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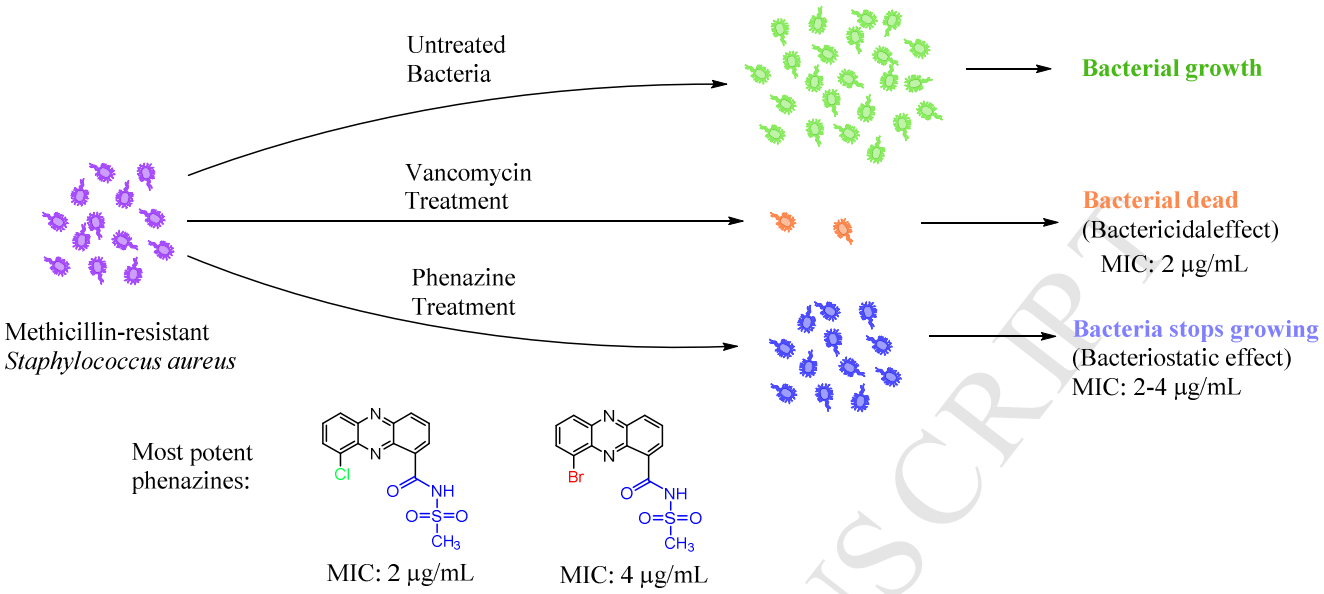

Most potent phenazines:

MIC: 2 µg/mL

MIC: 4 µg/mL

Simple Synthesis of Endophenazine G and Other Phenazines and Their Evaluation as Anti-Methicillin-Resistant Staphylococcus Aureus Agents

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1. Introduction

The number of bacterial infections continues to increase annually, and the emergence of resistance in most pathogens has rendered many clinically used antibiotics ineffective, causing a major health concern in humans.[1, 2] One such pathogen is methicillin-resistant S*taphylococcus aureus* (MRSA), which is listed as one of the ESKAPE pathogens. *S. aureus* infections used to occur more frequently in immunocompromised patients however, in recent years MRSA has become a serious threat to otherwise healthy individuals.[3, 4] It is estimated that half million Americans are hospitalized every year due to MRSA infections [5] accounting for thousands of deaths annually. The past decade has also seen the rise of vancomycin-resistant *S. aureus* strains (VRSA), one of the last resort antibiotics used for the treatment of many *S. aureus* infections. [6-8] It is clear that there is a critical need to find new antibiotics against this pathogen; however, the discovery of antibacterial drugs has seen a marked decline over the last 50 years.[9] Indeed, only seven new antibiotics have been approved by the FDA in the last decade.[10]

For centuries, scientists have looked to nature in order to identify molecules with antibacterial activity. An intriguing family of natural products known for its broad biological activities are the phenazines, secondary metabolites isolated mainly from *Pseudomonas* and *Streptomyces*.[11] Arguably, the most interesting characteristic of the phenazines are their antibacterial properties. Since the discovery of the phenazine antibiotic pyocyanin (**1**, Figure 1), many phenazines have been discovered and evaluated as antimicrobial agents.[11, 12] In recent years there has been a revival in the interest of phenazine antibiotics. Recent reports have introduced molecules such as *D*-Alanylgriseoluteic Acid [13] (**2**, Figure 1) and 2,4-dibromo-1-hydroxiphenazine[14] (**3**) with activities in the low μM range against bacteria such as *S. aureus*, *E. faecalis*, *E. coli*, and *Salmonella*.[13, 15-19] In particular, the work by the Huigens group has

yielded promising phenazines with intriguing activities against *MRSA* and other pathogens *in vitro* and *in vivo*.[15, 16, 20]

These reports prompted us to look further into phenazine natural products as anti-MRSA drugs. Our search led us to two natural products: endophenazine A1 (**4**) and endophenazine G (**5**), which were isolated from *Kitasatospora* (sp. HKI 714).[21] These phenazines are active against various pathogens including *P. aeruginosa, vancomycin-resistant Enterococcus faecalis (VRE), and MRSA*.[22] In this study, we report the first synthesis of endophenazine G (**5**) using an efficient route and a series of phenazine analogues. In addition, we describe the anti-MRSA activity of the synthesized compounds, basic tests to understand their mechanism of action, and computational studies to establish initial structure-activity relationships (SAR).

2. Results and Discussion

2.1.Chemistry. The synthesis of the natural product **5** is shown on Scheme 1. Briefly, we coupled 2-bromo-3-nitrobenzoic acid (**6**) with 2-bromoaniline (**7**) using Ullmann conditions.[15] The obtained intermediate **8** was cyclized under reductive conditions in basic media to afford phenazine **9**. Esterification of the compound provided molecule **10**, which was coupled with 1,1-dimethylallyl alcohol (**11**) using a Mizoroki−Heck reaction.[23] Hydrolysis of compound **12** provided the natural product endophenazine G in 88% yield. The double bond of **5** was further reduced in the presence of hydrogen using Pd-C as a catalyst to get analogue **13**.[24]

During preliminary antibacterial assays, it was found that precursor **9** was as active as the natural product **5** with minimum inhibitory concentrations (MIC) against MRSA of 32 μg/mL (Table 1). Therefore, we decided to explore the result of substitutions in both molecules, **5** and 9, to develop structure activity relationships (SAR). Using the same chemistry, the bromine atom of

9 was placed at different positions within the ring (**18** and **19**, Scheme 2), or replaced with a methyl group (**20** [25]), or chlorine atom (**21**)[15] to understand how electronic properties affect activity. Next, some of the compounds were esterified with methyl iodine under basic conditions (**22**,[26] **23** and **24**). Molecule **22** was reacted with the isoprenoid **11**, to provide an ester with a tail opposite to the ester group (**25**). Basic hydrolysis of the methyl ester provided the natural product **26**, which had been previously isolated from *Streptomyces cinnamonensis*.[27] The carboxylic acid was converted into a dimethylamide derivative, via an acyl chloride intermediate, and subjected to Heck coupling conditions to yield **27**.

To further understand the role of electronegativity and hydrogen bonding on antibacterial activity, the carboxylic acid was replaced by an *N*-(methylsulfonyl)amide bioisoster (**28**) using CDI and methane sulfonamide [28] or the electron withdrawing nitrile group (**29** and **31**), which was prepared by dehydration [29] of an amide [30] intermediate. Also, two secondary amine derivatives **30** and **32**, which do not have the ability to ionize or act as hydrogen bond donors, were prepared via an acyl chloride intermediate as shown in Scheme 3.[31] During preliminary tests, it was found the *N*-(methylsulfonyl)amide **28** presented promising activity (MIC 4 μg/mL, Table 1). Thus, analogue **33** (Scheme 3) was prepared from molecule **21**.[15] Our library was completed by substituting the bromide atom of molecule **9** with a fluorine atom (Scheme 4) providing analogue **35** [25] that was further functionalized to **37**.

2.2 Biological Evaluation. The biological activity of the synthesized compounds against a Community-Associated MRSA strain, LAC JE2, was evaluated by performing broth microdilution minimum inhibitory concentrations (MIC) assays and the results are shown in Table 1. Initially, we tried to understand the role of the alkyl moiety but found that **5** is more

active than analogue **26** (MIC of 32 μg/mL vs 128 μg/mL), which has an alkyl tail opposite to the carboxylic acid. Removing the unsaturation slightly decreases activity to 64 μg/mL (**5** vs **13**). Given that molecule **9** (bromine substitution) presented activity similar to the natural product; we decided to explore analogues of this molecule do to its ease of synthesis. It was found that moving the position of the Br increases MIC from 32 μg/mL (**9**) to 128 μg/mL (**18**) and 64 μg/mL (**19**). The Br was also exchanged by CH_3 and Cl giving analogues with MICs of 32 μg/mL (**20**), and 16 μg/mL (**21**), which validates our observation the alkyl chain is not needed for activity. The smaller substituents H (**35**) and F (**36**) both provide MICs of 64 μg/mL. The data suggest that an electron-withdrawing group at position *1* is not enough for activity as seen with primary amides (**36** and **38**), substituted amides (**27** and **32**), nitriles (**29** and **31**) and esters (i.e. **10**, **12**, and **37**). The best activity was observed with the *N*-(methylsulfonyl)amide derivatives **28** and **33**. Both exhibited low MIC values of 4 μg/mL and 2 μg/mL, respectively, comparable to that of vancomycin (2 μg/mL under our testing conditions).

The optimal anti-MRSA activity is obtained when the substituents are on the same side of the phenazine core. Placing substituents at other positions decreased activity. The alkyl tail is not key for activity although some bulkiness at position *9* is needed for good biological action. The results indicate that size is more important than electronics in this position as seen with **9**, **20**, **21**, **35**, **36**. This may indicate the activity is not linked to the ability of the phenazines to accept electrons. However, a larger library and computational studies are needed to confirm this observation. The result support the idea that electronegativity alone does not induce activity as seen with the esters and nitrile examples. It seems that electronegative-ionizable groups are key for the antibacterial activity of these molecules. It is worth to mention that the carboxylic acid and the *N*-(methylsulfonyl)amide are expected to have similar pKa values (around 4.5 in water).

