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ARTICLE

Antibacterial and Cytotoxic Activities of Schiff Base Analogues of 4-Aminoantipyrine

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Abstract Schiff base is the class of compounds showing wide range of biological activities having the azomethine (-N=CH-) active pharmacophore, which play major roles in their significant bio-activities. A series of Schiff base analogues of 4-amino-antipyrine analogues have been tested for bactericidal and cytotoxic activities against selected bacterial strains and brine shrimp (*Artemia salina*) nauplii, respectively. Of the compounds tested, two compounds showed a good inhibition of bacterial growth against *E. coli* and *C. sakazakii*, whereas three compounds demonstrated high cytotoxicity with LC_{50} values of 225, 480, and 581 ppm, in a short term bioassay using *A. salina*. Qualitative structure-cytotoxic activity relationships were studied using physicochemical parameters; a good correlation between clogP and cytotoxic activity was observed.

Keywords antibacterial activity · brine shrimp toxicity · crystal structure · pyrazole-3-ones · Schiff base

Introduction

Schiff base compounds containing an azomethine group (-CH=N-) have received much attention in the field of chemistry and biology due to their chemotherapeutic value (Ali et al., 2002; Cukurovali et al., 2002; Santos et al., 2001). Schiff base compounds belong to a widely used group of organic intermediates and are important for the production of special chemicals such as pharmaceuticals or rubber additives (Macho et al., 2004) and as amino protective groups in organic synthesis (Bey and Vevert, 1977). They are also used as effective ligands for metal ions and liquid crystals in analytical, medicinal, and polymer chemistry (Layer, 1963; Higuchi and Yamamoto et al., 1999; Adams, 2000; Jarrahpour et al., 2004; Agarwal and Prasad, 2005).

Antipyrine (1,5-dimethyl-2-phenylpyrazole-3-one) is a compound that possesses a pyrazolone moiety with a five-membered lactam ring containing two nitrogens and a ketone in the same molecule. Antipyrine and its 4-amino derivative (4-amino-1,5-dimethyl-2-phenylpyrazole-3-one) have shown outstanding pharmacological properties (Kees et al., 1996; Burdulene et al., 1999; Bashkatova et al., 2005; Costa et al., 2006; Ei Ashry et al., 2007; Santos et al., 2010) such as anti-inflammatory, analgesic, antiviral, antipyretic, antirheumatic, and antimicrobial activity. 4-Aminoantipyrine is an antipyrine that forms a variety of Schiff bases with aldehydes, and a remarkable number of compounds have recently been reported (Montalvo-González and Ariza-Castolo, 2003; Hu, 2006; Li and Zhang, 2006; Liu et al., 2006; Zhang et al., 2006; Zhao, 2007) with a wide range of biological activities and applications (Ismail, 2000; Abd Rehim et al., 2001; Yadav et al., 2003; Santos et al., 2010; Ali et al., 2012).

In our previous study, we reported the synthesis, as well as antioxidant and anti-inflammatory activities, of a series of Schiff base analogues of 4-aminoantipyrine (Alam et al., 2012) and a crystal structure of (*E*)-4-[benzylideneamino]-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (Alam and Lee, 2012). Here, we report on the antibacterial and cytotoxic activity of thirteen Schiff base analogues of 4-aminoantipyrine (**3a-m**) together with the crystal structures of **3g** and **3h**. The synthesis of compounds **3a-m** have been demonstrated in our previous studies (Alam et al., 2012; Alam and Lee, 2012); however, crystal structures of compound **3g** and **3h**, as well as antimicrobial and cytotoxic actions of compounds

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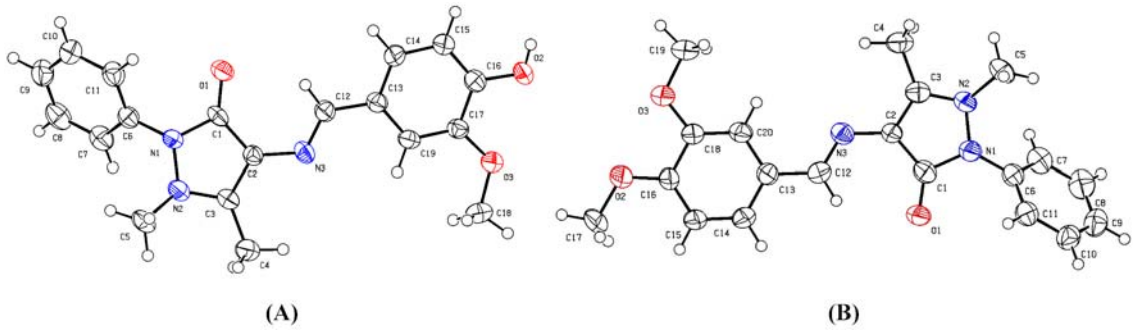


