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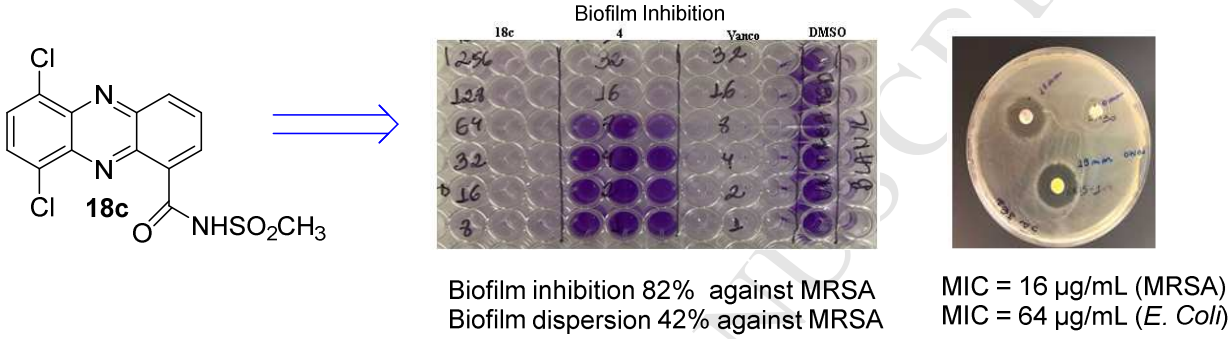

Synthesis, biological evaluation, and metabolic stability of phenazine derivatives as antibacterial agents

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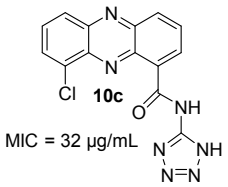
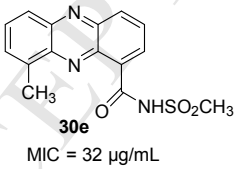
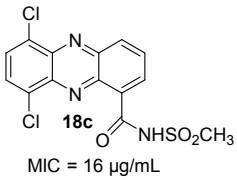
Abstract: Drug-resistant pathogens are a major cause of hospital- and community-associated bacterial infections in the United States and around the world. These infections are increasingly difficult to treat due to the development of antibiotic resistance and the formation of bacterial biofilms. In the paper, a series of phenazines were synthesized and evaluated for their *in vitro* antimicrobial activity against Gram positive (methicillin resistant *staphylococcus aureus,* MRSA) and Gram negative (Escherichia coli, *E. coli*) bacteria. The compound 6,9-dichloro-*N*-(methylsulfonyl)phenazine-1-carboxamide (**18c)** proved to be the most active molecule (MIC = 16 µg/mL) against MRSA whereas 9-methyl-*N*-(methylsulfonyl)phenazine-1-carboxamide (**30e)** showed good activity against both MRSA (MIC = 32 µg/mL) and *E. coli* (MIC = 32 µg/mL). This molecule also demonstrated significant biofilm dispersion and inhibition against *S. aureus*. Preliminary studies indicate the molecules do not disturb bacterial membranes and there activity is not directly linked to the generation of reactive oxygen species. Compound **18c** displayed minor toxicity against mammalian cells. Metabolic stability studies of the most promising compounds indicate stability towards phase I and phase II metabolizing enzymes.







Introduction:

Phenazines are nitrogen-containing heterocyclic compounds that differ in their chemical and physical properties based on the type and position of present functional groups. [1-3] Natural and synthetic phenazines possess various biological activities such as antihypertensives, antiparasitics, antimalarials, neuroprotectants, radical scavengers, anti-cancer and cancer preventive agents.[1, 2, 4] However, they are mainly known for their antibacterial activity against a broad spectrum of pathogens. Some anti-bacterial phenazines include tubermycin B (**1**, which possesses activity against Mycobacterium tuberculosis),[2] and 2,4-dibromo-1-hydroxyphenazine (**2**). Recently Huigens and co-workers reported various halogenated phenazines as potent biofilm-eradicating agents against diverse bacteria.[5-8] Their remarkable efforts have led to various lead compounds such as **3**[6] that presents minimum biofilm eradication concentration (MBEC) of 2.35 μM against MRSA and 0.20 μM against vancomycin-resistant *Enterococcus faecium* (VRE). Our group has recently synthesized the potent molecule 9-chloro-*N*-(methylsulfonyl)phenazine-1-carboxamide (**4)** and the natural product endophenazine G **5**.[9]

These literature reports prompted us to look further into phenazine analogues as new anti-bacterial agents against *E. coli* and *S. aureus*, two bacterial pathogens that can cause severe infections at surgical sites, in skin and soft tissues, and the urinary tract (UTIs).[10-12] UTIs can lead to nosocomial pneumonia, meningitis, and septicemia.[12, 13] In addition, these pathogens are a major clinical problem in biofilm-related diseases such as chronic wound infection, periodontitis, and ocular infections.[14, 15] Here in, we describe the synthesis of a series of new phenazines possessing and present the anti-bacterial activity of the compounds against *E. coli*

and MRSA, the kinetics of killing, the ability to inhibit biofilms, and the propensity to cause bacterial mutations. Also, we assessed the toxicity of the most promising compounds.

2. Results and discussion

2.1 Chemistry

Based on our data, which indicated an acidic proton was needed for activity,[9] we prepared chloro-substituted phenazines containing acid bioisosteres (Scheme 1). 9-Chlorophenazine-1-carboxylic acid (**6**)[15] was coupled with *O*-(*tert*-butyldimethylsilyl)hydroxylamine (**7**) in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), followed by treatment with trifluoroacetic acid in CH_2Cl_2, to afford 9-chloro-*N*-hydroxyphenazine-1-carboxamide (**9**) in 60% yield. The chloro carboxamide analogue **12a** was prepared by reacting **6** with oxalyl chloride followed by aqueous ammonia. This amide (**12a**) was then converted into nitrile **13** by a dehydration reaction with thionyl chloride at 100 °C in DMF. To understand the role of hydrogen bonding on anti-bacterial activity, the carboxylic acid was replaced by a cyanamide (**12b)** and a tetrazole group (**12c)** using CDI. Against our hypothesis, the preliminary anti-bacterial assays revealed that the bioisosteric modifications of **8** to the *N*-hydroxylamide **9** and the tetrazole amide **12c** did not improve MIC against MRSA when compared with the lead compound **6**. Thus we decided to explore different substitutions on the benzene ring to study the structure activity relationships (SAR) and find more active compounds.

Therefore, the dichlorophenazine carboxylic acid **17** (Scheme 2) was synthesized using an Ullmann coupling between 2-bromo-3-nitrobenzoic acid (**15**) and 2,5-dichloroaniline (**14**) as done before.[9] The obtained acid was then converted to the ester **18a** with methyl iodide or to the amide **18b** by treatment with oxalyl chloride and aqueous ammonia. The carboxylic acid

bioisostere *N*-(methylsulfonyl)amide **18c** was prepared by coupling methanesulfonamide using CDI and catalytic amounts of DMAP.

To further understand the influence of electronics on antibacterial activity, 9-bromo-6-methoxy (**21a**, Scheme 3) 7-bromo-9-methoxy (**21b)**, and 6,9-dimethoxyphenazine-1-carboxylic acids (**21c)** were synthesized using standard coupling and cyclization conditions in 32−48% yields. The carboxylic acid bioisosteres **22a−c** were prepared using the methods described in Schemes 1 and 2 in 51−59% yields.

The positional isomers **21b** and **22c** were prepared to study the effect of electron donating groups on antibacterial activity (Scheme 4). Besides the desired product **25**, the cyclization of **24** (interestingly) also yielded the dehalogenated product **26**[16] as an inseparable mixture. The mixture was converted to the corresponding esters **27** and **28**, purified, and hydrolyzed with sodium hydroxide to afford the desired acid **25** in 74% yield.

To further expand our library other analogues; **30a−f** (Scheme 5) were prepared as described in 22−75% yields. Finally, we prepared phenazine **31** (Scheme 6), an analogue of **6**, to further confirm the role of an acidic proton on antibacterial activity MRSA

Biological Evaluation: We studied the minimum inhibitory concentration (MIC), of the molecules against on two bacterial stains: *E. coli* K12 and *S. aureus* JE2 (Table 1). The mono chloro phenazine derivatives **9** and **12c** showed good activity against *S. aureus* (MIC = 32 µg/mL) confirming our previous observations regarding the role of an acidic proton. Meanwhile compounds **12a** and **12b**, with similar Log P values were relatively inactive against the organisms. These results were partially surprising, because **9** and **12a** should present similar pKa values, and as a result, similar activities, indicating the hydroxyl group attached to the amide nitrogen may play an additional role. As expected, the chloro-nitrile analogue **13** did not show

activity, maybe due to the lack of acidic protons confirming our previous observations. The dichlorophenazine **17** (MRSA MIC = 64 µg/mL) was slightly more active than the corresponding ester **18a** and carboxamide **18b** (MIC = 128 µg/mL). 6,9-Dichloro-*N*-(methylsulfonyl)phenazine-1-carboxamide **18c** (MIC = 16 µg/mL) was 4-fold more potent against *S. aureus* than **17** further validating the positive impact of the sulfonamide substituent. Still, this compound was less potent than our previously discovered lead compound **6**.[9] Our previous report suggested substituents at the *6* position decreased activity and this finding strengthens that observation. The other dichloro analogues (**18a** and **18c**) were inactive against MRSA due to the increased pKa values. More important, compound **18b** showed good activity against the Gram (-) pathogen *E.coli* (MIC = 32 µg/mL). The bromo-methoxy phenazine carboxylic acids (**21a−c**, **25** and **26**), their esters (**22c**, **27** and **28**), sulfonamide (**22a**) and cyanamide (**22b**) analogues were moderately active or inactive against both pathogens (MIC = 64−256 µg/mL) excluding **22b** which presented an MIC = 32 µg/mL against *E. coli*. We concluded that electron donating groups or the lack of substitutions at the 9- position are not beneficial for antibacterial activity even if an acidic group is present on the phenazine core. Other phenazine derivatives: fluoro **30c** and **30d**, and methoxy ester **30f** were also inactive against both microorganisms (MIC = 64−256 µg/mL). Compound **30e** displayed good activity against both pathogens with MIC = 32 µg/mL further highlighting the positive effect of the sulfonamide group. Compound **31** presented an S. aureus MIC ≥ 256 µg/mL against MRSA, which indicates removal of the acidic proton decreases activity even if the sulfonamide group is present. The SAR studies had demonstrated that the *N*-methylsulfonyl and 9-Cl are an essential part of the pharmacophore required for the Gram-positive bacteria activity. The best activity was obtained when the substituents are on the same side (1- and 9- positions) of the benzene ring in phenazine core. Introduction substituents at other

positions at 8- or 7- positons of the benzene ring decreased activity. These results and our previous findings suggest the size and positions of substituents on the benzene rings are more important than electronics for antibacterial action. At the moment there is no clear trend regarding the activity against Gram (-) pathogens. Also, the most active compounds against Gram (+) do not present the same trend of activity against Gram (-) bacteria.

