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INVESTIGATION ON BIOLOGICAL ACTIVITIES OF ANTHRANILIC ACID SULFONAMIDE ANALOGS

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ABSTRACT

In the previous studies, the cytotoxicities of anthranilate sulfonamides were investigated. Herein, the bioactivities of 4-substituted (X = NO_2, OCH_3, CH_3, Cl) benzenesulfonamides of anthranilic acid (**5-8**) are reported. The results revealed that all sulfonamides selectively exerted antifungal activity (25-50 % inhibition) against *C. albicans* at 4 μg/mL. Furthermore, compounds **6** and **8** show antioxidative (SOD) activity. These sulfonamides, except for **6**, selectively display cytotoxic effects toward MOLT-3 cells. It is interesting to note that sulfonamides with electron withdrawing substituent (**5**, X = NO_2) exhibited the highest cytotoxicity. This study provided preliminary structure-activity relationship of the anthranilic sulfonamides that is useful for further in-depth investigation.

Keywords: sulfonamides, anthranilic acid, antimicrobials, antioxidants, cytotoxicity

INTRODUCTION

Sulfonamides (**1**, R = heterocyclic rings, Figure 1) are compounds constituting diverse medicinal applications, widely used as antimicrobial (Genç et al., 2008; Ozbek et al., 2007), anticancer (Ghorab et al., 2009; El-Sayed et al., 2011), antiinflammatory (Borne et al., 1974) and antiviral agents as well as HIV protease inhibitors (De Clercq, 2001). Sulfonamide (**1**, R =H) is well recognized as an antimetabolite (Mengelers et al., 1997). It has a similar structure to *p*-aminobenzoic acid (PABA, **2**) which is an essential compound for the synthesis of tetrahydrofolate in bacteria (Mengelers et al., 1997). Anthranilic acid (*o*-aminobenzoic acid, **3**), an isomeric form of PABA, has been shown to be an interesting pharmacophore essential for biological activities. Its derivatives such as anthranilate sulfonamides have been recently reported to be cytotoxic (Shahlaei et al., 2010) acting as methionine aminopeptidase-2 (MetAP-2) inhibitors. Previously, anthranilate-hydroxamic acid sulfonamides were reported to act as matrix metalloproteinase (MMP) inhibitors. These com-

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pounds were potent inhibitors of MMP-9 and MMP-13 (Levin et al., 2001). MMP-9 was shown to be potential inhibitors of tumor metastasis, while MMP-3 provided protection against the cartilage degradation associated with osteoarthritis.

Considering the molecular structures of anthranilates, the MMP and MetAP-2 inhibitors, therefore, simple and small molecules of sulfonamide derivatives of anthranilic acid **5-8** (Figure 2) are interesting compounds for this investigation. Based on the literature, some bioactivities of these sulfonamides (**5-8**), namely antiinflammatory and aldose reductase inhibitory activities have been previously reported (Borne et al., 1974; DeRuiter et al., 1989; Wydysh et al., 2009). The present study reports antimicrobial, antioxidative and cytotoxic effects of the sulfonamides **5-8**.

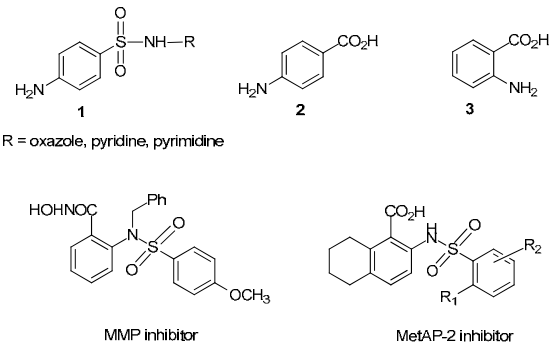

Figure 1: Chemical structures of sulfonamides and aminobenzoic acids

In general, sulfonamides are prepared from the reaction of amines and sulfonyl halides in organic solvents. In this study, an environmentally friendly method using water as the solvent, was conducted for the synthesis of sulfonamides (Figure 2).