Fig. 1 The molecular structure of (A) (*E*)-4-(4-hydroxy-3-methoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3g**) and (B) (*E*)-4-(3, 4-dimethoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3h**), showing 50% probability displacement ellipsoids, and the atom-numbering scheme.

3a-m, have not yet been reported. *In vitro* antibacterial activities were screened against six bacterial strains (e.g., *Klebsiella pneumonia*, *Staphylococcus aureus*, *Cronobacter sakazakii*, *Citrobacter freundii*, *Salmonella enterica,* and *Escherichia coli*). The cytotoxicities of the previously described compounds were screened using brine shrimp (*Artemia salina*) in a lethality bioassay. The brine shrimp nauplii usually are used as a preliminary screening tool for the determination of toxicity of synthesized compounds. Physicochemical calculations were also carried out to discuss the relationship between the electronic properties and cytotoxic activities of Schiff base analogues of 4-aminoantipyrine.

Materials and Methods

Synthesis. The Schiff base analogues of 4-aminoantipyrine (**3a-m**) used in the present study were prepared according to our previous report (Alam et al., 2012; Alam and Lee, 2012). Briefly, an anhydrous ethanol solution (10 mL) of 4-amino-1,5-dimethyl-2-phenylpyrazol-3-one (203 mg, 1 mmol) was added to an anhydrous ethanol solution (10 mL) of substituted benzaldehyde (1 mmol), and the mixture was refluxed at 80°C for 4–6 h under atmospheric conditions (Scheme 1). The progress of the reaction was monitored by thin layer chromatography. The precipitates formed were collected by filtration and purified by recrystallization with ethanol, and then dried *in vacuo* to produce the pure compound in high yield (80–94%). Single yellow crystals of compounds **3g** and **3h** suitable for X-ray analysis were obtained by slow evaporation of an ethanol solution. A single crystal of suitable size (0.37 mm ×0.25 mm×0.14 mm for **3g** and 0.37 mm×0.24 mm×0.19 mm for **3h)** was chosen for the X-ray diffraction studies. The data were collected at a temperature of 200(2) K on a Bruker *SMART* CCD area detector diffractometer (Bruker, 2000) with graphite monochromated radiation Mo$K\alpha$ (λ =0.71073 Å). The structure was solved by direct methods using SHELXTL (Sheldrick, 2008).

Antibacterial Screening. The bactericidal activities of the compounds **3a-m** *in vitro* were determined by the Kirby-Bauer disc diffusion method (Bauer et al., 1966**)**. Briefly, Trypticase Soy Agar medium (Sigma-Aldrich, USA) was used as basal medium for test bacteria. This agar medium was inoculated with 0.2 mL liquid cultures containing microorganisms (cultured for 24 h). Sample discs were gently placed on pre-inoculated agar plates, and *Klebsiella pneumonia*, *Staphylococcus aureus*, *Cronobacter sakazakii*, *Citrobacter freundii*, *Salmonella enterica*, and *Escherichia coli* incubated aerobically at 37°C for 24 h. Discs with only DMSO were used as a control and nalidixic acid was used as a positive control. Inhibitory activity was measured (in mm) as the diameter of the observed inhibition zones.

Cytotoxicity Assay. Brine shrimp nauplii (*A. salina*) were used in the *in vivo* cytotoxicity assay according to the method of Mayer et al. (1982), with some modifications. Brine shrimp nauplii were hatched in a small tank divided by a net, which contained artificial seawater (3.8% NaCl). The tank was partially exposed to incandescent light to attract the naupliis. The assay was performed 24 h after their hatching, and no food was added during the hatching and experimental periods. Test samples (3 mg) were dissolved in 0.6 mL of DMSO to obtain stock solutions of 5 mg mL^{-1} per sample. From the stock solutions, different concentrations of test samples were placed in separate vials, with the volume of each vial made up to 5 mL using artificial seawater to obtain the desired final concentrations. Twenty brine shrimp nauplii were then placed in each vial. The negative control was prepared in the same manner, except the sample was omitted. Gallic acid was used as standard. After 24 h of incubation, the vials were observed using a magnifying glass, and the number of survivors in each vial counted and noted. Tests were performed in triplicate, and the resulting data were transformed to probit analysis software (Finney, 1978) for determination of LC_{50} values in ppm.