2.3 Mechanism of action studies. Next, we decided to study the mechanism of action of the most active compound, **33**, and the natural product **5**. Although the phenazines can exert their biological effects by various mechanisms [13, 32-34] we focused in investigating membrane disruption or interference with redox cascades. We decided to study their ability to disrupt bacterial membranes. [18] because bacteria cannot (easily) alter their membrane composition. Thus, if the phenazine damage the membrane, they could be antibiotics that can overcome resistance. The other selection is based on the fact that interference with electron-flow and production of reactive oxygen species (ROS) [35] is the most common mechanism of action of the phenazines.

To assess whether our phenazines were disrupting the membrane, we monitored propidium iodide (PI) uptake of **5** and **33**. PI is a DNA intercalating agent that enters bacteria and binds to the chromosomal DNA only when its membrane is damaged. As shown in figure 2, MRSA treated with these compounds did not exhibit increased PI staining suggesting that the compounds were not disrupting the membrane.[36] In agreement with these data, confocal microscopy showed that compounds **5**, and **33** did not induce PI uptake on the MRSA strain (see SI Figure 1).

The next experiments sought to understand if interference with redox processes or generation of ROS were responsible for the antibacterial action. [13] Thus, we studied the interference with cellular redox cycling processes using an electron donor (NADPH) and an electron acceptor (cytochrome c, cyt-c).[13] Our experiments indicated a concentration-dependence on the rate of cyt-c reduction, a result that has been observed by the Bean and Bunz groups.[13, 18] However, compound **5**, which is less active than **33**, shows a higher rate of reduction. A similar result is observed with molecule **27** (see SI). It is assumed that cyt-c reduction occurs through a one-

electron transfer process.[13] Therefore, we tested the generation of ROS using the radical scavenger 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA).[13, 37] If radicals are present, the absorbance of the ABDA molecule would shift from the visible to the UV spectrum. The ABDA experiment did not indicate that radicals were generated. A single flow cytometry experiment using the ROS indicator hydroxyphenyl fluorescein (HPF)[38] also suggested that radicals were not generated (not shown). Thus, we concluded that even when the phenazines interfere with the redox processes, this mechanism does not (exclusively) lead to bacterial killing as observed by others.[13]

Given these results, we wondered whether our compounds had a static or cidal affect. Although pyocyanin (**1**) and other phenazine compounds have been reported to be bactericidal agents [11, 39], there have also been a few reports that indicate they could be bacteriostatic. [40, 41] Our results show that compounds **5** and **33** had a bacteriostatic effect on MRSA at 10X MIC concentrations (Figure 4), whereas the vancomycin control reduced the number of viable bacteria after 24 hrs (bactericidal compound). Thus, these phenazines are bacteriostatic in nature, in agreement with the lack of PI staining and ROS generation.

Finally, we studied the toxicity of some of the active phenazines against HaCat cells (immortal keratinocytes) to assess their potential as antibacterial drugs. The in vitro IC_{50} values for the compounds were 306 mM (molecule **5**), 345 mM (molecule **9**), 118 mM (**28**) and 193 mM (**33**). The obtained IC_{50} values coupled with the MIC results indicate that phenazines **5**, **9**, and **33** are promising leads for the development of MRSA drugs.

2.4 QSAR models of synthesized compounds. In order to develop a predictive model that can be used to guide future phenazine optimization we calculated physicochemical parameters, and

the linear analyses of the structures and their antimicrobial activities (Table 2). Three physicochemical parameters were included in each QSAR model. For the quantum chemical descriptors, such as the E_{HOMO} and E_{LUMO} energies, ΔE (the difference between E_{LUMO} and E_{HOMO} orbital energy), the dipole moments were first evaluated individually against ln (MIC). The results showed that only the E_{HOMO} energies were linearly correlated to the ln (MIC). Thus only the E_{HOMO} energies (eV) in the QM parameter set were included in the further QSAR studies.

The first QSAR model was based on the physicochemical parameters SMR (molecular refractivity, a description of the volume, or size of a compound), log S (solubility), and E_{HOMO} orbital energies (eV) following equation 1.

$$\ln(MIC) = 30.80 - 0.37 * SMR + 4.09 * E_{HOMO}(ev) - 0.35 * logS \quad \text{(Equation 1)}$$

$N = 16$, $R^2 = 0.63$, Pearson's Correlation $R = 0.79$, MAE= 0.65, RMSE = 0.71

Equation 1 suggested that the activities (ln (MIC)) was positively correlated with the increase of E_{HOMO} energies and negatively correlated with the increase of the logS and SMR, indicating that decreasing solubility and decreasing the size of molecules while increasing the E_{HOMO} energies would likely enhance the activities. The R^2 of 0.63 from the training set and the Q^2 of 0.40 from the test set, along with high Pearson's correlation (0.79), and small mean absolute error (0.65) and small root-mean-square (RMSE) error between the predicted and the observed activities suggested that predictability of model 1 (Figure 5).

We also used logP (a measurement of hydrophobicity), TPSA (topological polar surface area, a measurement of polarity), and the E_{HOMO} energies (eV) to develop a second QSAR model (model 2, Fig. 1, right).

$\ln(MIC) = 25.97 + 3.31 * E_{HOMO}(ev) - 0.03 * TPSA + 0.17 * logP$ (Equation 2)

$N = 16$, $R^2 = 0.68$, Pearson's Correlation $R = 0.82$, MAE= 0.60, RMSE = 0.67

Equation 2 suggested that the activities (ln (MIC)) were positively correlated with the increase of E_{HOMO} energies and the hydrophobicity (logP), but negatively correlated with the increase of the TPSA, indicating that decreasing hydrophilicity of molecules while increasing the HOMO energies and increasing the hydrophobicity of the molecules would likely lead to more potent compounds. The R^2 of 0.68 from the training set and the Q^2 of 0.73 from the test set, along with high Pearson's correlation (0.82), and small mean absolute error and small root-mean-square error (Table 3) between the predicted and the observed.

Though eq. 1 and eq. 2 used different sets of physicochemical parameters, the models supported each other. Table 4 showed that in both model 1 and 2, the Pearson's correlation (Pearson R) is very high for both training set, even for the test set, the Pearson R were pretty high, suggested strong linear relationship between the predicted activities and the observed activities. The mean absolute error and the root-mean-square errors in both models, both in training sets and in test sets were small, less than one unit of ln (MIC). Therefore, both models are predictive and thus can be used for ligand optimization. The QSAR study predicts that compounds with decreased hydrophobicity and E_{HOMO} energies, and increased water solubility may show better antibacterial activity.

3.Conclusions and perspectives

We are presenting, to the best of our knowledge, the first synthesis of a phenazine natural product endophenzine G (**5**) using a simple route that can be applied to the preparation of other endophenazines.[11] We have also identified two promising lead compounds containing an *N*-(methylsulfonyl)amide substituent with MICs comparable to vancomycin. Even when 7 phenazine-containing sulfonylamides are listed in Scifinder,[42] their preparation and biological activity has not yet been reported. Thus, this paper indicates the promising potential of phenazines containing this group. No membrane disruption was observed. Based in our experiments, it seems the phenazines interfere with redox cascades but those results do not correlate with the antibacterial activity. Our studies indicate the active phenazines are bacteriostatic. We also propose two simple QSAR models that can be used for the development of new phenazine antibacterials. Further directions will seek to increase the library for in-depth structure-activity-relationships and validation of the QSAR models, and further in-depth mechanistic studies.

4.Experimental Section

4.1. Chemistry

4.1.1. General Methods.

All reagents and solvents were obtained from commercial sources (Sigma-Aldrich, VWR or Fisher) and used without further purification. ^{1}H NMR spectra were recorded on a Bruker 500 MHz spectrometer. ^{1}H NMR chemical shifts are reported in ppm with the solvent as the internal standard ($CHCl_3$, 7.26 ppm). ^{13}C NMR chemical shifts are reported in ppm with the solvent as

the internal standard (DMSO-d6, 77 ppm). Compounds were analyzed for purity by HPLC. Purities of synthesized compounds were all found to be >95% by the following HPLC method: Agilent 1100 HPLC with VWD detectors, C18 column; Eluent: 20% water in methanol over 20 min; Flow rate 1.0 mL/min; Column temp. 25 °C; λ 254 nm. The Mass spectrometry (MS) was carried out on a Micromass Q-Tof Mass Spectrometer with an electrospray ionization source. Microscopy and flow cytometry were performed on a CARLZEISS LSM 710, Jena (Germany) and a BD LSR II. The melting points were measured with a EZ-Melt automated melting point apparatus from Stanford Research Systems.

4.1.2. Synthetic procedures:

4.1.3. **(E)-9-(3-Hydroxy-3-methylbut-1-en-1-yl)phenazine-1-carboxylic acid (5)**

To a solution of compound **12** (50 mg, 0.16 mmol) in THF-methanol (1:1, 10 mL) was added sodium hydroxide (25 mg, 0.62 mmol) dissolved in water (5 mL). The reaction mixture was heated to 40^{o} C for 2 hr, the solvent was concentrated to half its volume, and acidified to pH 2 with hydrochloric acid (1 N), the precipitate was filtered, and dried under vacuum to afford compound **5** as yellow solid (43 mg, 88%); mp: 151-153 ^{o}C. IR (KBr) 3448, 2953, 2917, 1695, 1155, 751 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 15.75 (s, 1 H), 8.98 (d, *J* = 6.5 Hz, 1 H), 8.54 (d, *J* = 7.5 Hz, 1 H), 8.26 (d, *J* = 8.5 Hz, 1 H), 8.09-8.04 (m, 2 H), 7.96 (t, *J* = 8 Hz, 1 H), 7.58 (d, *J* = 16 Hz, 1 H), 6.66 (d, *J* = 16 Hz, 1H), 1.58 (s, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$): 165.9, 144.1, 144.0, 143.1, 139.0, 138.0, 137.3, 135.6, 134.9, 131.7, 130.4, 129.1, 128.8, 124.9, 120.4, 71.3, 29.7; HRESIMS calcd for $C_{18}H_{16}N_2O_3$ 309.1234 (MH^+), found 309.0418 (MH^+). Anal. calcd for $C_{18}H_{16}N_2O_3$. 0.5 HCl: C, 66.20; H, 5.09; N, 8.58. Found: C, 66.15; H, 5.24; N, 8.31.

