{16}H_{12}ClNO_7S$ (397.79): C, 48.31; H, 3.04; N, 3.52. Found: C, 48.06; H, 3.34; N, 3.33. Log *P*: 1.65.

4.1.8.4. 7-[4-(2,4-Dichlorophenyl) aminosulphonyl]-6-hydroxy-4-methoxybenzofuran-5-carboxylic acid ***8d***. Yield 65%, mp 160–162 °C. IR (KBr, cm^{-1}): 3389 (NH), 3177 (OH), 2923 (COOH), 1615 (C=O), 1600 (NH bending), 1303, 1058 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 3.99 (s, 3H, OCH_3), 5.29 (s, 1H, NH exch.), 6.75 (d, 1H, furan H, *J*: 1.8 Hz), 6.91 (d, 1H, ArH, *J*: 8.1 Hz), 7.20 (s, 1H, ArH), 7.36 (d, 1H, ArH, *J*: 7.8 Hz), 7.85 (d, 1H, furan H, *J*: 2.0 Hz), 11.5 (s, 1H, OH exch.), 12.50 (s, 1H, COOH exch.). Anal. Calcd. For $C_{16}H_{11}Cl_2NO_7S$ (432.24): C, 44.46; H, 2.57; N, 3.24. Found: C, 44.31; H, 2.61; N. Log *P*: 2.21.

4.1.8.5. 6-Hydroxy-7-[4-(p-methoxyphenyl) aminosulphonyl]-4-methoxybenzofuran-5-carboxylic acid ***8e***. Yield 55%, mp 190–192 °C. IR (KBr, cm^{-1}): 3321 (NH), 3150 (OH), 2955 (COOH), 1660 (C=O), 1599 (NH bending), 1307, 1130 (SO_2). ^{1}H NMR (DMSO-d_6, D_2O) δ ppm: 4.02 (s, 3H, OCH_3), 4.08 (s, 3H, OCH_3), 5.00 (s, 1H, NH exch.), 6.98 (d, 1H, furan H, *J*: 2.0 Hz), 7.23 (d, 2H, ArH, *J*: 8.7 Hz), 7.43 (d, 2H, ArH, *J*: 8.7 Hz), 8.10 (d, 1H, furan H, *J*: 2.0 Hz), 11.5 (s, 1H, OH exch.), 12.50 (s, 1H, COOH exch.). ^{13}C NMR (DMSO-d_6): 55.4 (OCH_3), 61.58 (OCH_3), 99.5 (C5), 104.3 (C7),

109.8 (C3), 112.1 (C3a), 114.7 (C3Ar, C5Ar), 122.0 (C2Ar, C6Ar), 128.4 (C1Ar), 146.5 (C2), 151.6 (C4Ar), 153.1 (C7a), 156.8 (C4), 164.2 (C6), 176.6 (C=O). Anal. Calcd. For $C_{17}H_{15}ClNO_8S$ (393.38): C, 51.91; H, 3.84; N, 3.56. Found: C, 52.00; H, 3.83; N, 3.89. Log *P*: 0.97.

4.2. Pharmacology

4.2.1. Materials and methods

4.2.1.1. Animals. Adult male albino rats (180–200 g) and mice of both sexes (25–30 g) were used. Rats were housed singly in wire bottomed cages, while mice were housed randomly in groups in polypropylene cages. All animals were kept under uniform and controlled conditions of temperature and light/dark (12/12 h) cycles, fed with standard rodent diet and water ad libitum. Animals were allowed to adapt to the laboratory environment for one week before experimentation. The experimental tests on animals have been performed in accordance with the Institutional Ethical Committee approval.

4.2.1.2. Preparation of the tested chemical compounds. All the tested chemicals and the reference drug (sulphasalazine) were dissolved in DMSO before oral administration to the experimental animals.

4.2.1.3. Doses. In this investigation, sulphasalazine was given orally in a dose of 12.5 mmol kg^{-1} (=5 mg kg^{-1} b.wt). This dose was calculated by converting the therapeutic dose that used in human to rat's dose [37]. Other groups were administered the test compounds **2a**–**c**, **4**, **5a**–**c**, **6**, **7** and **8a**–**e** in a dose of 12.5 mmol kg^{-1} (2.8–5.8 mg kg^{-1} b.wt). Glacial acetic acid was diluted in distilled water to be 4% and injected into the colon of rats through a rubber catheter at the dose of 5 ml kg^{-1}.

4.2.2. Determination of LD_{50}

LD_{50} of each new compound was determined according to Finney's method [38]. For this purpose, albino mice (25–30 g) were divided into groups each was composed of 5 animals. Preliminary experiments were done for each compound to determine the minimal dose that kills all mice as well as the maximal dose that fails to kill any animal. Consequently, several oral doses at equal intervals were chosen in between these doses. Animals were kept under observation for 24 h during which symptoms of toxicity and rate of mortality in each group were recorded.

Calculations were performed using the following formula:

$$\text{Percent Mortality (PM)} = (I - S) \times 100/I.$$

where *I* am the initial number of animals and *S* is the number of survivors.

4.2.2.1. Data analysis.

1. The percent mortality was calculated using the formula listed above.
2. The concentration of the tested compound was plotted versus the percent mortality on a graph.
3. The value corresponding to LD_{50} was obtained from the graph.