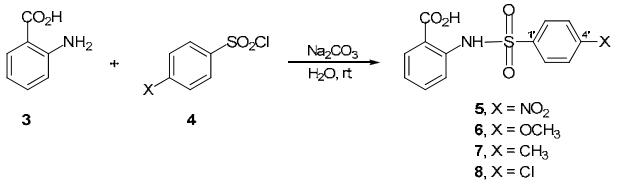

Figure 2: Chemical structures of sulfonamides **5-8**

MATERIALS AND METHODS

General

^{1}H-NMR spectra were recorded on a Bruker AVANCE 300 NMR spectrometer (operating at 300 MHz for ^{1}H). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Melting points were determined on an Electrothermal melting point apparatus (Electrothermal 9100) and are uncorrected. Column chromatography was carried out using silica gel 60 (0.063–0.200 mm). Analytical thin layer chromatography (TLC) was performed on silica gel 60 PF_{254} aluminium sheets (cat. No. 7747 E., Merck). Solvents were distilled prior to use. Reagents for cell culture were as follows: RPMI-1640 (Rosewell Park Memorial Institute medium, Gibco and Hyclone laboratories, USA), HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid), L-glutamine, penicillin, streptomycin, sodium pyruvate and glucose (Sigma, USA), Ham's/F12 (Nutrient mixture F-12), DMEM (Dulbecco's Modified Eagle's Medium) and FBS (fetal bovine serum, Hyclone laboratories, USA), gentamicin sulfate (Government Pharmaceutical Organization, Thailand), MTT (3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich, USA). Vitamin E, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and bovine erythrocyte superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (USA). DMSO was purchased from Fluka.

Tested sulfonamides

Sulfonamides (**5-8**) were prepared using the modified method (Kamal et al., 2008). A general procedure for the sulfonylation of amine (**3**) by benzenesulfonyl chlorides (**4**, X = NO_2, OCH_3, CH_3, Cl) is as follow.

A mixture of anthranilic acid (**3**, 10 mmol) and substituted arenesulfonyl chloride (**4**, 30 mmol) was suspended in water (20 mL). The suspension was basified (pH 8) with saturated Na_2CO_3 and further stirred at room temperature. The reac-

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tion was monitored by TLC chromatograms. The completed reaction was added conc. HCl, precipitates were collected by filtration and washed with 0.1 M HCl and cold water. Purification by silica gel column chromatography (hexane:EtOAc; 9:1-6:4) gave the desired sulfonamides **5-8**. Their IR and ^{1}H-NMR spectral data and melting points were performed. The compounds are listed as follow.

2-(4′-Nitrophenylsulfonamido)benzoic acid (**5**); pale-yellow powder (95.70 %), m.p. 209-212 °C (lit. m.p. 215-218 °C; Borne et al., 1974).

2-(4′-Methoxyphenylsulfonamido) benzoic acid (**6**); pale-yellow powder (98.07 %), m.p. 171-173 °C.

2-(4′-Methylphenylsulfonamido) benzoic acid (**7**); pale-yellow powder (95.86 %), m.p. 186-188 °C (lit. m.p. 229-231 °C; Borne et al., 1974; lit. m.p. 212 °C; Peifer et al., 2007).

2-(4′-Chlorophenylsulfonamido) benzoic acid (**8**); pale-yellow powder (80.49 %), m.p. 177-180 °C (lit. m.p. 201-203 °C; Borne et al., 1974).

Bioactivities

Antimicrobial assay

Antimicrobial activity was performed using the agar dilution method as previously described (Prachayasittikul et al., 2011). The compounds dissolved in DMSO were individually mixed with Müller Hinton (MH) broth. The solution was then transferred to the MH agar solution to yield the final concentrations of 256-2 μg/mL. Twenty one strains of microorganisms, cultured in MH broth at 37 °C for 24 h, were diluted with 0.9 % normal saline solution to adjust the cell density of 1×10^8 cell/mL. The organisms were inoculated onto each plate and further incubated at 37 °C for 24-48 h. Compounds which possessed high efficacy to inhibit bacterial cell growth were analyzed. The microorganisms used for the activity testing are shown in Table 1.

Radical scavenging: DPPH assay

Reaction between DPPH and tested compounds was investigated *via* spectrophotometric method (Prachayasittikul et al., 2010a). The DPPH assay was initiated by adding 1 mL of 0.1 mM solution of DPPH in methanol to a sample solution (0.45 mL, 1 mg/mL dissolved in DMSO). The reaction mixture was incubated for 30 min in a dark room. The absorbance at 517 nm (UV-1610, Shimadzu) was measured and the percentage of radical scavenging activity (RSA) was calculated from the following equation:

$$\text{RSA (\%)} = \left(1 - \frac{Abs._{sample}}{Abs._{control}}\right) \times 100$$

where $Abs._{control}$ is the absorbance of control without compounds and $Abs._{sample}$ is the absorbance of tested compounds. Vitamin E was used as a standard.