Computational Methods. The molecular geometries of the compounds **3a-m** were built with a standard bond length and angles using the ChemBio3D ultra Ver. 14 molecular modeling program (CambridgeSoft Corporation, USA). The energy was minimized by the semi-empirical molecular orbital PM3 method (Stewart, 2004). Physicochemical properties were calculated using mol inspiration cheminformatics software (Molinspiration

Cheminformatics, Slovak Republic). The method for calculation of clogP was developed by Molinspiration (milogP2.2-2005) based on group contributions and correction factors by fitting calculated logP with experimental logP for a training set more than twelve thousand, mostly drug-like molecules. Molecular polar surface area (PSA) was calculated based on the methodology published by Ertl et al. (2000), as a sum of fragment contributions. The maps of molecular lipophilicity potential (MLP) and PSA were viewed in Molinspiration Galaxy 3D Structure Generator (ver. 2010.02 beta) using an optimized structure generated by the semi-empirical molecular orbital PM3 method.

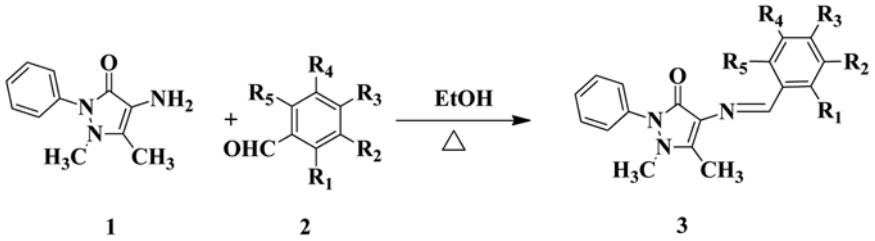
Results and Discussion

Synthesis and Crystal Structure. Schiff base analogues of 4-aminoantipyrine (**3a-m**) was synthesized according to our previous studies (Alam and Lee, 2012; Alam et al., 2012) as shown in Scheme 1. The X-ray diffraction crystal data and structure refinement for compounds **3g** and **3h** are shown in Table 1. The details of the X-ray data have been provided as supplementary materials. The ORTEP diagrams of **3g** and **3h** are shown in Fig. 1 with thermal ellipsoids drawn at a 50% probability. The packing arrangement in the crystal structure of compounds **3g** and **3h** are shown in Fig. 2, with intermolecular and intramolecular hydrogen bonding interactions.

Antibacterial Activity. The Schiff base derivatives (**3a-m**) of 4-aminoantipyrine were evaluated for their *in vitro* antibacterial activities against one Gram-positive bacteria (*Staphylococcus aureus*) and five Gram-negative bacteria (*Cronobacter sakazakii*, *Citrobacter freundii*, *Salmonella enterica*, *Klebsiella pneumonia*, and *E. coli*) by disc diffusion methods. Among the 13 Schiff bases of 4-aminoantipyrine tested, eight compounds inhibited three Gram-negative bacteria and four compounds resisted the growth of one Gram-positive bacteria (Table 2). Inhibition zones of compounds **3a-m** and nalidixic acid, a positive control, were measured at a dose of 500 and 50 μg disc^{-1}, respectively. Specifically, compounds **3a** and **3c**, only showed weak activity against one bacterial strain (*C. sakazakii*), whereas compounds **3g** and **3j** only showed weak activity against *S. aureus*. Compounds **3f**, **3h**, **3i,** and **3k** also demonstrated weak activity against *E. coli*. Compound **3l** exhibited weak activity against two bacterial strains (*S. aureus* and *E. coli*). Compound **3m** inhibited the growth of highest number of bacterial strains tested (*K. pneumonia*, *S. aureus*, *C. sakazakii*, and *E. coli*), whereas compounds **3b**, **3d**, and **3e** did not show any activity. However, compounds **3k** and **3m** demonstrated the highest activity against *E. coli* and *C. sakazakii*, respectively, among the compounds tested. As all the active compounds showed similar inhibition zones, structural differences observed may not play an important role in variations of the activity and mechanism of bactericidal action.