We than evaluated phenazines **4** and **18c** in biofilm dispersion and inhibition assays against MRSA. The eradication of formed biofilms and the prevention of their development remains a medical microbiology challenge. We assessed if phenazines **4** or **18c** for their ability to disperse established staphylococcal biofilms. The dose response curve for **18c** is presented in Figure 2. The percentage of biofilm dispersion of **18c** was 42% and 61% at MIC and 2 × MIC concentrations, respectively. In our assay those values were comparable to the vancomycin control,[17, 18] which produced 45% and 59% at MIC and 2 × MIC concentrations. Compound **4** showed no biofilm dispersion over the tested concentration range (1-32 μg/mL).

The biofilm-formation inhibition assay, is presented the in Figure 3. Compound **18c** exhibited significant activity against MRSA with 82% inhibition at 1X MIC. Meanwhile molecule **4** showed ~90% inhibition at 8X MIC.

Next, we tested whether our active phenazines were bacteriostatic or bactericidal against MRSA. Some phenazine compounds have been described as bactericidal agents[19] while others have been reported to be bacteriostatic.[9, 20] Our time-killing experiments show that compound **18c** had a bacteriostatic effect on MRSA (Figure 4) as we previously observed with **4**.[9]

Then, we decided to investigate the presence of resistant colonies using the Kirby Bauer method[21] (Figure 5 and SI). The presence of a colony within the inhibition area would suggest

a resistant colony. Compound **18c** showed a 19 mm inhibition zone, which is equipotent with the control streptomycin (18 mm at 10 µg concentration) while **4** presented an inhibition zone of 16 mm, **7** of 11 mm, and molecule **30e** of 10 mm. No colonies were detected inside the mentioned zones. Then, we monitored the presence of new colonies in the inhibition area (or a decrease in the diameter of the inhibition zone) by incubating the samples for 5 additional days. No changes were observed during this time, indicating that no resistance against the compounds was developed at the testing conditions.[22, 23]

We next tried to elucidate if our molecules' antimicrobial activity was related to membrane damage. Flow cytometry offers a good method for determining the amount of fluorescent dye that is uptaken by cells.[24] We decided to use propidium iodide (PI), a red fluorescent dye that is impermeable to the membrane, to assess permeability, which can be linked to disruption or damage.[25] Flow cytometry data of **4** and **18** compounds (not shown) indicated no PI penetration was observed. TEM studies (see SI) also indicated that *S. aureus* or *E.coli* did not presented cell membrane damage after treatment with compounds **4** or **18c**.

We tested the cytotoxicity of compound **18c** against HCEC (Human colonic epithelial) cells to evaluate their potential as anti-bacterial drugs. The *in vitro* IC_{50} values for **18c** was 208 µM, similar to what we observed for **4** (193 µM[9]). This indicates some window of selectivity for bacterial over mammalian cells for the phenazine sulfonamides. We also studied the *in vivo* toxicity of compounds **4** and **18c** in wax moth larvae model.[26] The results showed that 70% of larvae survival after treatment with **4** (3 µg/mL, 1.5 MIC) after 96 h. We were not able to treat the larvae with higher concentrations of compounds due to toxicity and some precipitation that clogged the needle. At this concentration, we did not observe any efficiency as antibacterial

agents. Due to solubility problems, we were not able to study **18c** (no toxicity was observed at 0.5 MIC).

Metabolic stability determination:

We determined the *in vitro* metabolic stability of compounds **4** and **18c** (see Figures 6, 7, and SI) using human liver S9 fraction and also studied the stability in simulated gastrointestinal fluids (SGF) and tris buffer (50 mM, pH 7.4). Compounds **18c** and **4** were not metabolized in the presence of phase I and phase II metabolizing enzymes (Figure 6). The stability of **4** and **18c** were evaluated in SGF and simulated intestinal fluids (SIF) at gastro-intestinal pH as well as at physiological condition pH 7.4 following literature protocols.[27] Both compounds were stable at all three tested pH conditions (1.2, 6.8 and 7.4).

In order to find a correlation between the compounds and their anti-bacterial activity, some physicochemical properties (total polar surface area, Log P, pKa) were calculated using molinspiration (www.molinspiration.com) and marvin sketch software (www.chemaxon.com). Although a low pKa is important for activity, having an acidic proton does not equal an active compound and other substituents also play a role on the action. An old report showed that as the pKa of the sulfonamides increases, the bacteriostatic activity passes through a maximum and then decreases.[28] Future directions of this project will focus in varying electronics to reach an optimal pKa value. Unfortunately, we were not able to find a correlation between activity and the other selected parameters i.e. the dipole moment. The activity of phenazine compounds is indirectly correlate to their reduction potential, which is itself related to the energy of LUMO.[29] Thus, phenazine with the lowest LUMO energy should be most active.[30] We calculated the LUMO energies and molecular electrostatic potential (MEP) of selected compounds (see SI) using DFT with the B3LYP/6-311G(*) basis set in Spartan'14.

Unfortunately, no significant correlation was deduced between LUMO energies and antibacterial activity of all tested compounds (in the future, we will expand to other bass sets for our calculations).

Conclusions:

In the present manuscript, we have synthesized a series of phenazine analogues and evaluated their antibacterial activity against MRSA and *E.coli*. We identified three new active compounds against MRSA; **18c**, **9**, and **12c**. We also validated the role of the *N*-(methylsulfonyl) substituent. In addition, three compounds **18b**, **22b**, and **30e** were found to be active against *E. coli*. Compound **18c** has the ability to disrupt established biofilms and also prevent their formation, and bacteria were not able to develop resistance towards the compound. The experiments indicated the primary mechanism of action is not related to membrane disruption. We are currently exploring other mechanisms of action including inhibition of PhzA/B[31], DNA binding,[32] or binding to specific metals.[33] The compounds **18c** and **4** were not metabolized by phase I and phase II enzymes under this conditions and were stable at physiological pH. These compounds are promising leads that can be further optimized as broad spectrum antibacterials. Future directions include the synthesis of new compounds with enhanced activity and water solubility that present less toxicity *in vitro* and *in vivo*.

4. Experimental Section

4.1 Chemistry

General Methods

^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. The chemical shifts are reported in parts per million (ppm) relative to internal standard tetramethylsilane or residual solvent peak. ^{13}C NMR chemical shifts are reported in ppm with the solvents ($CDCl_3$: 77. 16

ppm, DMSO-d_6: 39.52). The Mass spectrometry (MS) was carried out on a Micromass Q-Tof Mass Spectrometer with an electrospray ionization (ESI) source. Infrared spectra (IR) were recorded on a FT-infrared spectrometer (Bio-Rad). Thin-layer chromatography was carried out on aluminum backed silica gel plates (Merck 60 F254), with visualization of components by UV light (254 nm).

Analysis of sample purity was performed on an Agilent Technologies 1220 infinity LC HPLC system with a Diamonsil AAA 5u, C18 column (250 mm × 4.6 mm). HPLC conditions: solvent A = CH_3CN containing 0.1% TFA, solvent B = H_2O containing 0.1% TFA, flow rate = 1.0 mL/min; compounds were eluted with a gradient of 3-97% over 25 min. Purity was determined by total absorbance at 254 nm. All tested compounds have a purity ≥95%.

Confocal microscopy was performed on a CARLZEISS LSM 710, Jena (Germany). The melting points were measured with an EZ-Melt automated melting point apparatus from Stanford Research System and are not correct. All reagents and chemicals and solvents were purchased from commercial sources (Sigma-Aldrich, VWR or Fisher) and used without further purification.

Synthetic Procedures:

N-((tert-Butyldimethylsilyl)oxy)-9-chlorophenazine-1-carboxamide (**8**). To a stirred solution of **6** (0.10 g, 0.39 mmol) in CH_2Cl_2 (10.0 mL) was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.07 g, 0.43 mmol) at room temperature. After 2h, *O*-(*tert*-butyldimethylsilyl)hydroxylamine **7** (0.06 g, 0.43 mmol), trimethylamine (82.0 µL, 0.59 mmol) and DMAP (cat. amount) were added. The mixture was stirred at room temperature for 12 h and then the solvent was evaporated *in vacuo*. The residue was purified by silica gel column chromatography using EtOAc/Hexane (1:3) as eluent to afford the pure product as a yellow solid

(42 mg, 31%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.34 (s, 6 H), 1.08 (s, 9 H), 7.86 (t, 1 H, J = 8.5 Hz), 8.02−8.07 (m, 2 H), 8.25 (dd, 1 H, J = 1.0, 9.0 Hz), 8.42 (dd, 1 H, J = 1.5, 9.0 Hz), 9.04 (dd, 1 H, J = 1.5, 7.5 Hz), 13.12 (br s, 1 H); HRESIMS calcd for $C_{19}H_{23}ClN_3O_2Si$: 388.1170 (MH^+); found: 388.1244 (MH^+).

9-Chloro-N-hydroxyphenazine-1-carboxamide (**9**). To a stirred solution of **8** (0.03 g, 0.09 mmol) in CH_2Cl_2 (3 mL) at 0^0 C was added CF_3COOH (0.2 mL). The reaction mixture was allowed to warm to room temperature and stirred for 12 h. The reaction mixture was neutralized with aqueous $NaHCO_3$ and extracted with CH_2Cl_2 (2 × 25 mL). The CH_2Cl_2 solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness under reduced pressure to afford the titled compound as a yellow solid (14 mg, 60%); R_f: 0.65 (MeOH/ CH_2Cl_2, 1:9); mp: 249−251 °C. IR (KBr) 3408, 3322, 2962, 1704, 1666, 1254, 1143, 743 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.04 (t, 1 H, J = 7.5 Hz), 8.17 (t, 1 H, J = 7.5 Hz), 8.30−8.33 (m, 2 H), 8.49 (d, 1 H, J = 8.5 Hz), 8.76 (d, 1 H, J = 7.0 Hz), 9.72 (br s, 1 H), 12.57 (br s, 1 H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 128.92, 129.75, 131.23, 131.57, 131.61, 131.64, 132.90, 134.97, 137.60, 139.17, 143.30, 143.37, 162.13; HRESIMS calcd for $C_{13}H_9ClN_3O_2$: 274.0378 (MH^+); found: 273.9753 (MH^+).