4.1.4. **2-((2-Bromophenyl)amino)-3-nitrobenzoic acid** (**8**)

To a solution of 2-bromo-3-nitrobenzoic acid (**6**, 2.0 g, 8.1 mmol) and 2-bromoaniline (**7**, 2.1g, 12 mmol) in ethanol (100 mL) was added copper powder (26 mg, 0.4 mmol), copper (I) bromide (117 mg, 0.81 mmol) and *N*-ethyl morpholine (2.0 mL, 16 mmol). The reaction mixture was heated to reflux for 16 hr, diluted with aqueous ammonium hydroxide solution (0.1 N, 25 mL) and filtered over celite. The filtrate was acidified to pH 2 using hydrochloric acid (1 N), and the solvent removed by filtration. The obtained yellow precipitate was dried under vacuum to afford 2-((2-bromophenyl)amino)-3-nitrobenzoic acid **8** as yellow solid (1.6 g, 60 %, yield). Which was used for the next step without further purification.

4.1.5. **9-Bromophenazine-1-carboxylic acid** (**9**)

To a solution of 2-((2-bromophenyl)amino)-3-nitrobenzoic acid **8** (1.6 g, 4.7 mmol) in aqueous sodium hydroxide (2 N, 100 mL) was added sodium borohydride (718 mmol, 19.0 mmol). The reaction mixture was heated to 100 ^{o} C for 16 hr, cooled to room temperature, and acidified to pH 2 using hydrochloric acid (1 N), the solid precipitate was filtered, washed with water (2 X 20 mL), and dried the under vacuum to afford 9-bromophenazine-1-carboxylic acid **9** as yellow solid (800 mg, 56%); mp: 314-316 ^{o}C. IR (KBr) 3034, 2905, 1716, 1503, 1428, 1245, 1206, 927 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.08 (d, J = 7,1 Hz, 1 H), 8.60 (dd, J_1 = 9.0 Hz J_2 = 1.5 Hz, 1 H), 8.37 (dd, J_1 = 8.5, J_2 = 7.5 Hz, 2H), 8.13 (t, J = 7.5 Hz, 1 H), 7.88 (t, J = 7.5 Hz, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): 165.5, 144.4, 143.8, 140.1, 138.2, 137.8, 135.7, 134.7, 131.8, 131.2, 129.8, 125.2, 122.6; HRESIMS calcd for $C_{13}H_7N_2O_2Br$ 302.9764 (MH^+), found 302.9136 (MH^+).

4.1.6. **Methyl 9-Bromophenazine-1-carboxylate** (**10**)

To a solution of compound **9** (800 mg, 2.62 mmol) in dimethylformamide (5 mL) was added potassium carbonate (1.8 g, 13 mmol) followed by methyl iodide (822 µL, 13.2 mmol).

The reaction mixture was stirred under nitrogen atmosphere at room temperature for 24 hr. The solvent was removed under vacuum, the obtained residue was diluted with dichloromethane (20 mL), the solid filtered off, the liquid filtrate collected. The solvent was removed under vacuum and the obtained residue purified by silica gel column chromatography using ethyl acetate-hexane (1:10 to 10:1) to afford methyl 9-bromophenazine-1-carboxylate **10** as yellow solid (590 mg, 1.83 mmol, 71 %); mp: 119-121 ^{o}C. IR (KBr) 2929, 2831, 1716, 1507, 1417, 1372, 1262, 1209, 1017 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): 8.40 (d, *J* = 7.5 Hz, 1 H), 8.33 (d, *J* = 7.0 Hz, 1 H), 8.25 (t, *J* = 9.5 Hz, 2 H), 7.94 (t, *J* = 7.0 Hz, 1 H), 7.75 (t, *J* = 7.0 Hz, 1 H), 4.17 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): 167.2, 143.7, 143.0, 141.0, 140.9, 134.0, 133.0, 132.8, 131.9, 131.2, 130.0, 129.4, 125.3, 52.8; HRESIMS calcd for $C_{14}H_9N_2O_2Br$ 316.9120 (MH^+), found 316.9113 (MH^+).

4.1.7. **Methyl (E)-9-(3-Hydroxy-3-methylbut-1-en-1-yl)phenazine-1-carboxylate (12)**

A solution compound **10** (100 mg, 0.315 mmol**)** in dimethylformamide (15 mL) was added to a thick-wall tube and the solution purge with nitrogen gas for 5 min. Then, in a sequential order were added palladium acetate (3.54 mg, 15,7 μmol), triphenyl phosphine (8.31 mg, 31,5 μmol), potassium carbonate (87.2 mg, 0.63 mmol) and a catalytic amount of tetrabutylammonium bromide. Nitrogen gas was bubbled into the solution for 5 minutes, followed by addition of 3-methyl-2-buten-2-ol (**11**, 66 μL, 0.63 mmol). The tube was sealed with a teflon cap and the reaction mixture heated to 110 ^{o} C for 16 hr. The solvent was removed, the residue was diluted with dichloromethane (20 mL), filtered through a celite pad, the liquid filtrated collected, the solvent removed under vacuum, and the crude product was purified by silica gel column chromatography (ethyl actetate1hexane, 1:1) to afford compound **12** as yellow solid (81 mg, 78%); mp: 142-144 ^{o}C. IR (KBr) 3476, 2949, 1658, 1523, 1417, 1278, 1119, 980,

751 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): 8.38 (d, *J* = 8.5 Hz, 1 H), 8.28 (d, *J* = 7.2 Hz, 1 H), 8.13 (d, *J* = 8.5 Hz, 1 H), 7.97 (d, *J* = 6.5 Hz, 1 H), 7.89-7.83 (m, 3 H), 7.10 (d, *J* = 16.5 Hz, 1 H), 4.14 (s, 3H), 1.59 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): 167.5, 143.4, 142.4, 141.7, 141.7, 139.9, 136.3, 133.2, 132.2, 131.7, 131.3, 129.3, 128.3, 127.0, 121.7, 71.5, 52.7, 29.8; HRESIMS calcd for $C_{19}H_{18}N_2O_3$ 323.3715 (MH^+), found 323.3656 (MH^+).

4.1.8. **9-(3-Hydroxy-3-methylbutyl)phenazine-1-carboxylic acid (13)**

Compound **5** (45 mg, 0.14 mmol) was dissolved in ethanol (5 mL) and reduced by catalytic hydrogenation (1 atm H_2 prssure) using 10% palladium on carbon to afford the title compound. The catalyst was filtered off over a celite pad, the celite was washed with dichloromethane, and the solvent was removed under vacuum. Compound **13** was isolated as a yellow solid (30 mg, 70%), mp: 258-260 ^{o}C. IR (KBr) 3469, 2953, 2921, 1699, 1458, 1254, 1193, 1127, 874, 759 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.01 (d, *J* = 7.0 Hz, 1 H), 8.57 (d, *J* = 8.5 Hz, 1 H), 8.24 (d, *J* = 8.5 Hz, 1 H), 8.07 (t, *J* = 7.0 Hz, 1 H), 7.95-7.90 (m, 2 H), 3.52-3.49 (m, 2 H), 2.05-2.02 (m, 2 H), 1.45 (s, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.0, 144.6, 143.0, 140.4, 139.1, 139.0, 137.2, 135.0, 132.2, 131.9, 130.2, 128.2, 124.8, 70.8, 44.5, 29.5, 26.8; HRESIMS calcd for $C_{18}H_{18}N_2O_3$ 311.1390 (MH^+), found 311.0725 (MH^+).

4.1.9. **6-Bromophenazine-1-carboxylic acid (18)**

To a solution of 2-bromo-3-nitrobenzoic acid (**6**, 1.01 g, 4.06 mmol) and 3-bromoaniline (**14**, 1.05 g, 6.10 mmol) in ethanol (50 mL) was added copper powder (13.0 mg, 0.20 mmol), copper (I) bromide (58.3 mg, 0.41 mmol) and *N*-ethyl morpholine (1.00 mL, 8.12 mmol). The reaction mixture was heated at reflux for 16 hr, diluted with aqueous ammonium hydroxide (0.1 N, 12 mL) and filtered over celite. The filtrate was acidified to pH 2 using hydrochloric acid (1 N) and the solvent concentrated to half. The formed yellow precipitate was filtered, and dried

under vacuum to afford 2-((3-bromophenyl)amino)-3-nitrobenzoic acid as yellow solid (800 mg, 60%), which was used without further purification.. To a solution of 2-((3-bromophenyl)amino)-3-nitrobenzoic acid (800 mg, 2.37 mmol) in aqueous sodium hydroxide (2 N, 50 mL) was added sodium borohydride (359 mg, 9.50 mmol), and the reaction mixture was heated to 100 ^{o} C for 12 hr. The reaction mixture was cooled to room temperature, and acidified to pH 2 using hydrochloric acid (1 N). The formed precipitated was filtered, washed with water (2 X 20 mL) and dried under vacuum to afford 6-bromophenazine-1-carboxylic acid, **18**, as yellow solid (450 mg, 62%); mp: 288-290 ^{o}C. IR (KBr) 1711, 1544, 1462, 1254, 996, 987, 751 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): 14.18 (s, 1 H), 8.56 (d, J = 8.5 Hz, 1 H), 8.52 (d, J = 7.0 Hz, 1 H), 8.46 (d, J = 7.0 Hz, 1 H), 8.41 (d, J = 8.5 Hz, 1 H), 8.14 (t, J = 8.5 Hz, 1 H), 7.97 (t, J = 8.5 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6): 166, 148, 146, 143, 142, 141, 137, 136, 135, 134, 132, 130, 123; HRESIMS calcd for $C_{13}H_7BrN_2O_2$ 304.9743 (MH^+), found 304.9072 (MH^+).