Cytotoxic Activity. Cytotoxicity assays using brine shrimp (*A. salina*) napulii are an excellent tool for screening bioactive compounds with the potential ability to kill cancer cells and various pests (Mayer et al.,1982; Hartl and Humpf, 2000). The lethal dose (LC_{50}) obtained in the brine shrimp assay can be used to determine a more specific activity (Weidenbörner and Chandra Jha, 1993; Alam and Lee, 2011). The results of the *A. salina* toxicity assay for the Schiff base analogues of 4-aminoantipyrine (**3a-m**) are presented in Table 3. Among all compounds, **3b** showed the highest brine shrimp toxicity, with LC_{50} value of 225 ppm, followed by compound **3i** (LC_{50}=480 ppm) and **3f** (LC_{50}= 581 ppm); however, all compounds were less cytotoxic than the standard cytotoxic agent, gallic acid (LC_{50}=78 ppm). Compounds **3d** and **3e** showed moderate cytotoxicity, both with LC_{50} values of 730 ppm, whereas compounds **3e**, **3j**, **3l**, and **3m** exhibited similar weak activities (LC_{50} >1,000 ppm). Compounds **3a** and **3h** were inactive against *A. salina*.

Computational Studies. Bioactivity is generally governed by the frontier molecular orbitals and physicochemical properties of a molecule (Türkeer et al., 1990). Biological systems consist of a number of heterogeneous phases including water, serum protein, and lipid particles, among others. Therefore, drug must be transported through these types of phase barriers to reach the site of action, which are essentially a physicochemical process and more complex than the homogeneous equilibria. Lipophilicity is recognized as a meaningful parameter in structure-activity relationship studies. It has become the single most informative and successful physicochemical property in medicinal chemistry (Testa et al., 1996; Alam et al., 2013) and is used as major experimental and theoretical tool in drug design. To explain the qualitative structure-cytotoxic activity relationship of Schiff base analogues of 4-aminoantipyrine (**3a-m**), physicochemical calculations were carried out using molinspiration cheminformatics software. Physiochemical parameters of the compounds **3a-m** are presented in Table 4. The lipophilicity of a molecule depends on two



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Scheme 1 Synthesis of 4-aminoantipyrine analogues **3a-m**.

Table 1 Crystal data and structure refinement for (*E*)-4-(4-hydroxy-3-methoxybenzylidene amino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3g**) and (*E*)-4-(3, 4-dimethoxy benzylideneamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3h**)

Compound no.	3g	3h
Empirical formula	$C_{19}H_{19}N_3O_3$	$C_{20}H_{21}N_3O_3$
Formula weight	337.37	351.40
Temperature	200 (2) K	200 (2) K
Wavelength	0.71073 Å	0.71073 Å
Crystal system	Monoclinic	Monoclinic
Space group	Cc	$P2_1/c$
Hall symbol	-C2yc	-P2ybc
Unit cell dimensions	a=11.019 (2) Å	a=12.5666 (10) Å
	b=17.362 (4) Å	b=10.5366 (8) Å
	c=9.047 (2) Å	c=14.7293 (12) Å
	β=102.064 (6)°	β=108.843 (2)°
Volume	1692.6 (6) $Å^3$	1845.8 (3) $Å^3$
Z	4	4
Density (calculated)	1.324 Mg/m^3	1.265 Mg/m^3
Absorption coefficient	0.091 mm^{-1}	0.087 mm^{-1}
F (000)	712	744
Crystal size	0.37×0.25×0.14 mm^3	0.37×0.24×0.19 mm^3
Theta range for data collection	2.22 to 28.34°	2.42 to 28.31°
Index ranges	-14<=h<=9, -20<=k<=23, -12<=l<=12	-16<=h<=10, -11<=k<=14, -19<=l<=19
Reflections collected	6319	13515
Independent reflections	3587 [R(int)=0.0536]	4582 [R(int)=0.1005]
Completeness to theta	99.6 % (28.34°)	99.8 % (28.31°)
Absorption correction	None	None
Refinement method	Full-matrix least-squares on F2	Full-matrix least-squares on F2
Data / restraints / parameters	3587/2/230	4582/0/239
Goodness-of-fit on F2	0.817	0.637
Final R indices [I>2sigma (I)]	R1=0.0451, wR2=0.0532	R1=0.0432, wR2=0.0715
R indices (all data)	R1=0.1149, wR2=0.0757	R1=0.1834, wR2=0.0967
Largest diff. peak and hole	0.213 and -0.237 $e.Å^{-3}$	0.146 and -0.142 $e.Å^{-3}$