9-Chlorophenazine-1-carboxamide (**12a**). To a stirred suspension of 9-chlorophenazine-1-carboxylic acid **6** (0.14 g, 0.54 mmol) in CH_2Cl_2 (10 mL) at 0^0 C, was added one drop of DMF and oxalyl chloride (0.19 mL, 2.16 mmol) dropwise under N_2 atmosphere. The reaction mixture was allowed to warm to room temperature, stirred for 12 h, and the solvent was removed *in vacuo*. The residue was dissolved in CH_2Cl_2, cooled to 0^0 C and aqueous NH_3 (2.0 mL) was added. The mixture was stirred at room temperature for 4 h, and then the solvent was evaporated

in vacuo. The residue was suspended in H_2O (5 mL) for 30 min, filtered and washed with H_2O. The residue was recrystallized with methanol to afford the title compound as a yellow solid (58 mg, 42%); R_f: 0.67 (MeOH/ CH_2Cl_2, 1:9); mp: 275−277 °C. IR (KBr) 3326, 2913, 2831, 1688, 1577, 1385, 1270, 1119, 948, 882, 760 cm^{-1}; ^1H NMR (500 MHz, DMSO-d_6): δ 8.01 (t, 1 H, J = 8.0 Hz), 8.15 (t, 1 H, J = 8.0 Hz), 8.27−8.31 (m, 3 H), 8.48 (d, 1 H, J = 9.0 Hz), 8.84 (d, 1 H, J = 7.0 Hz), 10.12 (br s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6): 128.63, 129.74, 130.12, 131.14, 131.18, 131.23, 133.07, 135.48, 137.30, 139.66, 142.91, 143.06, 164.83; HRESIMS calcd for $C_{13}H_9ClN_3O$: 258.0356 (MH^+); found: 258.0420 (MH^+).

9-Chloro-N-cyanophenazine-1-carboxamide (**12b**). To a stirred solution of **6** (0.04 g, 0.15 mmol) in a mixture of DMF (5 mL) and CH_2Cl_2 (5 mL) were added 1,1'-carbonyldiimidazole (0.05 g, 0.31 mmol) and 4-dimethylaminopyridine (cat. amount) under N_2 atmosphere. The mixture was stirred at 70 ^{0}C for 2 h and then cooled to room temperature. Triethylamine (0.04 mL, 0.31 mmol) and cyanamide (**10**, 13.0 mg, 0.31 mmol) were added. The mixture was stirred at 70 ^{0}C for 24 h, and the solvents evaporated *in vacuo*. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (30:1) as eluent to afford the title compound as a yellow solid (21 mg, 49%); R_f: 0.41 (MeOH/ CH_2Cl_2, 1:50); mp: 251−253 °C. IR (KBr) 2921, 2251, 1703, 1397, 1127, 743 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.94 (t, 1 H, J = 8.0 Hz), 8.10−8.15 (m, 2 H), 8.30 (dd, 1 H, J = 1.0, 8.5 Hz), 8.59 (dd, 1 H, J = 1.0, 8.5 Hz), 9.10 (dd, 1 H, J = 1.0, 7.0 Hz), 14.12 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 106.58, 125.01, 128.81, 130.63, 131.19, 131.79, 135.82, 137.10, 137.88, 138.71, 139.11, 143.48, 144.03, 163.46; HRESIMS calcd for $C_{14}H_8ClN_4O$: 283.0308 (MH^+); found: 283.0331 (MH^+).

9-Chloro-N-(1H-tetrazol-5-yl)phenazine-1-carboxamide (**12c**). To a stirred solution of **6** (0.05 g, 0.19 mmol) in DMF (5 mL) was added 1,1'-carbonyldiimidazole (0.09 g, 0.58 mmol) and 4-dimethylaminopyridine (2.0 mg, 0.02 mmol) under N_2 atmosphere. The mixture was stirred at 60 ^{0}C for 2 h and then cooled to room temperature. Triethylamine (0.08 mL, 0.58 mmol) and 5-aminotetrazole (**11**, 0.05 g, 0.58 mmol) were added, the mixture was stirred at 70 ^{0}C for 48 h, and the solvents were evaporated *in vacuo*. The residue was treated with HCl (1 N, 5 mL) and filtered. The filtered solid was purified by silica gel column chromatography using CH_2Cl_2/MeOH (9:1) as eluent to afford the title compound as a yellow solid (35 mg, 58%); R_f: 0.53 (MeOH/ CH_2Cl_2, 1:9); mp: 305−306 °C. IR (KBr) 2909, 2840, 1716, 1589, 1438, 1287, 1198, 1128, 751 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): δ 7.92 (t, 1 H, J = 8.5 Hz), 8.08 (t, 1 H, J = 8.5 Hz), 8.13−8.17 (m, 2 H), 8.41 (d, 1 H, J = 8.5 Hz), 8.78 (d, 1 H, J = 7.0 Hz), 13.91 (s, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 126.84, 129.10, 131.49, 131.73, 132.24, 132.37, 135.27, 137.14, 137.25, 139.26, 143.48, 143.82, 149.85, 162.40; HRESIMS calcd for $C_{14}H_9ClN_7O$: 326.0552 (MH^+); found: 326.1498 (MH^+).

9-Chlorophenazine-1-carbonitrile (**13**). To a stirred suspension of **12a** (0.04 g, 0.16 mmol) in DMF (8 mL) at 0 ˚C, was added thionyl chloride (1.0 mL) dropwise under N_2 atmosphere. The mixture was heated at 100 °C for 3 h and then cooled to room temperature. The solvent was evaporated *in vacuo* and the residue was purified by silica gel column chromatography using CH_2Cl_2 as eluent to afford the title compound as a yellow solid (15 mg, 41%); R_f: 0.66 (EtOAc/Hexane, 1:4); mp: 258−260 °C. IR (KBr) 3043, 2900, 2218, 1948, 1454, 931, 759 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.86 (d, 1 H, J = 8.0 Hz), 7.95 (d, 1 H, J = 8.0 Hz), 8.07 (d, 1 H, J = 7.0 Hz), 8.22 (d, 1 H, J = 9.0 Hz), 8.35 (d, 1 H, J = 7.0 Hz), 8.51 (d, 1 H, J = 9.0 Hz); ^{13}C

NMR (125 MHz, $CDCl_3$): δ 114.15, 115.85, 128.74, 129.87, 131.34, 131.39, 134.06, 134.74, 137.70, 140.72, 141.65, 142.57, 144.81; HRESIMS calcd for $C_{13}H_7ClN_3$: 240.0250 (MH^+); found: 240.0404 (MH^+).

2-((2,5-Dichlorophenyl)amino)-3-nitrobenzoic acid (**16**). To a stirred solution of 2,5-dichloroaniline (**14**, 0.99 g, 6.10 mmol) and 2-bromo-3-nitrobenzoic acid (**15**, 1.00 g, 4.06 mmol) in ethanol (30 mL) was added copper powder (25 mg, 0.41 mmol), copper (I) bromide (58 mg, 0.41 mmol) and *N*-ethyl morpholine (1.00 mL, 8.12 mmol). The reaction mixture was heated at reflux for 14 h, diluted with aqueous ammonium hydroxide solution (0.1 N, 15 mL), and filtered over celite. The filtrate was acidified to pH 2 using hydrochloric acid (1 N), and the obtained precipitate was filtered. The obtained yellow solid was dried under vacuum to afford 2-((2,5-dichlorophenyl)amino)-3-nitrobenzoic acid (**16**) as a yellow solid (0.75 g, 57%, yield), which was used for the next step without further purification.

6,9-Dichlorophenazine-1-carboxylic acid (**17**). To a solution of 2-((2,5-dichlorophenyl)amino)-3-nitrobenzoic acid **16** (0.74 g, 2.26 mmol) in aqueous sodium hydroxide (2 N, 50 mL) was added sodium borohydride (0.34 g, 9.04 mmol). The reaction mixture was heated at 80 °C for 16 h, cooled to room temperature, and acidified to pH 2 using hydrochloric acid (1 N). The formed solid was filtered, washed with water (2 × 10 mL), and dried under vacuum the solid residue was purified by silica gel column chromatography using MeOH/CH_2Cl_2 (1:40) as eluent to afford the title compound as a yellow solid (280 mg, 42%); R_f: 0.49 (MeOH/ CH_2Cl_2, 1:50); mp: 265−267 °C. IR (KBr) 3367, 3081, 2946, 1683, 1409, 1283, 1009, 755 cm^{-1}; ^1H NMR (500 MHz $CDCl_3$): δ 8.07 (s, 2 H), 8.19 (t, 1 H, J = 8.0 Hz), 8.72 (d, 1 H, J = 8.5 Hz), 9.11 (d, 1 H, J = 7.0 Hz); ^{13}C

NMR (125 MHz, $CDCl_3$): δ 128.89, 130.68, 131.22, 131.77, 132.03, 132.60, 134.01, 136.26, 138.46, 140.20, 140.58, 143.21, 166.11; HRESIMS calcd for $C_{13}H_6Cl_2N_2O_2Na$: 314.9806 $(M+Na)^+$; found: 315.5114 $(M+Na)^+$.

Methyl 6,9-dichlorophenazine-1-carboxylate (**18a**). To a solution of acid **17** (0.09 g, 0.31 mol) in dimethylformamide (5 mL) was added potassium carbonate (0.09 g, 0.62 mmol) followed by methyl iodide (0.04 mL, 0.62 mmol). The reaction mixture was stirred under a nitrogen atmosphere at room temperature for 24 h. The solvent was removed under vacuum, the obtained residue was diluted with dichloromethane (10 mL), the solid filtered off, and the liquid collected. The solvent was removed under vacuum and the obtained residue purified by silica gel column chromatography using Hexane/EtOAc (4:1) as eluent to afford the title compound as yellow solid (70 mg, 77%); R_f: 0.57 (EtOAc/Hexane, 1:4); mp: 171−173 °C. IR (KBr) 3081, 1720, 1548, 1430, 1373, 1009, 849, 760 cm^{-1}; ^1H NMR (500 MHz $CDCl_3$): δ 4.15 (s, 3 H), 7.92 (s, 2 H), 7.97 (t, 1 H, J = 8.0 Hz), 8.38 (dd, 1 H, J = 1.0, 8.0 Hz), 8.54 (d, 1 H, J = 8.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): 52.86, 129.62, 129.85, 130.69, 131.67, 132.07, 133.15, 133.62, 133.77, 140.27, 140.52, 140.92, 142.77, 166.76; HRESIMS calcd for $C_{14}H_9Cl_2N_2O_2$: 307.0036 (MH^+); found: 307.0954 (MH^+).