Compounds **19**,[15] **20**,[25] **and 21**[15] were prepared following a similar procedure to the one used for **17**. Their spectroscopic data matched the literature reported values.

4.1.10. **Methyl 6-Bromophenazine-1-carboxylate (22)**

To a solution of compound **18** (400 mg, 1.3 mmol) in dimethylformamide (5 mL) was added potassium carbonate (915 mg, 6.61 mmol) followed by methyl iodide (411 µL, 6.63 mmol) and the reaction mixture was stirred under a nitrogen atmosphere for 24 hr at room temperature. The solvent was removed under vacuum, the residue diluted with dichloromethane, the solid filtered off, and the filtrate collected. The solvent was removed under vacuum and the compound purified the product by silica gel column chromatography (dichloromethane) to afford methyl 6-bromophenazine-1-carboxylate **22** as a yellow solid (310 mg, 74%), mp: 314-315 ^{o}C. IR (KBr) 1714, 1258, 1213, 1123, 1024, 927, 845, 743 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.55

(d, *J* = 9.0 Hz, 1 H), 8.34 (d, *J* = 9.0 Hz, 1 H) 8.33 (d, *J* =3.0 Hz, 1 H), 8.24 (d, *J* = 7 Hz, 1 H), 7.93 (t, *J* = 8.5 Hz, 1 H), 7.75 (t, *J* = 8.0 Hz, 1H), 4.14 (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$): 165.4, 144.4, 143.7, 140.1, 138.1, 137.7, 135.7, 134.7, 131.7, 131.2, 129.7, 125.2, 122.6; HRESIMS calcd for $C_{14}H_9BrN_2O_2$ 318.9900 (MH^+), found 318.9417 (MH^+).

4.1.11. **Methyl 9-Methylphenazine-1-carboxylate (24)**:

To a solution of compound **20** (70 mg, 0.29 mmol) in DMF (10 mL) was added methyl iodide (93 µL, 1.5 mmol), followed by K_2CO_3 (285 mg, 2.05 mmol), and the reaction mixture was stirred at room temperature for 24 hr under nitrogen. After that, the solvent was removed under vacuum, the residue was dissolved in dichloromethane (25 mL), the solids were filtered off, the filtrate was collected, the solvent removed under vacuum, and the crude product was purified by silica gel column chromatography (dichlorometane) to obtain compound **24** as yellow solid (62 mg, 84%); mp: 214-216 ^{o}C. The spectroscopic data of compound **24** matched with the literature reported values.[43, 44] IR (KBr) 2909, 1699, 1520, 1421, 1270, 1184, 1066, 1013,743 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.38 (dd, J_1= 8.5 Hz, J_2 = 1.0 Hz, 1 H), 8.20 (dd, J_1 = 7.0 Hz, J_2 = 1.0 Hz, 1 H), 8.06 (d, *J* = 8.5 Hz, 1 H), 7.85 (t, *J* = 7.0 Hz, 1 H), 7.76 (t, *J* = 6.5 Hz, 1 H), 7.69 (bd, 1 H), 4.11 (s, 3 H), 2.92 (s, 3 H).

4.1.12. **Methyl (*E*)-6-(3-Hydroxy-3-methylbut-1-en-1-yl)phenazine-1-carboxylate (25)**

Compound **22** (100 mg, 0.32 mmol**)** was dissolved dimethylformamide (10 mL) and added to a high-pressure (thick wall) tube. The solution was purged with nitrogen for 5 min, and then were sequentially added: palladium acetate (3.54 mg, 0.016 mmol), triphenyl phosphine (8.33 mg, 0.032 mmol), potassium carbonate (87.2 mg, 0.630 mmol) and a catalytic amount of tetrabutylammonium bromide. Nitrogen gas was bubbled into the solution for another 5 minutes, followed by the addition of 3-methyl-2-buten-2-ol (**11**, 66 µL, 0.63 mmol). The test tube was

sealed with a teflon cap and the reaction mixture heated at 110 ^{o} C for 16 hr. The solvent was removed under vacuum, the residue diluted with dichloromethane (10 mL) and filtered through celite pad, the filtrated collected, the celite was washed with dichloromethane (10 mL), the solvent removed under vacuum, and the crude product purified by silica gel column chromatography (ethyl acetate-hexane, 1-3) to afford compound **25** as yellow solid (81 mg, 78%); mp: 117-119 ^{o}C. IR (KBr) 3346, 2958, 1711, 1523, 1442, 1262, 1041, 804, 755 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.44 (d, J = 8.5 Hz, 1 H), 8.24 (dd, J_1 = 7.0 Hz, J_2 = 4.5 Hz, 2 H), 8.00 (d, J = 8.0 Hz, 1 H), 7.96 (d, J = 17 Hz, 1 H), 7.84 (t, J = 7 Hz, 2 H), 6.81 (d, J = 16.5 Hz, 1 H), 4.13 (s, 3 H), 1.58 (s, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 167.1, 143.7, 142.0, 141.5, 140.8, 140.8, 136.0, 134.0, 132.2, 131.2, 130.9, 129.4, 128.7, 126.2, 121.3, 71.5, 52.7, 29.9; HRESIMS calcd for $C_{19}H_{18}N_2O_2$ 323.1390 (MH^+), found 323.0830 (MH^+).

4.1.13. **(*E*)-6-(3-Hydroxy-3-methylbut-1-en-1-yl)phenazine-1-carboxylic acid (26)**

To a solution of compound **25** (50 mg, 0.155 mmol) in THF-methanol (1:1, 10 mL) was added a solution of sodium hydroxide (25 mg, 0.62 mmol) in water (5 mL) and the reaction mixture was heated at 40 ^{o} C for 2 hr. The solvent was concentrated to half its volume and the solution acidified with hydrochloric acid (1 N) to pH 2. The obtained precipitate was filtered and dried under vacuum to afford compound **26** as a yellow solid (40 mg, 83%); mp: 146-148 ^{o}C. IR (KBr) 3484, 2986, 1695, 1401, 1270, 1148, 972, 902,759 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.98 (d, J = 6.0 Hz, 1 H), 8.58 (d, J = 9.0 Hz, 1 H), 8.15 (d, J = 8.5 Hz, 1 H), 8.12 (d, J = 7.0 Hz, 1 H), 8.05-7.98 (m, 3 H), 6.86 (d, J = 16.5 Hz, 1 H), 1.60 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.0, 142.5, 142.1, 142.0, 139.9, 139.8, 137.4, 136.9, 135.5, 133.2, 130.0, 126.7, 126.6, 124.8, 120.6, 71.6, 30.0; HRESIMS calcd for $C_{18}H_{16}N_2O_3$ 309.1194 (MH^+), found 308.9907 (MH^+).

4.1.14. **6-Bromo-*N*,*N*-Dimethylphenazine-1-carboxamide**

To a solution of compound **18** (100 mg, 0.32 mmol) in dichloromethane (10 mL) was added a catalytic amount of dimethylformamide (2 drops), the reaction mixture was cooled to 0 ^{o} C, and oxalyl chloride (110 µL, 1.29 mmol) was added dropwise at 0 ^{o} C under nitrogen atmosphere. After completion of the addition, the reaction mixture was stirred at room temperature for 16 hr, the volatiles were removed under vacuum, the acid chloride intermediate was dissolved in dry THF (5 mL), the reaction mixture cooled to 0 ^{o} C, and diethyl amine in THF (2 M, 1 mL) was added. The reaction mixture was stirred at room temperature for 6 hr, the volatiles were removed under vacuum, and the crude product was used for next step without further purification to prepare compound **27**.

*4.1.15.***(*E*)-6-(3-Hydroxy-3-methylbut-1-en-1-yl)-*N*,*N*-dimethylphenazine-1-carboxamide (27)**

6-Bromo-N,N-Dimethylphenazine-1-carboxamide (45 mg, 0.136 mmol**)** was dissolved in dimethylformamide (2 mL) and the solution transferred to a thick wall tube. To this solution were sequentially added; palladium acetate (1.5 mg, 6.8 µmol), triphenyl phosphine (3.6 mg, 14 µmol), potassium carbonate (37.6 mg, 0.272 mmol) and a catalytic amount of tetrabutylammonium bromide. Nitrogen gas was bubbled into the solution for 5 minutes, then 3-methyl-2-buten-2-ol **6** (28 µL, 0.272 mmol) was added and the tube sealed with a teflon cap. The reaction mixture was heated to 110 ^{o} C for 16 hr, the solvent removed, the residue diluted with dichloromethane (10 mL), filtered through celite pad, and the celite was washed with dichloromethae (10 mL). The filtrated was collected, the solvent removed under vacuum, and the crude product was purified by preparative TLC (dichloromethane) to afford compound **27** as yellow solid (32 mg, 70%); mp: 199-201 ^{o}C. IR (KBr) 1703, 1352, 1206, 519 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.31 (t, *J* = 5.0 Hz, 1 H), 8.14 (d, *J* = 8.5 Hz, 1 H), 7.96 (d, *J* = 16.5 Hz, 1

H), 7.93 (d, *J* = 7 Hz, 1 H), 7.83 (d, *J* = 5.0 Hz, 2H), 7.78 (t, *J* = 7.5 Hz, 1 H), 6.78 (d, *J* = 16.5 Hz, 1 H), 3.35 (s, 3 H), 2.81 (s, 3 H), 1.57 (s, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 169.4, 143.6, 142.0, 141.7, 140.8, 140.1, 137.2, 136.1, 131.1, 130.5, 129.6, 129.2, 128.3, 126.0, 121.3, 71.5, 38.8, 35.0, 30.0; HRESIMS calcd for $C_{20}H_{21}N_3O_2$ 366.1707 (MH^+) 358.1526 (MNa^+), found 336.0933 (MH^+) 358.0727 (MNa^+).