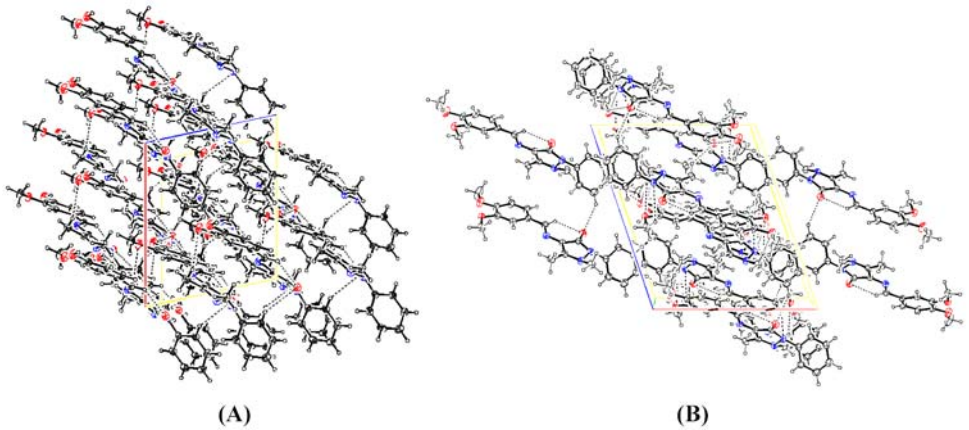
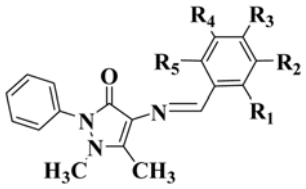


Fig. 2 Packing arrangement in the crystal structure of (A) (*E*)-4-(4-hydroxy-3-methoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3g**) and (B) (*E*)-4-(3, 4-dimethoxybenzylideneamino)-1,5-dimethyl-2-phenyl-1*H*-pyrazol-3(2*H*)-one (**3h**). *Dashed lines* indicate hydrogen bonds.

important factors, hydrophobicity and polarity, which help the molecule to cross or irreversibly damage the cellular membrane. Fig. **4** shows the MLP map and PSAs of selected inactive (**3a**), low active (**3j** and **3m**) and active (**3b** and **3i**) compounds. In the

Table 2 Antibacterial activities of Schiff base analogues of 4-aminoantipyrine (**3a-m**)



Comp. no	R_1	R_2	R_3	R_4	R_5	Growth inhibition area (mm)					
						K. p.	*S. a.*	*C. s.*	*C. f.*	*S. e.*	*E. c.*
3a	H	H	H	H	H	-	-	6	-	-	-
3b	H	H	Cl	H	H	-	-	-	-	-	-
3c	H	H	NMe_2	H	H	-	-	5	-	-	-
3d	H	H	OH	H	H	-	-	-	-	-	-
3e	H	H	OMe	H	H	-	-	-	-	-	-
3f	H	OH	OH	H	H	-	-	-	-	-	6
3g	H	OMe	OH	H	H	-	6	-	-	-	-
3h	H	OMe	OMe	H	H	-	-	-	-	-	5
3i	OH	H	OH	H	H	-	-	-	-	-	6
3j	OMe	H	OMe	H	H	-	5	-	-	-	-
3k	OH	H	OH	H	OH	-	-	-	-	-	9
3l	H	OMe	OH	OMe	H	-	5	-	-	-	7
3m	H	Me	OMe	H	H	6	6	9	-	-	5
Nalidixic acid						9	5	10	10	7	10

Inhibitory activity is expressed as the diameter (in mm) of the observed inhibition zone. Growth inhibitions were measured at 500 μg $disc^{-1}$ for compounds **3a-m** and 50 μg $disc^{-1}$ for standard nalidixic acid. K. b., *Klebsiella pneumonia*; S. a., *Staphylococcus aureus;* C. s., *Cronobacter sakazakii*; C. f. *Citrobacter freundii*; S. e., *Salmonella enterica*; E. c., *Escherichia coli*.