6,9-Dichlorophenazine-1-carboxamide (**18b**). This compound was prepared following the procedure used for **12b** with compound **17** as a starting material. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (21 mg, 42%); R_f: 0.29 (MeOH/ CH_2Cl_2, 1:30); mp: 267−269 °C. IR (KBr) 3350, 2905, 2831, 1687, 1646, 1397, 1054, 947, 760 cm^{-1}; ^1H NMR (500 MHz, DMSO-

d_6): δ 8.17-8.22 (m, 3 H), 8.26 (br s, 1 H), 8.51 (d, 1 H, J = 8.0 Hz), 8.85 (d, 1 H, J = 7.0 Hz), 9.94 (br s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6): 130.39, 130.93, 131.03, 131.23, 131.79, 132.49, 133.78, 136.77, 138.52, 139.80, 140.45, 143.32, 165.27; HRESIMS calcd for $C_{13}H_8Cl_2N_3O$: 291.9966 (MH^+); found: 291.6212 (MH^+).

6,9-Dichloro-N-(methylsulfonyl)phenazine-1-carboxamide (**18c**). To a stirred solution of **17** (0.05 g, 0.21 mmol) in DMF (5 mL) and CH_2Cl_2 (5 mL) added 1,1'-carbonyldiimidazole (0.07 g, 0.41 mmol) and 4-dimethylaminopyridine (2.0 mg, 0.02 mmol) under an N_2 atmosphere. The mixture was stirred at 70 ^{0}C for 2 h, and cooled to room temperature. Triethylamine (0.06 mL, 0.41 mmol) and methanesulfonamide (0.04 g, 0.41 mmol) were added, the mixture was stirred at 70 ^{0}C for 48 h, and the solvents were evaporated *in vacuo*. The residue was acidified using hydrochloric acid (1 N) and the yellow precipitated was filtered and dried under vacuum. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (50:1) as eluent to afford the title compound as a yellow solid (28 mg, 22%); R_f: 0.46 (MeOH/ CH_2Cl_2, 1:50); mp: 309−311 °C. IR (KBr) 3330, 3007, 2934, 1679, 1593, 1515, 1160, 1119, 747 cm^{-1}; ^{1}H NMR (500 MHz $CDCl_3$): δ 3.53 (s, 3 H), 7.99−8.02 (m, 2 H), 8.15 (t, 1 H, J = 8.0 Hz), 8.70 (dd, 1 H, J = 1.0, 9.0 Hz), 9.12 (dd, 1 H, J = 1.0, 9.0 Hz), 13.92 (s, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): 41.90, 126.65, 130.50, 130.97, 131.41, 131.84, 132.54, 135.95, 138.28, 138.41, 139.57, 140.60, 143.39, 162.92; HRESIMS calcd for $C_{14}H_{10}Cl_2N_3O_3S$: 371.2115 (MH^+); found: 371.4437 (MH^+).

9-Bromo-6-methoxyphenazine-1-carboxylic acid (**21a**). This compound was prepared following the same procedure used for **17** with compound **19a** as a starting material. The crude mixture

was recrystallized with methanol to afford the title compound as an orange solid (190 mg, 48%); R_f: 0.35 (MeOH/ CH_2Cl_2, 1:40); mp: 290−292 °C. IR (KBr) 3489, 3081, 2905, 1728, 1438, 1360, 1250, 1095, 756 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.22 (s, 3 H), 7.10 (d, 1 H, J = 8.5 Hz), 8.08 (t, 1 H, J = 8.5 Hz), 8.22 (d, 1 H, J = 8.5 Hz), 8.68 (dd, 1 H, J = 1.0, 8.5 Hz), 9.04 (dd, 1 H, J = 1.0, 7.0 Hz), 15.33 (br s, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6): 57.21, 108.35, 112.39, 125.18, 131.18, 135.25, 136.01, 138.05, 138.34, 138.62, 140.37, 142.45, 155.49, 165.71; HRESIMS calcd for $C_{14}H_{10}BrN_2O_3$: 332.9797 (MH^+); found: 332.9981 (MH^+).

7-Bromo-9-methoxyphenazine-1-carboxylic acid (**21b**). This compound was prepared following the same procedure used for **17** with compound **19b** as a starting material. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (42 mg, 31%); R_f: 0.36 (MeOH/ CH_2Cl_2, 1:40); mp: 202−204 °C. IR (KBr) 2909, 2831, 1716, 1626, 1581, 1434, 1287, 1123, 1000, 857 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.19 (s, 3 H), 7.26 (s, 1 H), 8.06 (t, 1 H, J = 8.0 Hz), 8.11 (s, 1 H), 8.48 (d, 1 H, J = 8.5 Hz), 8.96 (d, 1 H, J = 7.0 Hz), 15.55 (br s, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 57.14, 113.11, 123.53, 125.59, 127.06, 131.51, 132.41, 134.76, 137.24, 138.80, 144.01, 144.71, 154.42, 165.90; HRESIMS calcd for $C_{14}H_{10}BrN_2O_3$: 331.9797 (MH^+); found: 332.1489 (MH^+).

6,9-Dimethoxyphenazine-1-carboxylic acid (**21c**). This compound was prepared following the same procedure used for **17** with compound **19c** as a starting material. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a black solid (25 mg, 14%); R_f: 0.55 (MeOH/ CH_2Cl_2, 1:20); mp: 296−298 °C.

IR (KBr) 2938, 1736, 1589, 1479, 1205, 1013, 816, 751 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.53 (s, 3 H), 4.12 (s, 3 H), 4.17 (s, 3 H), 7.11 (d, 2 H, J = 5.5 Hz), 8.04 (t, 1 H, J = 8.0 Hz), 8.66 (d, 1 H, J = 8.5 Hz), 8.96 (d, 1 H, J = 7.0), 15.85 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 56.28, 56.42, 107.12, 108.29, 124.93, 130.34, 133.35, 134.86, 136.94, 137.37, 138.68, 141.86, 147.53, 148.54, 165.86; HRESIMS calcd for $C_{15}H_{13}N_2O_4$: 285.0797 (MH^+); found: 284.9408 (MH^+).

9-Bromo-6-methoxy-N-(methylsulfonyl)phenazine-1-carboxamide (**22a**). To a stirred solution of **21a** (0.10 g, 0.30 mmol) in a mixture of DMF (5 mL) and CH_2Cl_2 (5 mL) was added 1,1'-carbonyldiimidazole (0.10 g, 0.60 mmol) and 4-dimethylaminopyridine (4.0 mg, 0.03 mmol) under N_2 atmosphere. The mixture was stirred at 70 ^{0}C for 2 h, cooled to room temperature, and then triethylamine (0.08 mL, 0.60 mmol) and methanesulfonamide (0.06 g, 0.6 mmol) were added. The reaction mixture was stirred at 70 ^{0}C for 24 h and the solvents were evaporated *in vacuo*. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as an orange red solid (60 mg, 51%); R_f: 0.32 (MeOH/ CH_2Cl_2, 1:40); mp: 245−247 °C. 3350, 2909, 2831, 1679, 1516, 1315, 1234, 1136, 1086, 763 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.53 (s, 3 H), 4.22 (s, 3 H), 7.10 (d, 1 H, J = 8.5 Hz), 8.08 (t, 1 H, J = 8.5 Hz), 8.22 (d, 1 H, J = 8.5 Hz), 8.71 (d, 1 H, J = 8.5 Hz), 9.09 (d, 1 H, J = 7.0 Hz), 13.95 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 41.95, 56.96, 108.14, 113.34, 126.45, 130.46, 135.40, 135.97, 137.55, 137.90, 139.14, 139.83, 142.19, 155.06, 163.37; HRESIMS calcd for $C_{15}H_{13}BrN_3O_4S$: 409.9732 (MH^+); found: 409.9917(MH^+).

9-Bromo-N-cyano-6-methoxyphenazine-1-carboxamide (**22b**). To a stirred solution of **21a** (0.03 g, 0.09 mmol) in a mixture of DMF (5 mL) and CH_2Cl_2 (5 mL) was added 1,1'-carbonyldiimidazole (0.03 g, 0.18 mmol) and 4-dimethylaminopyridine (2.0 mg, 0.02 mmol) under N_2 atmosphere. The mixture was stirred at 70 ^{0}C for 2 h and then cooled to room temperature. Trimethylamine (0.03 mL, 0.18 mmol) and cyanamide (0.01 g, 0.18 mmol) were added, the reaction mixture was stirred at 70 ^{0}C for 24 h, and the solvents were evaporated *in vacuo*. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as an orange solid (18 mg, 56%); R_f: 0.33 (MeOH/ CH_2Cl_2, 1:50); mp: 280−282 °C. IR (KBr) 3350, 2909, 2831, 1679, 1516, 1315, 1234, 1136, 1086, 763 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.53 (s, 3 H), 4.22 (s, 3 H), 7.11 (d, 1 H, J = 8.5 Hz), 8.09 (t, 1 H, J = 7.0 Hz), 8.22 (d, 1 H, J = 8.5 Hz), 8.71 (d, 1 H, J = 8.5 Hz), 9.09 (d, 1 H, J = 7.0 Hz), 14.14 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 57.15, 107.04, 108.39, 112.60, 125.00, 130.71, 135.88, 136.35, 137.79, 138.43, 138.65, 139.41, 142.36, 155.42, 163.77; HRESIMS calcd for $C_{15}H_8BrN_4O_2$: 354.9907 (MH^-); found: 354.9658 (MH^-).

Methyl 7-bromo-9-methoxyphenazine-1-carboxylate (**22c**). This compound was prepared following the procedure used for **18a** with compound **21b** as a starting material. The crude product was purified by silica gel column chromatography using Hexane/EtOAc (4:1) as eluent to afford the title compound as a yellow solid (19 mg, 61%); R_f: 0.45 (EtOAC/Hexane, 1:4); mp: 195−197 °C. IR (KBr) 3073, 2917, 1720, 1519, 1270, 1111, 760, cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 4.10 (s, 3 H), 4.16 (s, 3 H), 7.14 (d, 1 H, J = 1.5 Hz), 7.84 (t, 1 H, J = 8.0 Hz), 8.02 (d, 1 H, J = 1.5 Hz), 8.35−8.39 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 52.72, 56.97, 111.69,

123.34, 126.23, 130.06, 130.74, 133.36, 133.72, 136.08, 139.81, 143.29, 143.94, 155.75, 166.17; HRESIMS calcd for $C_{15}H_{12}BrN_2O_3$: 347.0026 (MH^+); found: 347.1580 (MH^+).

9-Bromo-7-methoxyphenazine-1-carboxylic acid (**25**) and *7-Methoxyphenazine-1-carboxylic acid* (**26**). To a stirred solution of ester **27** or **28** (0.02 g, 0.06 mmol) in a mixture of MeOH (2 mL), THF (3 mL) and water (2 mL) was added NaOH (2 N, 0.23 mL). The mixture was stirred at 45 ºC for 2 h and then cooled to room temperature. The solvent was evaporated *in vacuo* and the solution was acidified with 2 N HCl to pH 2. The precipitates were filtered and dried to afford the titled compounds.