4.1.16. **General Procedure for the preparation Methanesulfonamide phenazines 28 and 33**. To a solution of the desired acid (0.13 mmol) in dimethylformamide (3 mL) was added carbonyl diimidazole (CDI, 26 mg, 0.16 mmol) and the reaction mixture was heated at 60 ^{o} C for 2 hr under nitrogen atmosphere. The reaction mixture was cooled to room temperature, methane sulfonamide (126 mg, 1.32 mmol) was added, followed by the addition of triethylamine (220 µL, 1.58 mmol) and a catalytic amount of dimethylaminopyridine (DMAP). After completion of the addition, the reaction mixture was stirred overnight at room temperature under nitrogen atmosphere. The solvent was removed under vacuum, and the residue was acidified using hydrochloric acid (1 N). The yellow precipitated was filtered and dried under vacuum. The crude product was purified by preparative TLC, using dichloromethane as mobile phase, to obtain the products as yellow solids.

4.1.16.A. **9-Bromo-*N*-(methylsulfonyl)phenazine-1-carboxamide (28)**. Yellow solid (21.1 mg, 43% yield); mp: 165-167 ^{o}C. IR (KBr) 2917, 1676, 1429, 1332, 1258, 1160, 751 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 13.97 (s, 1 H), 9.10 (q, J_1 = 7.5 Hz, J_2 =1.5 Hz,, 1 H), 8.59 (q, J_1 = 9.0 Hz, J_2 =1.5 Hz, 1 H) 8.33 (m, 2 H), 8.11 (dd, J_1 = 9.0 Hz, J_1 =1.5 Hz, 1 H), 7.85 (dd, *J* = 7.5 Hz, 1 H), 3.56 (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 163.3, 144.1, 143.7, 139.7, 138.8, 137.6, 135.6, 135.4, 131.8, 130.7, 129.5, 126.7, 123.7; HRESIMS calcd for $C_{14}H_{10}BrN_3O_3S$ 379.9699 (MH^+), found 379.8847 (MH^+).

4.1.16.B **9-Chloro-*N*-(methylsulfonyl)phenazine-1-carboxamide (33)**. See general procedure for the synthesis of **28** and **33**. Yellow crystals (20 mg, 46% yield); mp: 193-195 ^{o}C. IR (KBr) 2923, 2840, 1675, 1428, 1110, 760 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 14.12 (s, 1 H), 9.05 (d, J = 6.5 Hz, 1 H), 8.54 (d, *J* = 7.5 Hz, 1 H), 8.24 (d, *J* = 9.0 Hz, 1 H), 8.06 – 8.09 (m, 2 H), 7.87-7.90 (dd, J_1 = 7.5 Hz, J_2 =1.5 Hz, 1 H), 3.52 (s, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 163.2, 144.1, 143.5, 139.3, 137.7, 137.5, 135.6, 132.8, 131.7, 131.3, 130.8, 128.7, 126.6; HRESIMS calcd for $C_{14}H_{10}ClN_3O_3S$ 334.7545 (MH-), found 334.7024 (MH-).

4.1.17. **9-Bromophenazine-1-carbonitrile (29)**

To a solution of compound **9** (100 mg, 0.321 mmol) in dichloromethane (10 mL) was added a catalytic amount of dimethylformamide (2 drops). The reaction mixture was cooled to 0 ^{o} C, and oxalyl chloride (110 µL, 1.29 mmol) was added dropwise under nitrogen atmosphere. After completion of the addition, the reaction mixture was stirred at room temperature for 16 hr, and the volatiles were removed under vacuum. The obtained acid chloride was dissolved in dry dichloromethane (5 mL), the reaction mixture was cooled to 0 ^{o} C, aqueous ammonia (30%, 0.5 mL) was added dropwise, and the reaction mixture stirred for 4 hr. The solvent was removed under vacuum, the residue diluted with water (10 mL), the solid filtered, and dried under vacuum to afford **9-bromophenazine-1-carboxamide** as brownish yellow solid. Due to the difficulty of purification of the compound, we proceed to the next step. To the crude compound from the previous step (80 mg), was added thionyl chloride (10 mL), and the reaction mixture was heated to reflux for 12 hr. The thionyl chloride was removed under vacuum, and the crude nitrile compound was purified by silica gel column chromatography (ethyl acetate-hexane, 1:10 to 1:1) to afford the title compound as a yellow solid (41 mg, 53% yield over 2 steps); mp: < 400 ^{o}C. IR (KBr) 2222, 1508, 1487, 1413, 1277, 1119, 759 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.52 (d, *J*

= 8.5 Hz, 1 H), 8.37 (d, *J* = 7 Hz, 1 H), 8.30 (d, *J* = 7 Hz, 1 H), 8.26 (d, *J* = 8.5 Hz, 1 H), 7.97 (t, *J* = 7.0 Hz, 1 H), 7.81 (t, *J* = 8.5 Hz, 1 H); ^{13}C NMR (125 MHz, $CDCl_3$): 144.7, 142.6, 141.9, 141.3, 137.7, 135.1, 134.6, 131.9, 129.9, 129.5, 125.0, 115.8, 114.0; HRESIMS calcd for $C_{13}H_6BrN_3$ 284.9725 (MH^+), found 285.0769 (MH^+).

4.1.18. **9-Bromo-*N*,*N*-dimethylphenazine-1-carboxamide (30)**

To a solution of compound **9** (100 mg, 0.32 mmol) in dichloromethane (10 mL) was added a catalytic amount of dimethylformamide (2 drops), the reaction mixture was cooled to 0 ^{o} C, and oxalyl chloride (110 µL, 1.29 mmol) was added dropwise at 0 ^{o} C under nitrogen atmosphere. After completion of the addition, the reaction mixture was stirred at room temperature for 16 hr, the volatiles were removed under vacuum, the acid chloride intermediate was dissolved in dry THF (5 mL). The reaction mixture was cooled to 0 ^{o} C, and diethyl amine in THF (2 M, 1 mL) was slowly added. The reaction mixture was stirred at room temperature for 6 hr, the volatiles were removed under vacuum, and the crude product was purified by silica gel column chromatography (dichloromethane) to afford compound **30** as yellow solid (81 mg, 74%); mp: 148-150 ^{o}C. IR (KBr) 1617, 1274, 755 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.31 (d, *J* = 8.5 Hz, 1 H), 8.23 (d, *J* = 9.0 Hz, 1 H), 8.19 (d, *J* = 7 Hz, 1 H), 7.98 (d, *J* = 6.5 Hz, 1 H), 7.94 (t, *J* = 7.5 Hz, 1 H), 7.72 (t, *J* = 7.5 Hz, 1 H), 3.38 (s, 3 H), 2.90 (s, 3 H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 168.8, 143.9, 142.9, 140.6, 140.0, 137.8, 133.8, 131.1, 131.0, 130.3, 129.6, 129.5, 125.0, 39.2, 35.2; HRESIMS calcd for $C_{15}H_{12}BrN_3O$ 332.0216 (MH^+), found 331.9575 (MH^+).

4.1.19. **(*E*)-9-(3-Hydroxy-3-methylbut-1-en-1-yl)phenazine-1-carbonitrile (31)**

To a solution compound **29** (45 mg, 0.16 mmol**)** in dimethylformamide (5 mL) placed in a test tube were sequentially added: palladium acetate (1.78 mg, 8 µmol), triphenyl phosphine (4.2 mg, 16 µmol), potassium carbonate (44 mg, 32 µmol) and a catalytic amount of

tetrabutylammonium bromide. Nitrogen gas was bubbled into the solution for 5 minutes, compound **29** was added, the test tube sealed with a teflon cap, and the reaction mixture heated to 110 ^{o} C for 16 hr. The solvent was removed, the residue diluted with dichloromethane (20 mL), the mixture filtered through celite pad, the pat was washed with dichloromethane (10 mL), the filtrated collected, and the solvent removed under vacuum. The crude product was purified by silica gel column chromatography to afford compound **31** as yellow solid (19 mg, 42%); mp: 152-154 ^{o}C. IR (KBr) 2970, 2905, 2210, 1515, 759 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.51 (d, *J* = 8.5 Hz, 1 H), 8.32 (d, *J* = 7.0 Hz, 1 H), 8.18 (d, *J* = 8.5 Hz, 1 H), 8.04 (d, *J* = 6.5 Hz, 1 H), 7.95-7.91 (m, 2 H), 7.79 (d, *J* = 16.5 Hz, 1 H), 7.35 (d, *J* = 16 Hz, 1 H), 1.60 (s, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$): 144.5, 143.3, 142.1, 141.9, 141.1, 136.7, 136.3, 134.7, 132.0, 129.2, 128.6, 128.3, 121.5, 116.3, 113.9, 71.6, 29.8; HRESIMS calcd for $C_{18}H_{15}N_3O$ 312.1107 (MNa^+), found 312.0390 (MNa^+).

*4.1.20.***(*E*)-9-(3-Hydroxy-3-methylbut-1-en-1-yl)-*N*,*N*-dimethylphenazine-1-carboxamide (32)**

To a solution compound **31** (55 mg, 0.166 mmol**)** in dimethylformamide taken in a test tube were sequentially added palladium acetate (1.86 mg, 0.0083 mmol), triphenyl phosphine (4.4 mg, 17 μmol), potassium carbonate (46 mg, 33 μmol) and a catalytic amount of tetrabutylammonium bromide. Nitrogen gas was bubbled into the solution for 5 minutes, then compound **6** (53 μL, 0.5 mmol) was added, the test tube sealed with a teflon cap, and the reaction mixture heated to 110 ^{o} C for 16 hr. The solvent was removed under vacuum, the residue diluted with dichloromethane (10 mL), filtered through celite pad, the filtrated collected, the solvent was removed under vacuum, and the crude product was purified by preparative TLC (dichloromethane) to afford compound **32** as yellow solid (33 mg, 59%); mp: 149-151 ^{o}C. IR

(KBr) 3344, 2917, 1609, 1409, 1257, 1151, 910, 766 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.23 (d, *J* = 8 Hz, 1 H), 8.11 (d, *J* = 9.0 Hz, 1 H), 7.92-7.88 (m, 4H), 7.80 (d, *J* = 15.5 Hz, 1 H), 6.80 (d, *J* = 16 Hz, 1H), 3.36 (s, 3 H), 2.83 (s, 3 H), 1.54 (s, 6 H); ^{13}C NMR (125 MHz, $CDCl_3$): 169.3, 143.6, 142.5, 141.4, 141.0, 139.1, 137.5, 136.2, 131.0, 130.4, 130.2, 128.6, 128.4, 126.0, 121.4, 71.3, 39.0, 35.1, 30.0, 29.7; HRESIMS calcd for $C_{20}H_{21}N_3O_2$ 336.4145 (MH^+) 358.1526 (MNa^+), found 336.0933 (MH^+) 358.0635 (MNa^+).