Table 3 The results of cytotoxic effect of compounds **3a-m** and Gallic acid against *A. salina*

Comp. no	Mortality, LC_{50} (ppm)	[c]Regression Equation	[d]R^2
3a	NA	-	-
3b	225 (180-279)[a]	Y=1.566 X+1.031	0.79
3c	>1000 (20[b])	-	-
3d	729 (526-1107)[a]	Y=0.93 X+2.33	0.91
3e	730 (526-1105)[a]	Y=0.9233 X+2.344	0.91
3f	581 (441-853)[a]	Y=1.0567 X+2.076	0.90
3g	754 (584-1170)[a]	Y=1.3167 X+1.132	0.92
3h	NA	-	-
3i	481 (383-642)[a]	Y=1.1867 X+1.816	0.91
3j	1036 (763-1657)[a]	Y=1.15 X+1.482	0.94
3k	ND	-	-
3l	>1000 (20[b])	-	-
3m	>1000 (30[b])	-	-
Gallic Acid	78 (65-94)[a]	Y=2.5933 X–2.4888	0.88

[a]95% confidence limits; [b]Mortality percentage at the concentration of 1000 ppm; [c]Obtained from Log (conc.) *vs* probit correlation. [d]Log (conc.) *vs* probit correlation coefficient; NA-not active; ND-not determined (Difficult to solubilize in DMSO).

present study, compound **3b** was the most active, followed by compounds **3i**, **3f**, **3d**, **3e**, **3g,** and **3j** with clogPs of 3.537, 2.096, 1.950, 2.479, 2.815, 2.298, and 2.987, respectively. Although a

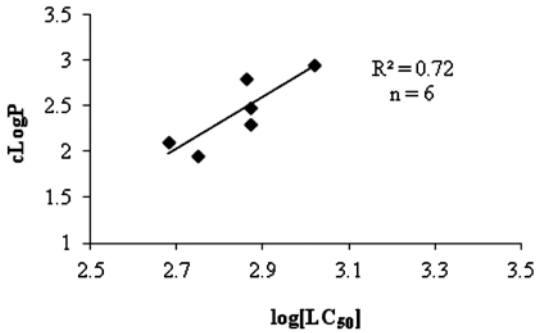


Fig. 3 Correlation between cytotoxic activity and calculated octanol-water partition coefficient (clogP) of active Schiff base analogues (**3d-g**, **3i**, **3j**) of 4-aminoantipyrine against *A. salina*.

small number of compounds were used in the present study, a good correlation was observed in which cytotoxic activity decreased with increasing logP (Fig. 3). The correlation coefficients (r^2) between the clogPs and cytotoxic potencies of selected compounds against *A. salina* were found to be 0.72 (n=6) in the absence of **3c.** Furthermore, the above correlations should be treated with caution, because there were few exceptions, *e.g.* clogP of an inactive compound **3a** (2.859) were the same as a moderately active compounds **3e** (2.815). In addition, clogP of a very low active compound **3l** (2.214) were the similar to the moderately active compounds **3g** (2.298), whereas **3h** (2.605) were inactive with

Table 4 Physico-chemical properties of Schiff base analogues of 4-aminoantipyrine (**3a-m**)

Comp.	MW (g/mol)	clogP[a]	TPSA[b]	OH-NH interact[c]	O-N interact[d]	nrotb[e]	volume
3a	291.354	2.859	39.303	0	4	3	272.961
3b	325.799	3.537	39.303	0	4	3	286.497
3c	334.423	2.961	42.541	0	5	4	318.867
3d	307.353	2.479	59.531	1	5	3	280.979
3e	321.38	2.815	48.537	0	5	4	298.506
3f	323.352	1.95	79.759	2	6	3	288.996
3g	337.379	2.298	68.765	1	6	4	306.524
3h	351.406	2.605	57.771	0	6	5	324.052
3i	323.352	2.096	79.759	2	6	3	288.996
3j	351.406	2.987	57.771	0	6	5	324.052
3k	339.351	2.212	99.987	3	7	3	297.014
3l	367.405	2.214	77.999	1	7	5	332.07
3m	335.407	3.292	48.537	0	5	4	315.068

[a]Calculated octanol/water partition coefficient; [b]Molecular polar surface area; [c]Number of hydrogen-bond donors; [d]Number of hydrogen-bond acceptors ; [e]Number of rotatable bond.