(**25**) Yellow solid (14 mg, 74%); R_f: 0.38 (MeOH/ CH_2Cl_2, 1:40); mp: 270−272 °C. IR (KBr) 3064, 2909, 2835, 1736, 1617, 1589, 1409, 1192, 750 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.10 (s, 3 H), 7.50 (d, 1 H, J = 2.5 Hz), 8.04−8.08 (m, 2 H), 8.48 (d, 1 H, J = 8.5 Hz), 8.96 (dd, 1 H, J = 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): 56.49, 104.70, 123.22, 125.21, 125.32, 125.48, 131.21, 131.54, 133.91, 136.39, 138.43, 158.19, 161.77; HRESIMS calcd for $C_{14}H_8BrN_2O_3$: 330.9797 (MH^-) found: 330.9683 (MH^-).

(**26**) Yellow solid (18 mg, 86%); R_f: 0.36 (MeOH/ CH_2Cl_2, 1:40); mp: 290−292 °C. IR (KBr) 3370, 2909, 1740, 1483, 1205, 820, 745 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.08 (s, 3 H), 7.45 (d, 1 H, J = 2.5 Hz), 7.68 (dd, 1 H, J = 2.5, 9.5 Hz), 8.00 (t, 1 H, J = 8.0 Hz), 8.14 (d, 1 H, J = 9.5 Hz), 8.44 (dd, 1 H, J = 1.0, 7.0 Hz), 8.88 (dd, 1 H, J = 1.0, 7.0 Hz), 15.51 (br s, 1 H); HRESIMS calcd for $C_{14}H_{11}N_2O_3$: 255.2530 (MH^+); found: 255.2351 (MH^+).

*Methyl 9-bromo-7-methoxyphenazine-1-carboxylate and (***27***) and Methyl 7-methoxyphenazine-1-carboxylate (***28***).* The crude acids **25** and **26** were prepared according to the procedure used for

17 with compound **23** as a starting material. To a stirred mixture of crude compound **25** and **26** (0.14 g, 0.42 mmol) in DMF (10 mL) were added K_2CO_3 (0.11 g, 0.84 mmol) and MeI (0.12 g, 0.84 mmol). The mixture was stirred at room temperature for 24 h and then filtered. The filtrated was evaporated to dryness under reduced pressure. The residue was diluted with H_2O (50 mL) and extracted with EtOAc (2 ×50 mL). The EtOAc extracts were combined, and solution was washed with brine (25 mL), dried with Na_2SO_4, filtered, and evaporated to dryness under vacuum. The residue was purified by preparative TLC using Toluene/EtOAc (4:1) as eluent to afford the two esters.

(**27**). Yellow solid (48 mg, 22%); R_f: 0.57 (EtOAC/Hexane, 1:4); mp: 175−177 °C. IR (KBr) 2909, 2831, 1716, 1626, 1581, 1434, 1287, 1123, 1000, 857, cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.99 (s, 3 H), 4.13 (s, 3 H), 7.31 (d, 1 H, J = 2.5 Hz), 7.82−7.87 (m, 2 H), 8.20 (d, 1 H, J = 7.0 Hz), 8.26 (dd, 1 H, J = 1.0, 8.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): 52.73, 56.19, 104.34, 125.97, 129.78, 130.02, 131.22, 131.83, 132.25, 138.12, 139.33, 142.94, 144.71, 161.32, 167.31; HRESIMS calcd for $C_{15}H_{11}BrN_2O_3$: 347.0026 (MH^+); found: 347.0050 (MH^+).

(**28**). Yellow solid (32 mg, 29 %); R_f: 0.52 (EtOAC/Hexane, 1:4); mp: 147−149 °C. IR (KBr) 2918, 1720, 1679, 1581, 1438, 1336, 1279, 1119, 743, cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.02 (s, 3 H), 4.09 (s, 3 H), 7.37 (d, 1 H, J = 2.5 Hz), 7.51 (dd, 1 H, J = 2.5, 9.5 Hz), 7.81 (t, 1 H, J = 7.0 Hz), 8.13 (d, 1 H, J = 7.0 Hz), 8.16 (d, 1 H, J = 9.5 Hz), 8.28 (d, 1 H, J = 8.5 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): 52.64, 55.96, 104.17, 127.00, 129.04, 130.52, 131.46, 132.62, 139.41, 141.11, 142.67, 145.04, 161.85, 167.30; HRESIMS calcd for $C_{15}H_{12}N_2O_3$: 267.0848 (MH^+); found: 267.0450 (MH^+).

N-(Methylsulfonyl)phenazine-1-carboxamide (**30a**). This compound was prepared following the procedure used for **18c** with compound **29a** as a starting material. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (25 mg, 40%); R_f: 0.43 (MeOH/ CH_2Cl_2, 1:50); mp: 185−187 °C. IR (KBr) 3240, 2921, 2840, 1740, 1675, 1462, 1123, 735 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.52 (s, 3 H), 7.96−8.05 (m, 3 H), 8.31−8.33 (m, 2 H), 8.55 (dd, 1 H, J = 1.0, 9.0 Hz), 9.02 (dd, 1 H, J = 1.0, 7.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 41.90, 126.04, 128.82, 129.79, 129.84, 131.85, 132.92, 136.13, 136.89, 139.70, 140.77, 143.39, 143.77, 163.36; HRESIMS calcd for $C_{14}H_{12}N_3O_3S$: 302.0521 (MH^+); found: 302.1628 (MH^+).

N-Methyl-N-(methylsulfonyl)phenazine-1-carboxamide (**30b**). To a stirred solution of **30a** (0.02 g, 0.07 mmol) in *N*,*N*-dimethylformamide (3.0 mL) were added K_2CO_3 (0.02 g, 0.13 mmol) and CH_3I (10 µL, 0.13 mmol). The reaction mixture was stirred at room temperature for 12 h and the solvent was evaporated *in vacuo*. The residue was diluted with H_2O (10 mL), and extracted with EtOAc (2 × 20 mL). The combined EtOAc extracts were washed with HCl (1 N, 10 mL), and brine 20 mL). The organic layer was dried with Na_2SO_4, filted, and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using Hexane/EtOAc (3:1) as eluent to afford the title compound as a brown solid (15 mg, 75%); R_f: 0.42 (EtOAc/Hexane, 1:4); mp: 199−201 °C. IR (KBr) 3003, 2913, 1666, 1519, 1340, 1156, 959, 747 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.27 (s, 3 H), 3.47 (s, 3 H), 7.87−7.91 (m, 4 H), 8.16 (dd, 1 H, J = 1.0, 8.5 Hz), 8.26−8.28 (m, 1 H), 8.35 (dd, 1 H, J = 2.0, 8.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 33.83, 40.80, 128.78, 129.57, 129.76, 129.91, 131.34, 131.41, 131.78, 135.72,

139.98, 142.59, 143.24, 143.92, 169.85; HRESIMS calcd for $C_{15}H_{14}N_3O_3S$: 316.0678 (MH^+); found: 316.1716 (MH^+).

N-Cyano-9-fluorophenazine-1-carboxamide (**30c**). To a stirred solution of **29b** (0.04 g, 0.16 mmol) in a mixture of DMF (5 mL) and CH_2Cl_2 (5 mL) were added 1,1'-carbonyldiimidazole (0.05 g, 0.33 mmol) and 4-dimethylaminopyridine (2.0 mg, 0.02 mmol) under a N_2 atmosphere. The mixture was stirred at 60 ºC for 2 h, cooled to room temperature, and trimethylamine (0.05 mL, 0.33 mmol) and cyanamide (0.02 g, 0.33 mmol) were added. The reaction mixture was then stirred at 60 ºC for 24 h, and the solvents evaporated *in vacuo*. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (18 mg, 41%); R_f: 0.41 (MeOH/ CH_2Cl_2, 1:40); mp: 234−236 °C. IR (KBr) 3354, 2917, 2251, 1683, 1450, 710 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 7.71 (t, 1 H, J = 8.5 Hz), 7.93−7.96 (m, 1 H), 8.11 (t, 1 H, J = 7.0 Hz), 8.19 (d, 1 H, J = 8.5 Hz), 8.59 (d, 1 H, J = 8.5 Hz), 9.10 (d, 1 H, J = 7.0 Hz), 13.74 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 106.67, 115.37, 115.51, 125.27, 125.85, 125.90, 130.76, 131.01, 131.07, 136.37, 138.15, 143.80, 144.22, 163.64; ^{19}F NMR (469 MHz, $CDCl_3$):-124.13; HRESIMS calcd for $C_{14}H_8FN_4O$: 267.0604 (MH^+); found: 266.7151 (MH^+).

9-Fluoro-N-(methylsulfonyl)phenazine-1-carboxamide (**30d**). To a stirred solution of **29b** (0.05 g, 0.21 mmol) in a mixture of DMF (5 mL) and CH_2Cl_2 (5 mL) were added 1,1'-carbonyldiimidazole (0.07 g, 0.41 mmol) and 4-dimethylaminopyridine (2.0 mg, 0.02 mmol) under a N_2 atmosphere. The reaction mixture was stirred at 60 ºC for 2 h and then cooled to room temperature. Triethylamine (0.06 mL, 0.41 mmol) and methanesulfonamide (0.04 g, 0.41 mmol)

were added, the mixture was stirred at 70 ºC for 48 h, and the solvents were evaporated *in vacuo*. The residue was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (12 mg, 27%); R_f: 0.39 (MeOH/ CH_2Cl_2, 1:40); mp: 229−231 °C. ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.51 (s, 3 H), 7.66 (t, 1 H, J = 8.0 Hz), 7.89−7.93 (m, 1 H), 8.07 (t, 1 H, J = 8.0 Hz), 8.15 (d, 1 H, J = 9.0 Hz), 8.54 (dd, 1 H, J = 1.0, 8.5 Hz), 9.05 (dd, 1 H, J = 1.0, 7.0 Hz), 14.05 (1 H, br s); ^{13}C NMR (125 MHz, $CDCl_3$): δ 42.03, 115.21, 115.35, 125.76, 125.81, 126.77, 130.90, 131.10, 131.17, 136.09, 137.65, 143.90, 144.26, 163.24; ^{19}F NMR (469 MHz, $CDCl_3$): -123.04; HRESIMS calcd for $C_{14}H_{11}FN_3O_3S$: 320.0427 (MH^+); found: 319.6411 (MH^+).