4.1.21. **9-Fluorophenazine-1-carboxylic acid (35)** [25]

To a solution of 2-bromo-3-nitrobenzoic acid (**6**, 1.01 g, 4.06 mmol) and 2,6-difluoroaniline (**34**, 1.05 g, 6.10 mmol) in methanol (50 mL) was added copper powder (13.0 mg, 0.20 mmol), copper (I) bromide (58.3 mg, 0.41 mmol) and *N*-ethyl morpholine (1.00 mL, 8.12 mmol). The reaction mixture was heated at reflux for 16 hr, diluted with aqueous ammonium hydroxide (0.1 N, 12 mL) and filtered over celite. The solvent concentrated to half its volume under vacuum and then acidified to pH 2 using hydrochloric acid (1 N). The formed yellow precipitate was filtered, and dried under vacuum to afford 2-((2,6-difluorophenyl)amino)-3-nitrobenzoic acid as yellow solid (800 mg, 60%). To a solution of 2-((2,6-difluorophenyl)amino)-3-nitrobenzoic acid (650 mg, 2.21 mmol) in aqueous sodium hydroxide (2 N, 50 mL) was added sodium borohydride (336 mg, 8.84 mmol), and the reaction mixture was heated to reflux for 1 hr. The reaction mixture was cooled to room temperature, and acidified to pH 2 using hydrochloric acid (1 N). The formed precipitated was filtered, washed with water (2 X 20 mL) and dried under vacuum to afford 9-fluorophenazine-1-carboxylic acid **35**, as yellow solid (224 mg, 31%); mp: 233-235 ^{o}C. IR (KBr) 3047, 1728, 1515, 1458, 1246, 894, 763 cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ 15.17 (s, 1 H), 9.02 (d, *J* = 7.0 Hz, 1 H), 8.54 (d, *J* = 9.0 Hz, 1 H), 8.16 (d, *J* = 9.0 Hz, 1 H), 8.09 (t, *J* = 9 Hz, 1 H), 7.95-7.91 (m, 1 H), 7.69 (t, *J* = 8.5 Hz, 1 H),

^{13}C NMR (125 MHz, $CDCl_3$): 165.4, 144.4, 143.8, 138.1, 135.0, 131.2, 130.8, 130.8, 125.9, 125.8, 125.3, 115.5, 115.3; ^{19}F NMR (470 MHz, $CDCl_3$): δ -123.7; HRESIMS calcd for $C_{13}H_7N_2O_2F$ 242.0492 (MH^+), found 242.9999 (MH^+)

4.1.22. **9-Fluorophenazine-1-carboxamide (36)**

Compound **35** (121 mg, 0.50 mmol) was dissolved in dichloromethane (10 mL), the reaction mixture was cooled to 0 ^{o} C, and oxalyl chloride (110 µL, 1.29 mmol) was added dropwise under nitrogen atmosphere. Then, a catalytic amount of dimethylformamide (2 drops) was added. After completion of the addition, the reaction mixture was stirred at room temperature for 16 hr, the volatiles were removed under vacuum. The obtained acid chloride was dissolved in dichloromethane (5 mL), the reaction mixture cooled to 0 ^{o} C, aqueous ammonia (30%, 0.5 mL) was added dropwise, and the reaction mixture was stirred for 4 hr. The solvent was removed under vacuum, and the compound purified by silica gel column chromatography. The title compound was obtained as an orange solid (87 mg, 0.36 mmol, 72% yield); mp: 270-272 ^{o}C. IR (KBr) 3354, 1668, 1580, 1410, 1067, 755 cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6): δ 9.68 (brs, 1 H), 8.73 (d, J = 7Hz, 1 H), 8.45 (d, J = 9Hz, 1 H), 8.18 (brs, 1 H), 8.13 (t, J = 7 Hz, 2 H), 8.02 (q, J = 7.5 Hz, 1 H), 7.89 (t, J = 9.5 Hz, 1 H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 165.8, 157.4, 155.3, 143.7, 143.6, 140.1, 135.3, 133.6, 133.1, 133.0, 131.7, 131.4, 131.3, 126.0, 115.1, 115.0; ^{19}F NMR (470 MHz, $CDCl_3$): δ -125.6 HRESIMS calcd for $C_{13}H_8FN_3O$ 242.0724 (MH^+) 264.0544 (MNa^+), found 242.0189 (MH^+) 263.9966 (MNa^+).

Compounds **37- 39**[15, 26] were prepared according to literature procedures and their data was identical to published reports.

4.2. Biology

4.2.1. Minimum Inhibitory Concentration Assays

Broth microdilution assays were performed as described in the third edition of the ASM Clinical Microbiology Procedures Handbook. Briefly, a stock solution of the compound at pH 7 was prepared and then serial 2-fold dilutions were made in Muller Hinton II broth, cation-adjusted, CAMHB, (Becton, Dickinson and Company, Sparks, MD), containing 5% DMSO in Costar 96-well microtiter plates (Corning, Kennebunk, ME). Bacterial cultures were prepared using the direct colony suspension method to 0.5 McFarland units and each well was inoculated with 10 ul. Plates were incubated statically at 37˚C for 24 h. MIC values are reported as the lowest concentration of antibiotic at which no growth was observed. Vancomycin was used as positive control and media was used as negative control.

4.2.2. Killing Assays

Killing assays were performed by diluting an overnight culture 1:1000 into fresh CAMHB. After two hours of growth at 37˚C with agitation, 1-ml aliquots were removed and treated with 10X MIC of the compounds. Samples were removed every two hours and bacterial viability was quantified by serial dilution and plating on Tryptic Soy Agar (TSA).

4.2.3. Membrane disruption experiments

Staphylococcus aureus JE2 was resuspended in PBS to an OD_{600} of 1.0 and then treated with 1X, 2X or 10X MIC of compounds **5** and **33**. 200 ul of each sample was placed into wells of a black 96-well microtiter plate (Corning Inc, Corning, NY), and the relative fluorescent units (RFU) was measured in 15 min intervals for 4 hr in a Tecan Infinite 200 PRO plate reader (Tecan Trading AG, Switzerland).

4.2.4. Rate of reduction of cytochrome-c

This procedure was based on previous literature precedents[18] and modified accordingly by addition of DMSO and 0.1 M NaOH to increase solubility of the tested compounds. A

solution of DMSO and NaOH was prepared with 95% DMSO and 5% NaOH (0.1 M) by volume. Each compound was then dissolved in the DMSO/NaOH solution to make a 10 mM stock solution. A 200 µM solution of each compound was prepared by diluting the aforementioned solution with Dulbecco's Phosphate-Buffered Saline (DPBS, 1 X). In order to maintain an overall 5% DMSO/NaOH to 95% DPBS ratio, the needed volume of DMSO/NaOH solution was added. The solutions were then serially diluted (100 µM, 50 µM, 25 µM). To the dilutions containing, which were placed in a 96-well plate, was added NADPH (100 µM) followed by with cytochrome-c derived from equine heart (20 µM). The extinction of cyt-c (peak at 550nm) was monitored over time using a photospectrometer for a period of 900 s at 30 s intervals. As a reference for baseline correction, the extinction coefficient at 700 nm (a wavelength were neither the phenazine or cyt-c absorbs) was also monitored in order to account for lap-signal fluctuations.[18] Solutions containing only cytochrome-c (20 µM) in 95% DPBS-5% DMSO/NaOH buffer, cyt-c (20 µM) and NAPDH (100 µM) in PBS/DMSO/NaOH buffer, and 95% DPBS-5% DMSO/NaOH were used as controls.

4.2.5. Superoxide scavenging activity

The generation of superoxides was monitored by using (9,10- Anthracenediyl-bis(methylene)dimalonic acid, ABDA). ABDA has an absorbance shift upon reaction with superoxides in solution. Therefore, the decay of the ABDA was measured at 479 nm and monitored over the period of 500 s at 30 s intervals using a photospectrometer. We followed the exact same protocol of Hayden and co-workers, “The extinction at 600 nm was also plotted and used as a reference baseline to account for any fluctuations in the lamp signal, as neither the phenazine compounds nor ABDA has any extinction at this wavelength. These measurements were performed at 3 concentrations for each compound (0.11, 0.22, 0.33, 0.44, and 0.55 mM)

with three repeats per concentration. ABDA (5 μM) and NADPH (0.01 mM) concentrations were maintained constant throughout"[18]. The only difference was in the sample preparation. A stock solution of DMSO and NaOH was prepared with 95% DMSO and 5% NaOH (0.1 M) by volume. Each compound was then dissolved in the DMSO/NaOH solution to make a 10 mM solution. The solutions were serially diluted while re-adding the DMSO/NaOH solution after each serial dilution to maintain a 5% DMSO/NaOH concentration. In a 96-well plate, solutions containing the experimental compounds (0 mM, 0.11 mM, 0.22 mM, 0.33 mM, 0.44 mM, 0.55 mM) and NADPH (100 μM) were injected with ABDA (5 μM, final concentration) and monitored for ADBA signal extinction. Solutions containing ABDA (5 μM) in 95% DPBS-5% DMSO/NaOH buffer and 95% DPBS-5% DMSO/NaOH were used as controls.

4.2.6. Cytotoxicity experiments

Selected phenazine samples (~1.3 mg) were dissolved in 100 μL of a 2:3 mixture of NaOH (0.01 M) and DMSO. The samples were diluted with Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS and 1% penicillin streptomycin to a final concentration of 4 mM (3% final DMSO concentration). Then, an aliquot of this stock solution was taken and serial dilutions performed using DMEM buffer. Cytotoxicity was then performed using the celltiter blue assay[45] and following the providers' protocols (promega).[46] Cell death was measured by observing the change if absorbance of resazurin using a spectrophotometer. The results were plotted as Absorbance vs phenazine concentration and the IC_{50} (concentration that kills 50% of the cell population) calculated.