Superoxide scavenging: SOD assay

Inhibition of the photoreduction of nitro blue tetrazolium (NBT) was performed to measure the SOD activity (Suksrichavalit et al., 2009) The photochemically excited riboflavin was first reduced by methionine into a semiquinone, which donated an electron to oxygen to form a superoxide source. The superoxide readily converted the NBT into a purple formazan product. As a result, the SOD activity was inversely related to the amount of formazan formation. Purified SOD from bovine erythrocytes was used as a control.

Cytotoxic assay

Cytotoxic assay was evaluated as described (Tengchaisri et al., 1998). Cancer cells were grown in Ham's/F12 medium containing 2 mM L-glutamine supplemented with 100 U/mL penicillin, streptomycin and 10 % FBS, except for HepG2 cell which was grown in DMEM. Briefly, cell lines suspended in RPMI-1640 containing 10 % FBS were seeded with 1×10^4 cells (100 μL) per well in a 96-well plate. The incubation was performed at 37 °C

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under humidified atmosphere (95 % air, 5 % CO_2) for 24 h. Additional medium (100 μL) containing the test compound and vehicle was added to a final concentration of 50 μg/mL, 0.2 % DMSO and further incubated for 3 days. Cells were fixed with 95 % EtOH, stained with crystal violet solution and lysed with a solution of 0.1 N HCl in MeOH. The absorbance was measured at 550 nm. On the other hand, HuCCA-1, A549 and HepG2 cells were stained with MTT. IC_{50} values were determined as the drug and sample concentrations at 50 % inhibition of the cell growth. The tested cell lines were T-lymphoblast (MOLT-3, acute lymphoblastic leukemia), human hepatocellular carcinoma cell line (HepG2), human cholangiocarcinoma cancer cell (HuCCA-1) and human lung carcinoma cell line (A549).

RESULTS AND DISCUSSION

Chemistry

Sulfonamides **5-8** were successfully prepared in high yields (80-98 %) using the base catalyzed sulfonylation reaction of anthranilic acid with 4-substituted benzenesulfonyl chlorides (**4**) under the modified environmentally friendly method (Kamal et al., 2008). Their structures were confirmed by ^{1}H-NMR and IR spectra. Although, the synthesis of sulfonamides **5-8** were previously reported (Borne et al., 1974; Wydysh et al., 2009; Deng and Mani, 2006), in this study relatively higher yield products were achieved.

Biological activities

Antimicrobial activity

Compounds **5-8** were tested for their antimicrobial action using the agar dilution method against twenty-one strains of microorganisms (gram-positive and gram-negative bacteria and diploid fungus). It was found (Table 1) that the tested compounds selectively displayed growth inhibition (25-50 %) against *C. albicans* ATCC 90028 at 4 μg/mL. When tested at higher concentration (128 μg/mL), the sulfonamide **5** exhibited 50 % inhibition against the *C. albicans*. The tested sulfonamides were found to be inactive antibacterials.

It was reported that the degree of ionization of sulfonamido group ($-SO_2NH-C_6H_4-Y$) which gave anionic N-atom ($-SO_2\bar{N}-C_6H_4-Y$), was important for antibacterial activity (Genç et al., 2008; Mengelers et al., 1997). The anionic form enhanced its solubility and caused ionic interaction with receptors e. g. dihydropteroate synthetase or dihydrofolate synthetases (Mengelers et al., 1997). In this respect the sulfonamide requires an electron withdrawing substitutent (Y) on the phenyl ring, particularly where $Y = NO_2$ at *p*-position provided the compound with high antibacterial activity as compared to the *m*-position (Genç et al., 2008). On the other hand, the hydrophobic effect was not important for the activity (Mengelers et al., 1997). The sulfonamides **5-8** were shown to be inactive antibacterials, which could be due to their sulfonamido moieties ($-SO_2NH-C_6H_4-CO_2H$-*ortho*) that requires the electron withdrawing group at the *p*-position of the phenyl ring ($C_6H_4-CO_2H$).

Antioxidative activities

Studies were performed to investigate the superoxide scavenging (SOD) and radical scavenging (DPPH) activities of the sulfonamides. Compounds **6** and **8** (Table 2) exhibited weak SOD activity with 15.7 and 6.1 % NBT inhibition at 300 μg/mL. Whereas sulfonamides **5** and **7** were inactive superoxide scavengers. On the other hand, all compounds showed no radical scavenging activity (DPPH). So far, antioxidant properties of these anthranilic sulfonamides were not found in the literature. Study of the molecular structures revealed that the SOD activity depended on the asymmetric charge localization of the compound, where high asymmetric charge afforded the compound with high SOD activity (Prachayasittikul et al., 2010b).