9-Methyl-N-(methylsulfonyl)phenazine-1-carboxamide (**30e**). This compound was prepared following the procedure used for **18c** with compound **29c** as a starting material. The crude product was purified by silica gel column chromatography using CH_2Cl_2/MeOH (40:1) as eluent to afford the title compound as a yellow solid (14 mg, 22%); R_f: 0.42 (MeOH/ CH_2Cl_2, 1:40); mp: 242−244 °C. IR (KBr) 3403, 3072, 2950, 1712, 1548, 1503, 1426, 1258, 1205, 1115, 731 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.98 (s, 3 H), 3.52 (s, 3 H), 7.82−7.86 (m, 2 H), 8.02 (t, 1 H, J = 7.5 Hz), 8.15 (d, 1 H, J = 8.5 Hz), 8.54 (d, 1 H, J = 9.0 Hz), 9.01 (d, 1 H, J = 7.0 Hz), 14.51 (s, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 18.27, 41.91, 126.14, 127.77, 129.67, 131.86, 132.37, 135.97, 136.61, 136.99, 138.77, 140.66, 143.04, 144.11, 163.52; HRESIMS calcd for $C_{15}H_{14}N_3O_3S$: 316.0678 (MH^+); found: 316.0996 (MH^+).

Methyl 9-methoxyphenazine-1-carboxylate (**30f**). This compound was prepared following the procedure used for **18a** with compound **29d** as a starting material. The crude product was

purified by silica gel column chromatography using Hexane/EtOAc (4:1) as eluent to afford the title compound as a yellow solid (25 mg, 65%); R_f: 0.52 (EtOAc/Hexane, 1:4); mp: 149−151 °C. IR (KBr) 2909, 1723, 1262, 1086, 1021, 796 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 4.11 (s, 3 H), 4.17 (s, 3 H), 7.09 (d, 1 H, J = 7.0 Hz), 7.78−7.84 (m, 2 H), 7.88 (dd, 1 H, J = 2.0, 8.5 Hz), 8.38−8.41 (m, 2 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 52.67, 56.62, 107.20, 121.11, 129.40, 130.65, 131.51, 133.12, 133.77, 137.25, 139.87, 142.89, 144.00, 155.51, 166.40; HRESIMS calcd for $C_{15}H_{13}N_2O_3$: 269.0848 (MH^+); found: 269.0119 (MH^+).

9-Chloro-N-methyl-N-(methylsulfonyl)phenazine-1-carboxamide (**31**). To a stirred solution of **6** (0.02 g, 0.06 mmol) in DMF (2.0 mL) were added K_2CO_3 (20 mg, 0.12 mmol) and methyl iodide (12 µL, 0.18 mmol). The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated *in vacuo*, the residue was diluted with H_2O (20 mL), and extracted with EtOAc (2 × 20 mL). The combined EtOAc extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness under reduced pressure. The residue was purified by silica gel column chromatography using Hexane/EtOAc (4:1) as eluent to afford the title compound as yellow solid. (14 mg, 70%); R_f: 0.38 (EtOAc/Hexane, 1:4); mp: 220−222 °C. IR (KBr) 2991, 2909, 1716, 1663, 1430, 1336, 1254, 747 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ 3.29 (s, 3 H), 3.52 (s, 3 H), 7.82 (t, 1 H, J = 8.0 Hz), 7.94−8.01 (m, 3 H), 8.22 (d, 1 H, J = 9.0 Hz), 8.37 (dd, 1 H, J = 1.2, 9.0 Hz); ^{13}C NMR (125 MHz, $CDCl_3$): δ 33.99, 40.61, 129.15, 129.67, 130.72, 130.78, 130.85, 131.50, 133.10, 136.07, 139.56, 139.70, 142.81, 144.40, 169.46; HRESIMS calcd for $C_{15}H_{13}ClN_3NaO_3S$: 372.0186 $(M+Na)^+$; found: 372.1805 $(M+Na)^+$.

4.2. Strains and Growth conditions

Staphylococcus aureus USA 300 JE2 and *Escherichia coli* K12 were kindly provided by Dr. Kenneth W. Bayles, Department of Pathology and Microbiology, University of Nebraska Medical Center. Strains were maintained as frozen stocks at -80 °C in Tryptic Soy Broth (BD Diagnostics) with 50% of glycerol, which were plated onto nutrient agar and incubated at 37°C overnight to obtain single colonies. Experimental cultures were prepared by inoculating single colonies in Muller Hinton broth (Difco ™ BD Diagnostics) at 37 °C overnight.

4.3. Minimal inhibitory concentration (MIC) determination

Bacteria strains used in this experiment were *Staphylococcus aureus* USA 300 JE2 and *Escherichia coli* K12. The MICs of the phenazines were determined using a broth microdilution method as described in the third edition of the ASM Clinical Microbiology Procedures Handbook. Briefly, a stock solution of each compound was prepared in DMSO at 1 mg/mL and then serial 2-fold dilutions were made in Muller Hinton broth (Difco ™ BD Diagnostics), containing 5% DMSO in Cellstar 96-well microtiter plates (Greiner, Bio-One). Bacterial cultures were prepared using the direct colony suspension method to 0.5 McFarland units and each well was inoculated with 10 µL of the suspension there after, an aliquot of the phenazine solution (or control) were added the each well the plates were incubated statically at 37 °C for 24 h. MIC values were taken at the lowest concentration at which no growth was observed by the unaided eye and a microplate reader. The O.D value at 600 nm was recorded using AccuSkan, MultiSkan FC (Thermo Fisher Scientific). Vancomycin (Sigma) and Gentamicin (Alfa Aesar) were used as positive control and media was used as negative control. The assay was performed in triplicate.

4.4. Time killing assays

Staphylococcus aureus JE2 USA 300 was cultured in Muller Hinton Broth and incubated at 37 ºC for 6 hours and adjusted to approximately 7.5×10^6 CFU/mL. Bacteria solutions were treated

with compound **18c** at three times MIC value and incubated at 37 ºC. At different times (0, 1, 2, 3, 4, 6 and 24 hours), 100 μL aliquots from those solutions were serially diluted 10-fold in 0.9% saline in 96-well plates. Then, 10 μL from the dilutions were plated on Muller Hinton Agar and incubated at 37 ºC for 24 hours. The bacterial colonies were counted and results are represented in logarithmic scale (CFU/mL). Experiments were performed in triplicate.

4.5. Sample preparation for transmission electron microscopy (TEM)

Staphylococcus aureus JE2 USA 300 was cultured in Muller Hinton Broth and incubated at 37 ºC. The resultant mid-log phase cultures were diluted to a final concentration of 1.5×10^8 CFU/mL (0.5 McFarland). Bacteria cells were treated with phenazines **4** or **18c** at twice the MIC value and incubated for 18 h at 37 °C. A control was prepared where no compound was added. After treatment, cells were immediately washed three times with Phosphate Buffered Saline solution (PBS, GE Healthcare Life Science) and fixed with 2.0% (v/v) glutaraldehyde and 2% (v/v) of paraformaldehyde in 0.1 M phosphate buffer.

4.6. Sample Preparation for flow cytometry

Staphylococcus aureus JE2 USA 300 was cultured in Muller Hinton Broth and incubated at 37 ºC and adjusted to a final concentration of 3×10^8 CFU/mL. Bacteria cells were treated with phenazines **4** or **18c** at 1 × MIC value and incubated for 18 h at 37 °C. *S. aureus* JE with Propidium Iodide (PI) and *S. aureus* JE2 treated with Ethanol 70% were used as controls. After incubation, 50 μL of Propidium Iodide (Biotium, 1mg/ml) were added and maintained in the dark for 15-20 minutes. Cells were immediately washed three times, re-suspended in Phosphate Buffered Saline solution (GE Healthcare Life Science).

4.7. Sample preparation for confocal laser scanning microscopy (CLSM)[11]

Staphylococcus aureus JE2 USA 300 was cultured in Muller Hinton Broth and incubated at 37 ºC and adjusted to a final concentration of 6×10^9 CFU/mL. Bacteria cells were treated with phenazines **4** or Vancomycin at 1 × MIC value and incubated for 18 h at 37 °C. Controls is untreated with Propidium Iodide (PI), and treated with *S. aureus***.** After treatment, 50 µL of Propidium Iodide (Biotium) were added and maintained in the dark for 15-20 minutes. Cells were immediately washed three times, re-suspended in Phosphate Buffered Saline solution (GE Healthcare Life Science) and slides were prepared using 10 µL of antifade reagent with DAPI.

4.8. Mutant resistance studies in agar plates

Staphylococcus aureus JE2 USA 300 was cultured in Muller Hinton Broth and incubated at 37 ºC for 4 hours and diluted for 1.5×10^8 CFU/mL (0.5 McFarland). Inocula were spread on Muller Hinton Agar plates, after the samples dried, standard 6 mm paper discs (BD BBL™ Taxo™ Blank Discs), loaded with phenazines solution in DMSO **4**, **9**, **18c** and **30e** at 10 × MIC value and incubated at 37 ºC. After 24 hours the diameter of the inhibition zone was determined and plates were maintained for 6 days. No colony grew up during this time but if any colony grow in inhibition zone, it will be culture to determine MIC.

4.9. Biofilm Dispersion Assay for Staphylococcus aureus JE2[32]

Briefly, sterile 96-well flat-bottomed polystyrene microtiter plates (Thermo Scientific) were pretreated with 200 µL 0.1% gelatin solution overnight, and then gelatin was removed. Staphylococcus *aureus* JE2 USA 300 was cultured overnight in Tryptic Soy Broth (Difco ^{TM} BD Diagnostics) with 0.5% of glucose, diluted to 1×10^6 CFU/ mL and wells were filled with 100 µL of the bacterial suspension. The plates were incubated for 24 hours at 37 °C. Following this, the contents of the wells were discarded and 200 µL of two-fold dilutions of phenazines in fresh TSB with 0.5% glucose were delivered into each well at test concentrations ranging between

256−8 μg/mL for compound **18c** and 32−1 μg/mL for compound **6** and vancomycin**.** The plates were incubated for another 24 hours at 37 °C. After this incubation time, contents of the wells were discarded and remaining biofilms were then fixed with 200 μL of methanol for 15 minutes and the plates were emptied and left to on air dry. After drying, 100 μL of 0.1% crystal violet was added to each well for 10 minutes, then washed with water and again air dried. 100 μL of bleaching solution (methanol:glacial acetic acid:water (v/v/v) = 4:1:5) was added to all 96-wells to dissolve the remaining crystal violet stained biofilm. The O.D value at 540 nm was recorded using AccuSkan, MultiSkan FC (Thermo Fisher Scientific).