4.3. Computational Methods

To generate the physicochemical parameters, we built the 3D models of all compounds in MOE. The physicochemical parameters such as logP, TPSA, SMR, and logS were calculated in

MOE. The E_{HOMO} and E_{LUMO} energies (in eV) were calculated using Gaussian09 after each compound was energetically minimized using the density functional theory (DFT) method B3LYP at the 6-31G(d) level of theory. All minimized structures were further subject to frequency calculations which showed no negative frequencies, an indication of a true minimum being found. After all physicochemical parameters being assigned, all compounds were randomly divided into a training set and a test set. The activity data were converted to ln (MIC). Correlations between selected physicochemical parameters and the ln (MIC) were established using the Partial Least Square Fit (PLS) method. To evaluate the QSAR models, the resulting QSAR models were used to predict the activity data of the compounds in the test set.

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ABBREVIATIONS

Cyt-c: Cytochrome c; HPF: Hydroxyphenyl fluorescein; LogP: Partition coefficient; MIC:

Minimum inhibitory concentration; MRSA: Methicillin-resistant S*taphylococcus aureus*; ROS:

Reactive oxygen species; SAR: Structure-activity relationships.

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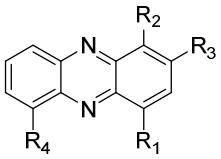
TABLES

Table 1. Antimicrobial activity of the compounds



#	R_1	R_2	R_3	R_4	MIC (μg/mL)
5	$HOC(CH_3)_2(CH)_2$	H	H	CO_2H	32
9	Br	H	H	CO_2H	32
10	Br	H	H	CO_2CH_3	128
12	$HOC(CH_3)_2(CH)_2$	H	H	CO_2CH_3	128
13	$HOC(CH_3)_2(CH_2)_2$	H	H	CO_2H	64
18	H	Br	H	CO_2H	128
19	H	H	Br	CO_2H	64
20	CH_3	H	H	CO_2H	32
21	Cl	H	H	CO_2H	16
22	H	Br	H	CO_2CH_3	>128
24	CH_3	H	H	CO_2CH_3	64
25	H	$HOC(CH_3)_2(CH)_2$	H	CO_2CH_3	128
26	H	$HOC(CH_3)_2(CH)_2$	H	CO_2H	128
27	H	$HOC(CH_3)_2(CH)_2$	H	$CON(CH_3)_2$	>128
28	Br	H	H	$CONHSO_2CH_3$	4
29	Br	H	H	CN	>128
30	Br	H	H	$CON(CH_3)_2$	>128
31	$HOC(CH_3)_2(CH)_2$	H	H	CN	128
32	$HOC(CH_3)_2(CH)_2$	H	H	$CON(CH_3)_2$	>128
33	Cl	H	H	$CONHSO_2CH_3$	2
35	F	H	H	CO_2H	64
36	F	H	H	CO_2NH_2	>128
37 [15, 26]	H	H	H	CO_2CH_3	128
38 [15]	H	H	H	CO_2NH_2	64
39 [15]	H	H	H	CO_2H	64
Vancomycin **(Control)**					2

The MIC is the highest value of four independent runs

Table 2. Physicochemical Properties of the Synthesized Compounds in the Training Set.

Compd	MIC	logP	TPSA	SMR	Homo(ev)	logS	PRED_M1	PRED_M2	ln (MIC)
5	32	3.59	83.31	8.93	-5.878	-3.40	4.60	4.40	3.47
9	32	3.40	63.08	7.17	-6.252	-3.56	3.78	3.79	3.47
13	64	3.48	83.31	8.86	-6.224	-3.16	3.12	3.23	4.16
18	128	3.40	63.08	7.17	-6.296	-3.56	3.60	3.65	4.56
21	16	3.19	63.08	6.90	-6.310	-3.20	3.51	3.56	2.77
22	128	2.90	63.08	6.87	-6.120	-2.63	4.10	4.14	3.47
24	64	3.16	52.08	7.31	-6.109	-3.04	4.13	4.59	4.16
26	128	3.59	83.31	8.93	-5.931	-3.40	4.38	4.22	4.85
28	4	2.58	89.02	8.71	-6.604	-3.96	1.91	1.64	1.39
30	128	3.86	72.31	9.37	-5.895	-3.81	4.51	4.75	4.85
31	128	3.58	69.80	8.71	-6.032	-3.78	4.19	4.33	4.85
33	2	2.37	89.02	8.44	-6.671	-3.60	1.61	1.39	0.69
37	16	2.82	61.31	7.49	-6.476	-2.93	2.53	3.02	2.77
38	64	1.87	68.87	6.56	-6.358	-2.75	3.29	3.00	4.16
39	64	2.60	63.08	6.40	-6.272	-2.47	3.60	3.59	4.16

PRED_M1: predicted ln (MIC) based on the model 1 (Equation 1); PRED_M2: predicted lm (MIC) based on the

Table 3. Physicochemical Properties of the Synthesized Compounds in the Test Set.

Compd	MIC	logP	TPSA	SMR	Homo(ev)	logS	PRED_M1	PRED_M2	ln (MIC)
10	128	3.66	52.08	7.61	-6.271	-3.97	3.68	4.13	4.85
12	128	3.86	72.31	9.37	-5.835	-3.81	4.76	4.94	4.85
35	64	2.76	63.08	6.40	-6.262	-2.76	3.75	3.65	4.16
37	128	2.87	52.08	6.84	-6.230	-2.88	3.76	4.14	4.72
19	64	3.44	63.08	7.17	-6.429	-3.56	3.05	3.21	4.16

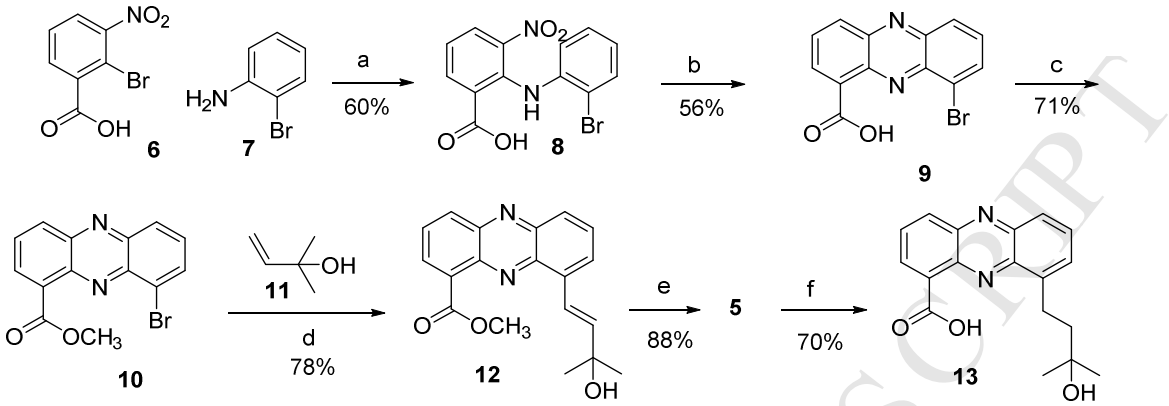
PRED_M1: predicted ln (MIC) based on the model 1; PRED_M2: predicted lm (MIC) based on the model 2

Table 4. QSAR Development of Model 1 and 2.

Model	Pearson R	R^2	MAE	RMSE
1				
Training	0.79	0.63	0.65	0.71
Test	0.63	0.40	0.75	0.86
2				
Training	0.82	0.68	0.60	0.67
Test	0.85	0.73	0.57	0.63