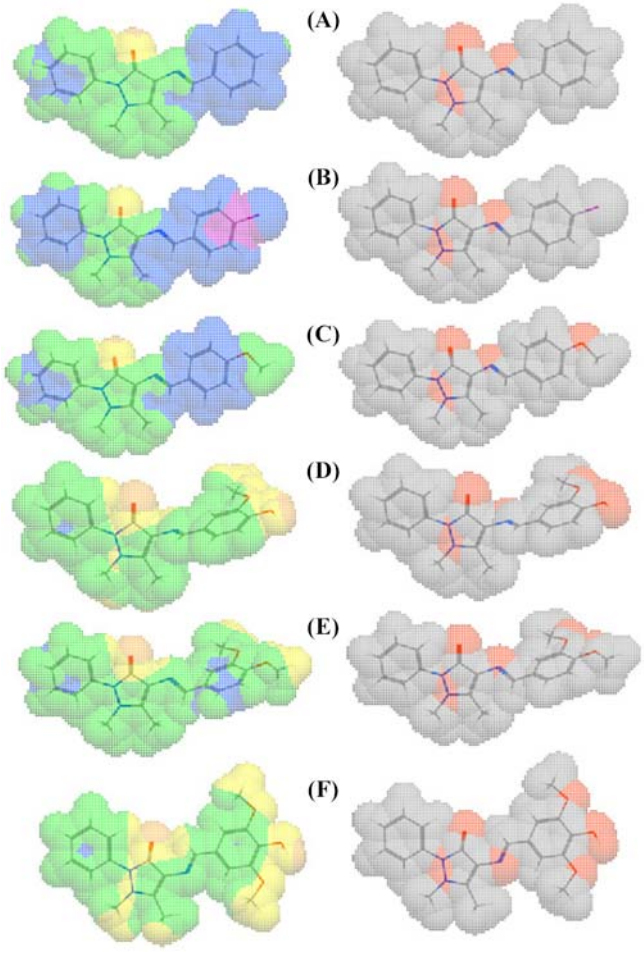


Fig. 4 Molecular lipophilicity potential (left) and polar surface area (right) of **3a** (A), **3b** (B), **3e** (C), **3g** (D), **3h** (E), and **3l** (F) showing the most lipophilic area (blue color), intermediate lipophilic area (pink color), most hydrophilic area (yellow color), intermediate hydrophilic area (green color), nonpolar area (gray white color), and polar area (red color).

lower clogP compared to that of **3e** (2.815). Therefore, maps of PSA and lipophilicity potentials were compared for compounds **3a**, **3b**, **3e**, **3g**, **3h,** and **3l** (Fig. 4). Although **3a** and **3e** have similar logP values, their hydrophobicity and polar surface area distribution are different. On the other hand, compound **3g** showed low polar surface (68.765) and hydrophilic area, whereas **3l** showed more polar surface (77.999) and hydrophilic area. Similarly, compound **3e** showed low polar surface (48.537) and hydrophilic area compared to that of **3h** (57.771). Although, the lipophilicity and hydrophilicity play an important role in determining logP of a molecule, their distribution in the molecular surface is also important to exert their biological activity. The design and synthesis of additional Schiff base analogues of 4-aminoantipyrine containing lipophilic and hydrophilic substituents are in progress in order to support our present study.

In conclusion, among 14 Schiff base analogues of 4-amino-antipyrine (**3a-m**), **3k** and **3m** demonstrated the highest antibacterial activity against *E. coli* and *C. sakazakii*, respectively. Compound **3b** (LC_{50}=225 ppm) exhibited the highest toxicity against brine shrimp nauplii (*A. salina*) followed by compounds **3i** (LC_{50}=480 ppm) and **3f** (LC_{50}=581 ppm). Physicochemical calculations indicate that the cytotoxicities of the tested compounds correlated well with the calculated logP. However, hydrophilicity and polar surface distribution on the molecular surface area also play important roles.

Supplementary Material. Crystallographic data for the structures of compound **3g** and **3h** have been deposited in the Cambridge Crystallographic Data Center (Deposition number CCDC-871055 and CCDC-871056, respectively). The data can be obtained, free of charge, upon request from Cambridge Crystallographic Data Center UK.

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