4.10. Biofilm Inhibition Assay for *S. aureus* JE2

Briefly, sterile 96-well flat-bottomed polystyrene microtiter plates (Thermo Scientific) were pretreated with 200 μL 0.1% gelatin solution overnight, and then gelatin was removed. 200 μL of two-fold dilutions of phenazines in fresh TSB with 0.5% glucose were delivered into each well at test concentrations ranging between 256−1 μg/mL for compound **18c** and 32−1 μg/mL for compound **4** and 32−0.0125 μg/mL to vancomycin. To each well, 100 μL of TSB with 0.5% glucose containing 2×10^6 CFU/mL Staphylococcus aureus cells, prepared from fresh culture was added. The plates were incubated at 37 °C for 24 hours. The wells were gently rinsed and then biofilms were fixed with 200 μL methanol for 15 minutes. After the plates were air dried, the biofilms were stained with 100 μL of 0.1 of % crystal violet for 10 minutes. The plates were rinsed with water and 100 μL of bleaching solution (methanol:glacial acetic acid:water (v/v/v) = 4:1:5) was added to all 96-wells to dissolve the remaining crystal violet stained biofilm. The O.D value at 540 nm was recorded using AccuSkan, MultiSkan FC (Thermo Fisher Scientific).

5.0. Cytotoxicity assay

The human pancreatic cancer cell line S2-013 was obtained from the American Type Culture

Collection (Rockville, MD, USA). The cells were cultured in high glucose-dulbecco's modified eagle's medium (HG-DMEM) that was supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in a humidified incubator set at 5% CO_2. The cells were plated at a concentration of 5000 cells/well in a 96-well plate and incubated for 24 h at 37 °C and 5% CO_2. On the following day, cells were treated with 400, 300, 200, 100, 50, 25 or 0 (vehicle control) µg/mL of the indicated compounds and incubated at 37 °C and 5% CO_2 for 24 h. Thereafter, the cells were treated with an MTT solution (Sigma-Aldrich, St. Louis, MO) and incubated at 37 °C with the 5% CO_2 for 2 h. Cell viability is assessed by the conversion of the yellow MTT into purple formazan crystals. The media was then removed and DMSO was added to each well to dissolve the formazan crystals. The absorbance of the solution was determined at 570 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, US).

The Human colonic epithelial cell line HCEC was purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were cultured in HCEC media at 37 °C in a hypoxia incubator chamber. The cells were plated 6000 cells/well in a 96-well plate and incubated for 24 h at 37 °C in a hypoxia incubator chamber. The cells were treated with 200, 100, 50, 25, 12.5, 6.25, or 0 (vehicle control) µg/mL of the tested compounds and incubated at 37 °C in a hypoxia incubator chamber for 24 hrs and thereafter the cells were treated with MTT stock solution of 2mg/mL (Sigma). The plates were incubated for 2 h then the resulting purple formazan was solubilized by DMSO with shaking for 10 minutes then the absorbance of solution was determined at 570 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific, US).

5.1. *In vivo* antimicrobial studies – *Galleria mellonella assay*

Galleria mellonella were purchased from www.corolina.com and were maintained on wood chips under the dark until use. Experiments were conducted according to Ramarao *et al* [33]. Briefly, larvae with (2-3 cm long and 200-300 mg in weight) were selected 24 hours before infection and placed into an empty petri dish. Bacterial overnight cultures were pelleted by centrifugation (5000 rpm for 5 min), washed twice and re-suspended in PBS (HyClone™ Dulbecco's Phosphate Buffered Saline solution GE Healthcare Life Science) 10 μL of bacteria suspension were injected on the first right proleg using a microsyringe (LS, Innovative Labor Systems). After 2 hours, 10 μL of a stock solution of **4** or **18c** compounds in DMSO at 1 mg/mL that was diluted in PBS to 300 μg/mL (to obtain 15 mg kg^{-1} body weight) were injected into different prolegs and the larvae incubated at 37 ºC. Insects were examined for the production of pigmentation (a black color indicates the worm is sick or death) and death was monitored over 96 hours. If the larvae did not move in response to touch they were considered dead. All tests in *G. mellonella* were performed in triplicate (n = 10 larvae per each group). As controls we used groups of *G. mellonella* infected, untreated, injected with 30 % DMSO in PBS or treated with vancomycin. The lethal dose, was determined to be 1.5×10^8 CFU/ mL used for *Staphylococcus aureus* JE2 USA 300.

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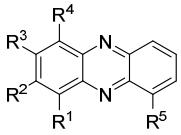
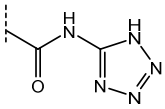


Table 1. **Antimicrobial activity of the compounds**

Compound Number	R^1	R^2	R^3	R^4	R^5	MIC (µg/mL)	
						S. aureus JE2	*E.coli* K12
4	Cl	H	H	H	H	2	128
9	Cl	H	H	H	CONHOH	32	128
12a	Cl	H	H	H	$CONH_2$	>256	128
12b	Cl	H	H	H	CONHCN	128	128
12c	Cl	H	H	H		32	128
13	Cl	H	H	H	CN	>256	64
17	Cl	H	H	Cl	CO_2H	64	128
18a	Cl	H	H	Cl	CO_2CH_3	128	64
18b	Cl	H	H	Cl	$CONH_2$	128	32
18c	Cl	H	H	Cl	$CONHSO_2CH_3$	16	64
21a	Br	H	H	OCH_3	CO_2H	128	64
21b	OCH_3	H	Br	H	CO_2H	128	128
21c	OCH_3	H	H	OCH_3	CO_2H	64	64
22a	Br	H	H	OCH_3	$CONHSO_2CH_3$	128	64
22b	Br	H	H	OCH_3	CONHCN	256	32
22c	OCH_3	H	Br	H	CO_2CH_3	128	128
25	Br	H	OCH_3	H	CO_2H	64	256
26	H	H	OCH_3	H	CO_2H	256	64
27	Br	H	OCH_3	H	CO_2CH_3	128	128
28	H	H	OCH_3	H	CO_2CH_3	128	64
30a	H	H	H	H	$CONHSO_2CH_3$	128	128
30b	H	H	H	H	$CONCH_3SO_2CH_3$	256	128
30c	F	H	H	H	CONHCN	128	128
30d	F	H	H	H	$CONHSO_2CH_3$	64	128
30e	CH_3	H	H	H	$CONHSO_2CH_3$	32	32
30f	OCH_3	H	H	H	CO_2CH_3	128	128
31	Cl	H	H	H	$CONCH_3SO_2CH_3$	>256	64
Gentamicin							0.25
Vancomycin						2	

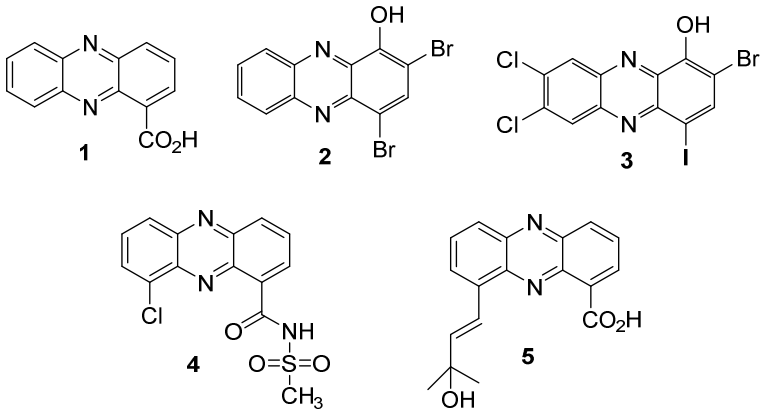
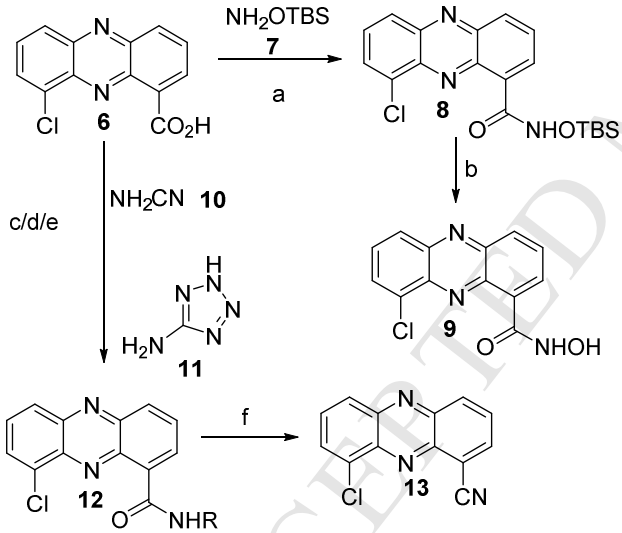


Figure 1. Representative Phenazines synthetic and natural products with antibacterial activity.

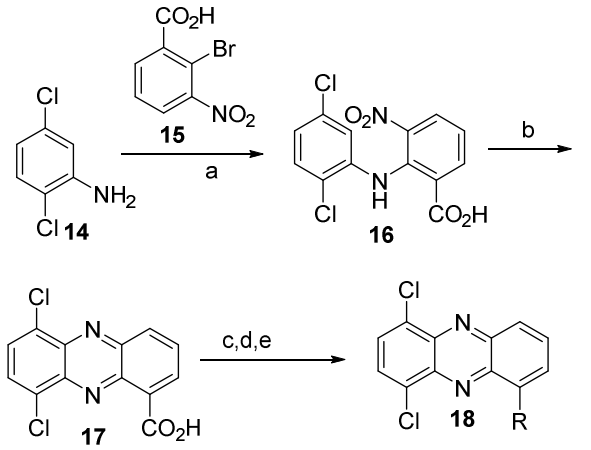
Scheme 1



12a: R = H
12b: R= CN
12c: R = 5(*H*)-tetrazole

Reagents and Conditions: (a) EDCI·HCl, DMAP Et_3N, **9**, CH_2Cl_2, rt; (b) CF_3COOH, CH_2Cl_2, rt; (c) (i) $(COCl)_2$, CH_2Cl_2, DMF, 0 °C to rt, (ii) aq. NH_3; (d) **10**, CDI, Et_3N, DMAP, CH_2Cl_2, DMF, 70 °C; (e) **11**, CDI, Et_3N, DMAP, DMF 70 °C; (f) $SOCl_2$, DMF 100 °C

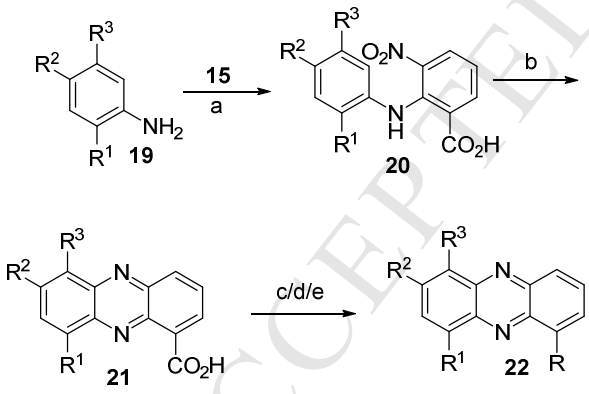
Scheme 2



18a R = CO_2Me
18b: R = $CONH_2$
18c: R = $CONHSO_2Me$

Reagents and conditions: (a) CuBr, Cu powder, EtOH, reflux; (b) 2N NaOH, $NaBH_4$, 80 °C; (c) CH_3I, K_2CO_3, DMF, rt; (d) (i) $(COCl)_2$, CH_2Cl_2, DMF, 0 °C to rt, (ii) aq. NH_3; (e) $CH_3SO_2NH_2$, CDI, Et_3N, DMAP 70 °C.

