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Design, Synthesis and Biological Evaluation of Antimicrobial Diarylimine and –amine Compounds Targeting the Interaction between the Bacterial NusB and NusE Proteins

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ABSTRACT: Discovery of antimicrobial agents with a novel model of action is in urgent need for the clinical management of multidrug-resistant bacterial infections. Recently, we reported the identification of a first-in-class bacterial ribosomal RNA synthesis inhibitor, which interrupted the interaction between the bacterial transcription factor NusB and NusE. In this study, a series of diaryl derivatives were rationally designed and synthesized based on the previously established pharmacophore model. Inhibitory activity against the NusB-NusE binding, circular dichroism of compound treated NusB, antimicrobial activity, cytotoxicity, hemolytic property and cell permeability using Caco-2 cells were measured. Structure-activity relationship and quantitative structure–activity relationship were also concluded and discussed. Some of the derivatives demonstrated improved antimicrobial activity than the hit compound against a panel of clinically important pathogens, lowering the minimum inhibition concentration to 1-2 μg/mL against *Staphylococcus aureus*, including clinical strains of methicillin-resistant *Staphylococcus aureus* at a level comparable to some of the marketed

antibiotics. Given the improved antimicrobial activity, specific inhibition of target protein-protein interaction and promising pharmacokinetic properties without significant cytotoxicity, this series of diaryl compounds have high potentials and deserve for further studies towards a new class of antimicrobial agents in the future.

Keywords: antimicrobial activity, diarylimine, diarylamine, protein-protein interaction, inhibitor, methicillin-resistant *Staphylococcus aureus*

1. Introduction

Antibiotic-resistant *Staphylococcus aureus* such as methicillin-resistant *S. aureus* (MRSA) strains re-emerged to become problematic causes of severe infections. Bacterial transcription is a valid but underutilized target for antibiotic discovery [1]. We previously discovered inhibitors interrupting the protein-protein interaction (PPI) between the core enzyme in bacterial transcription, RNA polymerase and the initiation factor sigma [2,3]. Recently, we reported a first-in-class inhibitor as a hit compound identified by *in silico* screening of a pharmacophore model designed based on the crystal structures of two bacterial proteins, NusB and NusE [4]. These two proteins are essential and highly conserved transcription factors specifically involved in the bacterial ribosomal RNA (rRNA) transcription. By examining the protein-protein interaction (PPI) interface of the crystal structure of *Escherichia coli* NusB-NusE complex [5], the interactions between NusB Glu81 and NusE His15, NusB Tyr18 and NusE Asp19, NusB Glu75 and NusE Arg16, and NusB Leu22 and NusE Val26 (*E. coli* amino acid residue numbering) were chosen to design the pharmacophore model (Figure 1), followed by *in silico* screening [4]. One of the short-listed compounds, (*E*)-2-{[(3-ethynylphenyl)imino]methyl}-4-nitrophenol (**1**) was able to specifically inhibit the NusB-

NusE interaction, decrease the level of bacterial rRNA and demonstrate antimicrobial activities with a minimum inhibition concentration (MIC) at 8 μg/mL against an antibiotic susceptibility control strain *S. aureus* ATCC 25923, as well as a number of clinical MRSA strains (MIC 16-64 μg/mL) without significant cytotoxicity against human A549 and HaCaT cell lines. It was the first time the NusB-NusE interaction has been used for inhibitor discovery to identify a small molecule with promising antimicrobial activities and cytotoxicities as a hit compound. Herein we extend our study to the structure-activity relationship (SAR) of this compound for comprehensive understanding of the inhibitory mechanism, and the possibility of further development as antimicrobial drug candidates.