4.2.3. Anti-ulcerogenic effect

Eighty five male albino rats weighing 190–200 g were divided into 17 equal groups. The first (sham) and the second (control) groups were given the solvent only in a dose of 5 ml kg^{-1}. Animals in the third group (reference) were given sulphasalazine in a dose of 12.5 mmol kg^{-1} (=5 mg kg^{-1} b.wt). Other groups were administered the test compounds **2a**–**c**, **4**, **5a**–**c**, **6**, **7** and **8a**–**e** in a dose of 12.5 mmol kg^{-1} (2.8–5.8 mg kg^{-1} b.wt.). All medications were administered intragastrically *via* the aid of an orogastric cannula for 5 successive days and the last dose was administered 2 h before colitis induction.

4.2.3.1. Induction of ulcerative colitis. Rats were fasted for 36 h with access to water ad libitum after which they were lightly anesthetized with ether. A flexible plastic rubber catheter with an outside diameter of 2 mm was inserted 8 cm into the colon through the anus. Ulcerative colitis was induced by injecting glacial acetic acid (4%) into the colon of all rats (except the sham group) through the rubber catheter [29] in a dose of 5 ml kg^{-1}. Injected rats were maintained in a head-down position for 2 min to prevent solution leakage. In sham group, equivolume of normal saline was injected into the colon instead of diluted glacial acetic acid. After 24 h of colitis induction, all rats were sacrificed using ether anesthesia.

4.2.3.2. Assessment of colon macroscopic damage. The tissue of colon, 8 cm in length and 3 cm proximal to the anus was excised, opened longitudinally and washed in saline buffer. The specimens were weighted and wet weight/length ratio was measured for all the rats. Macroscopic damage of the colon was recorded according to a previously validated scoring system from 0 to 4 [28].

The lesion scores were: 0 = no ulcer, 1 = mucosal erythema only, 2 = mild mucosal edema, slight bleeding or slight erosion, 3 = moderate edema, bleeding ulcers or erosions, 4 = severe ulceration, erosions, edema and tissue necrosis. *Ulcer area* was measured using plane glass square. Each cell on the glass square was 1 mm^2 in area and the number of cells was counted and the ulcer area was determined for each colon. *Ulcer index* was the last parameter, measured by summing the lesion score and the ulcer area for each tissue specimen [39]. The results were listed in Table 1.

4.3. Antimicrobial activity (in vitro)

Antimicrobial susceptibilities of the tested compounds were determined by agar-diffusion methods following the general recommendations of the Clinical and Laboratory Standards Institute (CLSI) (formerly the National Committee for Clinical Laboratory Standards).

4.3.1. Medium

A solid medium, namely Mullere Hinton agar (MHA; beef infusion 300 g/L, casein acid hydrate 17.5 g/L, starch 1.5 g/L, agaragar 17 g/L, and distilled water 1000 ml, adjusted to pH 7.4).

4.3.2. Test microorganisms

Two Gram-positive bacteria namely *S. aureus* (MTCC 3160) and *B. subtilis* (MTCC 1456). Two Gram-negative bacteria namely *E. coli* (ATCC 8739) and *P. aeruginosa* (MTCC 3541) and the fungus *C. albicans* (ATCC 10231).

4.3.3. Primary screening

Primary screening of 16 compounds **1**, **2a**–**c**, **3**, **4**, **5a**–**c**, **6**, **7** and **8a**–**c** was done by the agar diffusion assay technique [35]. Twenty-four-hour-old bacterial cultures of all test microorganisms were used as inoculums, which was adjusted to 0.5 McFarland Standard. The stock solutions of all the test compounds (10 mg/ml) were prepared by dissolving 10 mg of the test compound in DMF (1 ml). Sulphasalazine and DMF were used as positive and negative controls, respectively. Twenty milliliters of molten and cooled MHA and 500 ml of each test bacterial culture were mixed (separate flasks were used for each bacterial culture) and poured in sterilized and labeled Petri plates. The wells of 6 mm were punched in the

solidified Petri plates aseptically. Fifty microliters from stock solutions of all the compounds as well as controls was added to each well of labeled Petri plates and incubated at 35 °C for 24 h. The diameter of the zone of growth inhibition around each well was measured after incubation using a Vernier Caliper. The results were listed in Table 2.

4.4. Molecular docking study

All the molecular modeling studies were carried out on an Intel Pentium 1.6 GHz processor, 512 MB memory with Windows XP operating system using Molecular Operating Environment (MOE 2008.10; Chemical Computing Group, Canada) [36] as the computational software. All the minimizations were performed with MOE until a RMSD gradient of 0.05 kcal mol^{-1} $Å^{-1}$ with MMFF94X forcefield and the partial charges were automatically calculated.

The X-ray crystallographic structure of murine COX-2 complexes with celecoxib (PDB ID: 6COX) was obtained from the protein data bank. The enzyme was prepared for docking studies where: (i) Ligand molecule was removed from the enzyme active site. (ii) Hydrogen atoms were added to the structure with their standard geometry. (iii) MOE Alpha Site Finder was used for the active sites search in the enzyme structure and dummy atoms were created from the obtained alpha spheres. (iv) The obtained model was then used in predicting the ligand–enzymes interactions at the active site, Table 3.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejmech.2010.05.071.

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