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Table 1: Antimicrobial activity[a] (*C. albicans*) of sulfonamides **5**-**8**

Compound	**Growth inhibition** (%) at 4 μg/mL
5	25[b]
6, 7	25
8	50

[a]Twenty-one strains of tested microorganisms were **gram-negative bacteria**; *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Serratia marcescens* ATCC 8100, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 15442, *Pseudomonas stutzeri* ATCC 17587, *Shewanella putrefaciens* ATCC 8071, *Achromobacter xylosoxidans* ATCC 27061 and *Plesiomonas shigelloides*, **gram-positive bacteria**; *Staphylococcus aureus* ATCC 29213, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecalis* ATCC 33186, *Micrococcus lutens* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes* and *Listeria monocytogenes* and **diploid fungus**; *Candida albicans* ATCC 90028 and *Saccharomyces cerevisiae* ATCC 2601. Ampicillin at 10 μg/mL was used as a control of antimicrobial testing system, it showed 100 % inhibition against *S. aureus* ATCC 29213, *S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *P. shigelloides* and *S. pyogenes.*
[b]At 128 μg/mL showed 50 % inhibition.

Table 2: Antioxidative activities of sulfonamides **5**-**8**

Compound[a]	**SOD** (%)	**DPPH** (%)
5	NA	NA
6	15.7	NA
7	NA	NA
8	6.1	NA

[a] Compounds were tested at 300 μg/mL.
IC_{50} of standard SOD is 0.75 μg/mL.
NA = no activity.

The sulfonamide **6**, with OCH_3 group at C-4′ displayed the highest SOD activity when compared to the compound **8** bearing Cl at C-4′. This could presumably explain that the stroger electron donating effect of OCH_3 contributed asymmetric charge on 4′-methoxybenzenesulfonyl moiety of the molecule (**6**).

Cytotoxicity

Cytotoxicity was evaluated against four cell lines (Table 3) using etoposide and/or doxorubicin as the reference drugs. Results showed that the cytotoxic activity was observed for compounds **5**, **7** and **8** toward MOLT-3 cell with IC_{50} values of 15.71, 32.88 and 33.96 μg/mL, respectively. Interestingly, the sulfonamide **5** with NO_2 substituted at C-4′ position exerted the highest cytotoxicity when compared to compounds **7** and **8** with CH_3 and Cl groups at C-4′, respectively. Both compounds **7** and **8** had comparable cytotoxic effect, but with the IC_{50} values of approximately two folds higher than the compound **5**. On the other hand, sulfonamide **6** bearing OCH_3 group at C-4′ was shown to be inactive compound. However, all sulfonamides were inactive cytotoxics when tested with HuCCA-1, A549 and HepG2 cells. The results suggested that the substituents at C-4′ position of compounds **5**-**8** governed their cytotoxicities. It is evident that an electron withdrawing group (NO_2) at C-4′ exhibited the highest cytotoxicity, whereas electron releasing groups; CH_3 and Cl provided the compounds with lower cytotoxicity. In case of the stronger electron releasing group, OCH_3 which exerted no cytotoxicity as noted for the sulfonamide **6**.

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Table 3: Cytotoxicity of sulfonamides **5**-**8**

Compound	IC_{50} (μg/mL)[a,b]			
	MOLT-3	HepG2	HuCCA-1	A549
5	15.71±0.70	inactive	inactive	inactive
6	inactive	inactive	inactive	inactive
7	32.88±1.75	inactive	inactive	inactive
8	33.96±2.06	inactive	inactive	inactive
Etoposide	0.021±0.002	19.00±1.73	-	-
Doxorubicin	-	0.53±0.06	0.40±0.282	0.35±0.00

[a] The assays were performed in triplicates, using etoposide and/or doxorubicin as reference drugs.

[b] an IC_{50} > 50 μg/mL indicates inactive.

CONCLUSION

4-Substituted (X) benzenesulfonamides of anthranilic acid (**5**-**8**) were prepared in high yields. Bioactivity testings revealed that all sulfonamides selectively exert antifungal action (25-50 % inhibition) against *C. albicans* with low concentration at 4 μg/mL. Some of the tested compounds (**6** and **8**) show SOD activity, whereas **6** is shown to be the highest antioxidant. These sulfonamides, except for **6**, selectively display cytotoxic effect toward MOLT-3 cells. The sulfonamide with electron withdrawing substituent (**5**, X = NO_2) exhibited the highest cytotoxicity, but with no antioxidant property. This study provided preliminary structure-activity relationship of the anthranilic sulfonamides useful for further in-depth investigation.

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