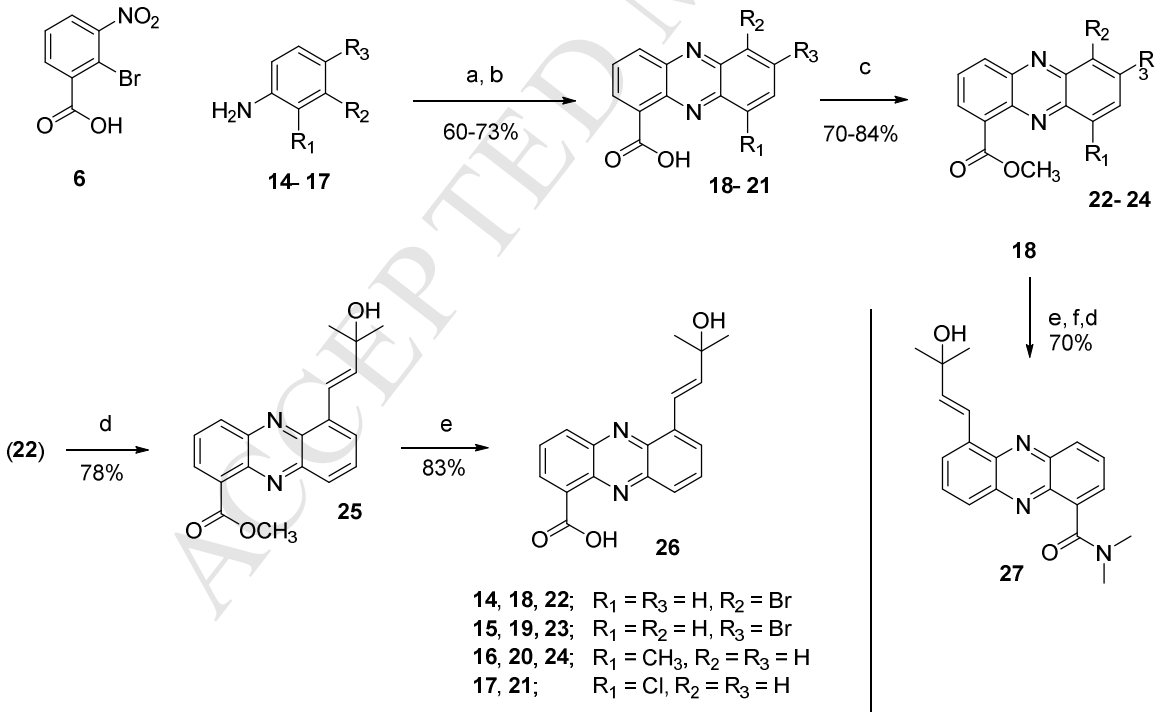
SCHEMES

Scheme 1. Synthesis of Endophenazine A1



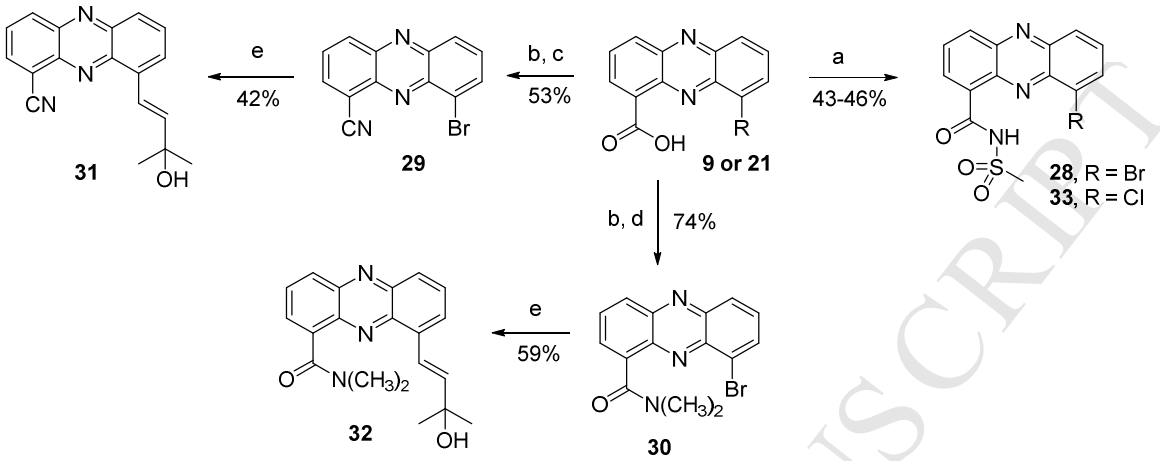
Reagents and Conditions: (a) Cu, CuBr, *N*-Et-morpholine, EtOH, reflux; (b) NaOH, $NaBH_4$, reflux; (c) MeI, K_2CO_3, DMF; (d) $Pd(OAc)_2$, PPh_3, K_2CO_3, TBA-Br (cat.), DMF, 110 ^oC; (e) NaOH, H_2O, THF, MeOH; (f) H_2, Pd-C, EtOH.

Scheme 2. Synthesis of natural phenazine **26** and other analogues



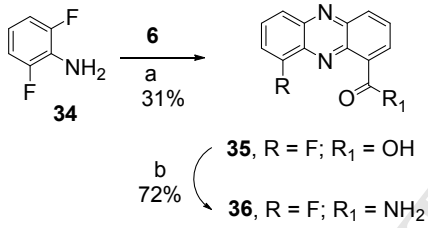
Reagents and Conditions: (a) Cu, CuBr, N-ethylmorpholine, EtOH, reflux; (b) NaOH, $NaBH_4$, reflux; (c) MeI, K_2CO_3, DMF; (d) **11**, $Pd(OAc)_2$, PPh_3, K_2CO_3, TBA-Br (cat.), DMF, 110 ^oC; (e) NaOH, H_2O, THF, MeOH; (f) i. $(COCl)_2$, DMF, DCM; ii. $NH(CH_3)_2$, Et_3N, 0 ^oC.

Scheme 3. Preparation of derivatives of **5** and **9**



Reagents and Conditions: (a) i. CDI, heat, ii. $CH_3SO_2NH_2$, Et_3N, DMAP, DMF; (b) $(COCl)_2$, DMF, DCM; (c) i. NH_3 (aq), ii. $SOCl_2$, reflux; (d) $NH(CH_3)_2$, Et_3N, THF, 0 ^oC; (e) **11**, PPh_3, DMF, $Pd(OAc)_2$, TBA-Br, K_2CO_3.

Scheme 4. Synthesis of fluoro-containing phenazines and by-products



Reagents and Conditions: (a) Cu, CuBr, MeOH, *N*-Et-morpholine, reflux; (b) i. $(COCl)_2$, DMF, DCM; ii. NH_3 (aq).

FIGURES

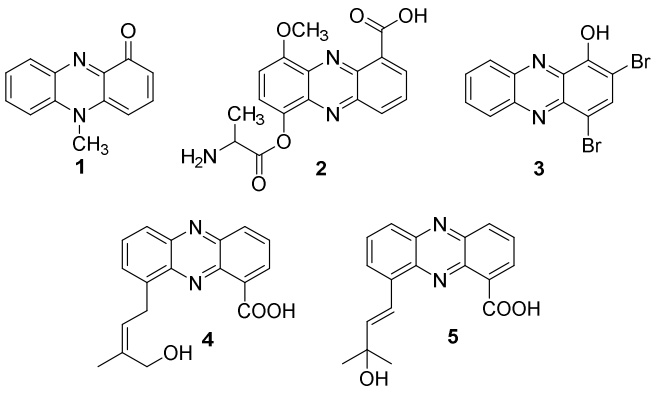


Figure 1. Phenazine natural products with antibacterial activity

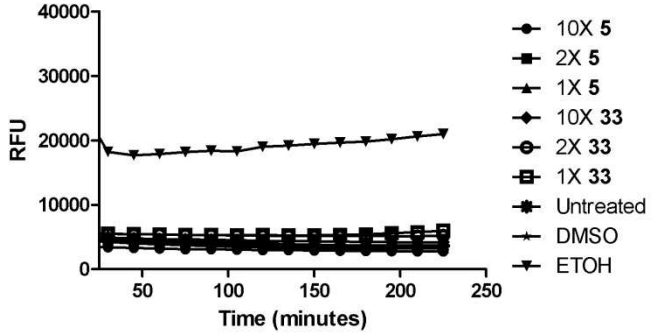


Figure 2. PI penetration upon treatment with molecules **5** and **33** at various concentrations

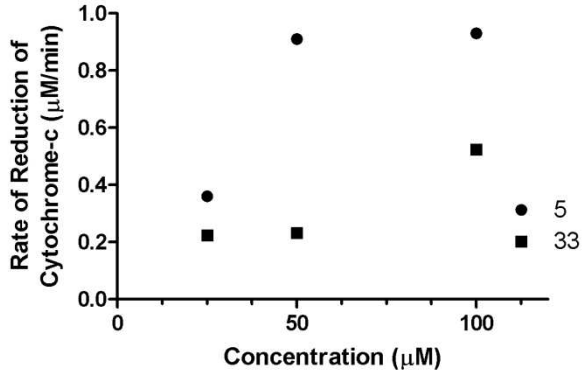


Figure 3. Effect of phenazines **5** and **33** concentration on cyt-c activity

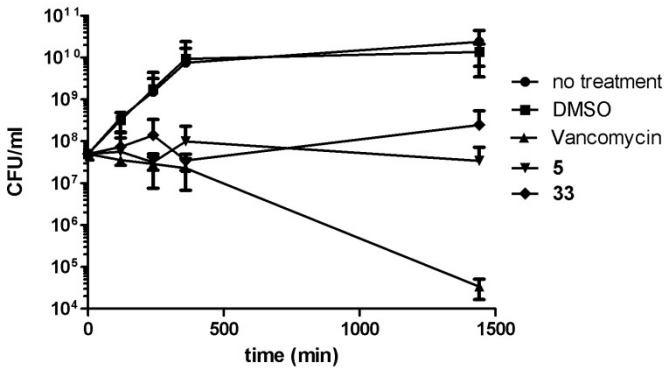


Figure 4. Phenazine **5** and **33** mode of action.

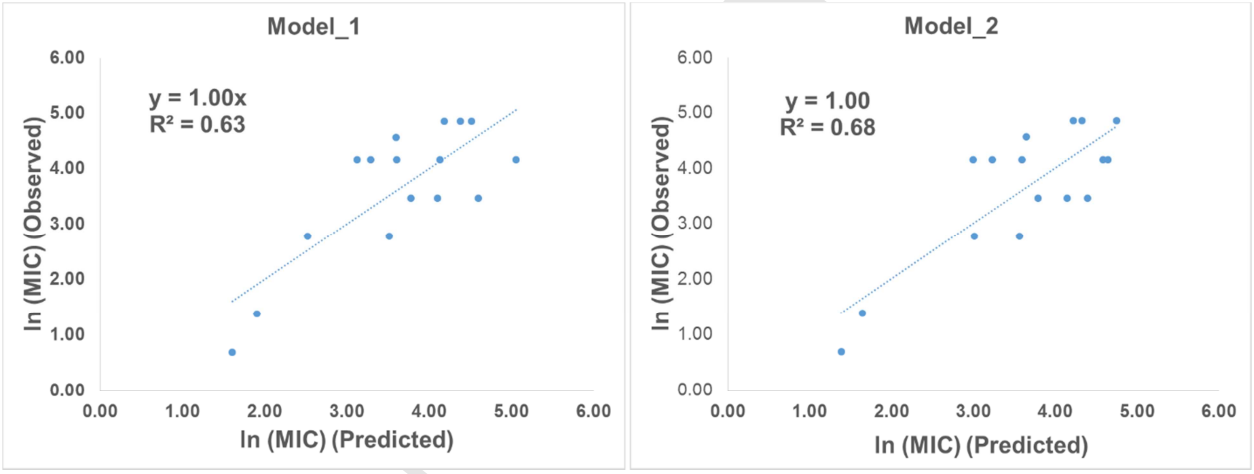


Figure 5. Plots of the predicted versus observed activities (ln (MIC)) for model 1 (left) and model 2 (right).

Highlights for review

- Simple total synthesis of the ntural product Endophenazine G
- Synthesis of new *N*-(methylsulfonyl)phenazines
- The *N*-(methylsulfonyl)phenazines display low MIC (2 and 4 μg/mL) values against MRSA
- The active phenazines are bacteriostatic agents