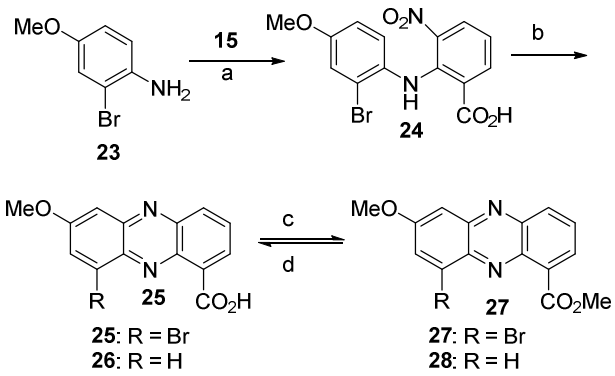
Scheme 3



19a, 21a, 22a: R^1 = Br, R^2 = H, R^3 = OMe, R = $CONHSO_2Me$
19b, 21b, 22b: R^1 = Br, R^2 = H, R^3 = OMe, R = CONHCN
19c, 21c, 22c: R^1 = OMe, R^2 = Br, R^3 = H, R = CO_2Me

Reagents and conditions: (a) **15**, CuBr, Cu powder, EtOH, reflux; (b) NaOH, $NaBH_4$, 80 °C; (c) $CH_3SO_2NH_2$, CDI, Et_3N, DMAP 70 °C; (d) NH_2CN, CDI, Et_3N, DMAP, CH_2Cl_2, DMF, 70 °C (e) CH_3I, K_2CO_3, DMF, rt.

Scheme 4

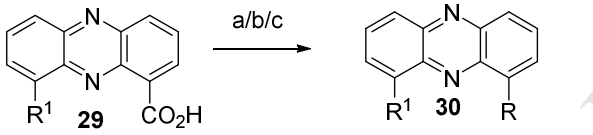


25: R = Br
26: R = H

27: R = Br
28: R = H

Reagents and conditions: (a) CuBr, Cu powder, EtOH, reflux; (b) 2N NaOH, $NaBH_4$, 80 °C; (c) CH_3I, K_2CO_3, DMF, rt; (d) NaOH, H_2O, MeOH, THF.

Scheme 5



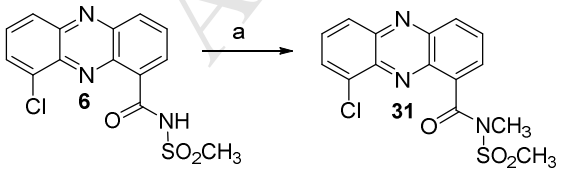
29a: R^1 = H
29b: R^1 = F
29c: R^1= CH_3
29d: R^1 = OCH_3

30a: R^1 = H, R = $CONHSO_2CH_3$
30b: R^1 = H, R = $CONCH_3SO_2CH_3$
30c: R^1 = F, R = CONHCN
30d: R^1 = F, R = $CONHSO_2CH_3$
30e: R^1= CH_3, R = $CONHSO_2CH_3$
30f: R^1 = OCH_3, R = CO_2CH_3

b

Reagents and conditions: (a) $CH_3SO_2NH_2$, CDI, Et_3N, DMAP, DMF; (b) CH_3I, K_2CO_3, DMF, rt; (c) NH_2CN, CDI, Et_3N, DMAP, CH_2Cl_2, DMF, 70 °C.

Scheme 6



Reagents and conditions: (a) CH_3I, K_2CO_3, DMF, rt;

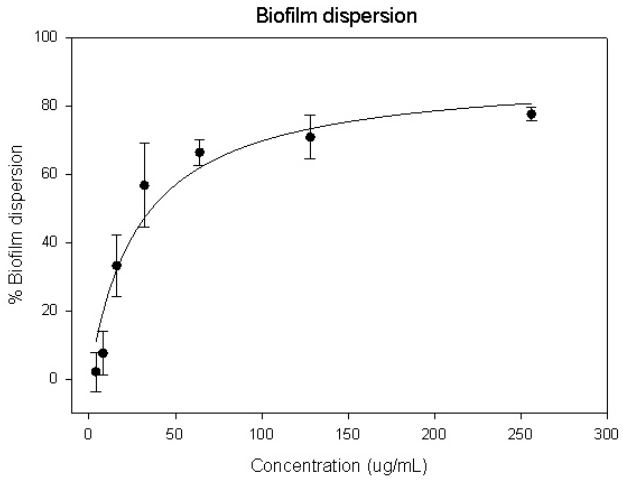


Figure 2. A dose response curve is presented for the compound **18c** against MRSA.

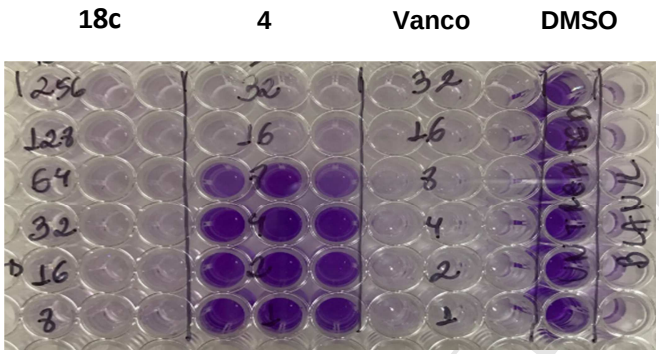


Figure 3. Biofilm inhibition assay for the compounds **18c**, **4** and Vancomycin.

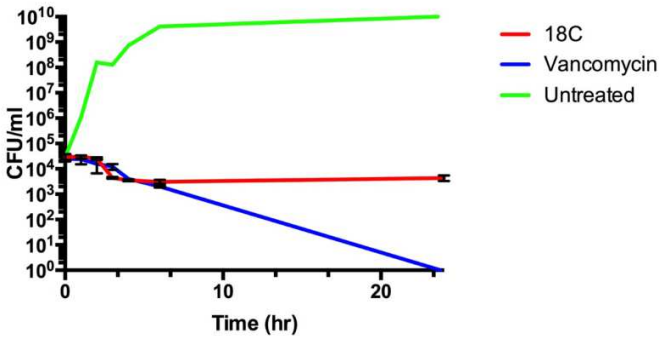


Figure 4. Mode of action of phenzine **18c**.

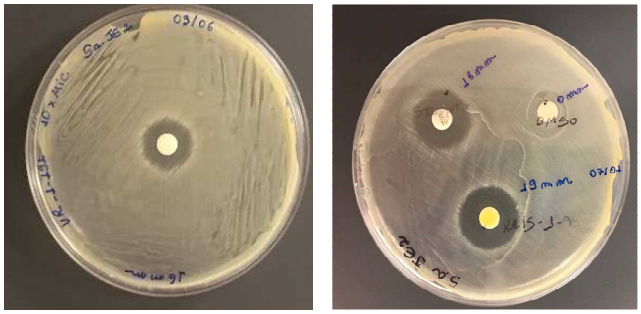


Figure 5. Mutant resistance studies of compound **4** (Left) and **18c** (Right): Compound **18c** (light yellow circle), streptomycin (control, white circle with inhibited clear zone on top left of the right figure)) and DMSO (white circle, no inhibited zone, top right of the same figure).

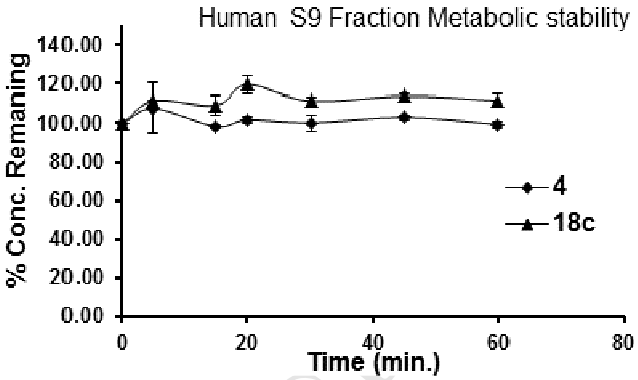


Figure 6. Time-dependent metabolic depletion of **4** and **18C** in human S9 fraction. Metabolic elimination profiles (% turnover or amount remaining vs. incubation time). Data shown as mean ± S.D (n#3)

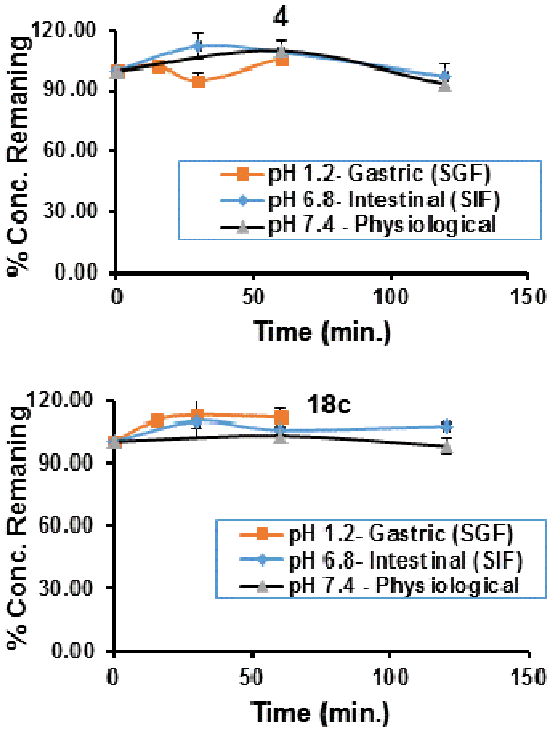


Figure 7. Stability of **4** (top) and **18c** (bottom) at three different pHs: 1.2, 6.8 and 7.4. Mean ± SD of triplicate determinations.

Highlights for review

- Synthesis of diverse phenazines that are active against gram (+) and gram (-) bacteria
- Active compounds inhibit biofilms formation
- Compounds are stable towards phase I and phase II enzymes