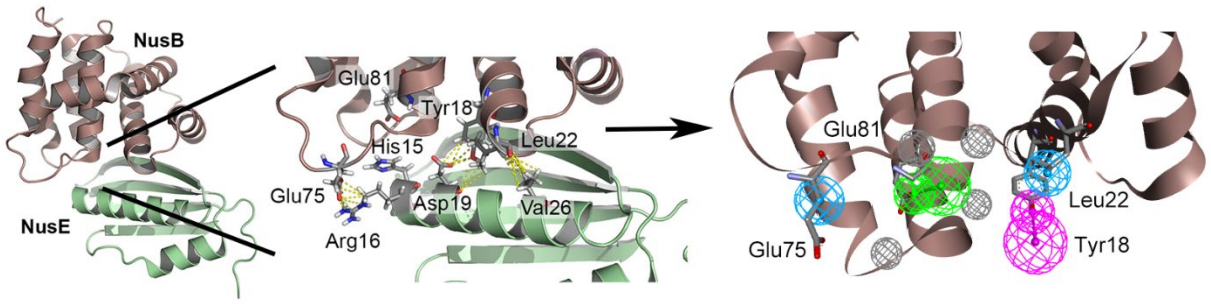


Figure 1. Structure of the *E. coli* NusB-NusE co-crystal complex (left; PDB: 3D3B), conserved interactions across bacterial species at the interface (middle) and pharmacophore model construction (right).

2. Chemistry

2.1 Derivative Design

The bioactive hit compound, **1** has a relatively low molecular weight of 263 and a specific conjugated diphenyl imine system. As predicted by the pharmacophore model, **1** falls into a basin-like surface of NusB, which is a hydrophobic semi-pocket (Figure 2, left). As indicated in Figure 2, the Glu75 residue of NusB forms a hydrophobic interaction with the ethynyl group of **1**, Glu81 bonds to the phenolic hydroxyl group of **1** by hydrogen bond, and the nitro group of **1** accepts a hydrogen bond donation from Tyr18 of NusB.

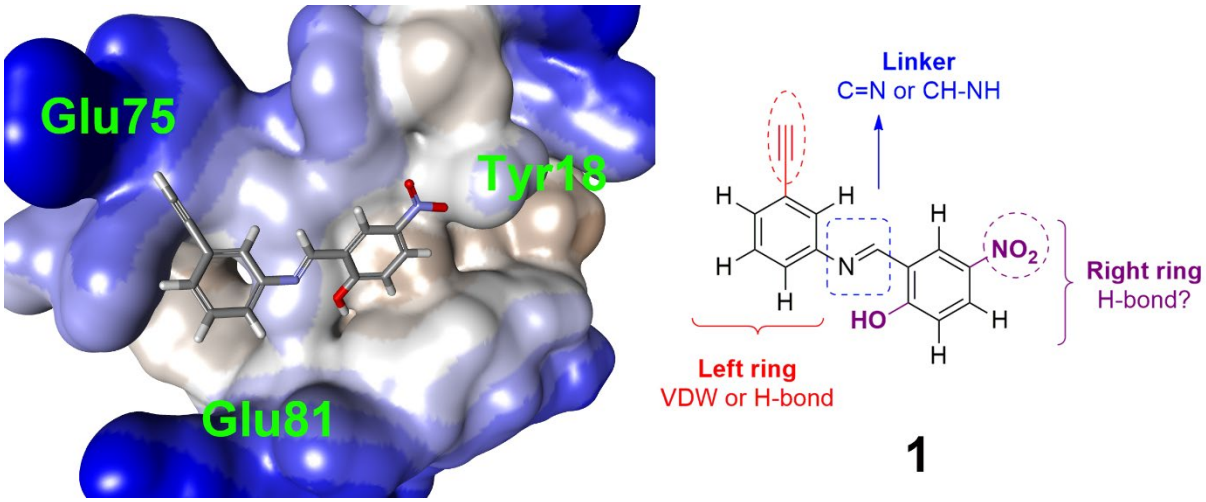


Figure 2. The docking model showing the interaction of the hit compound **1** with *E. coli* NusB (PDB:3D3B) with the hydrophilicity of NusB surface is colored in blue (left) and the modification plan of the hit compound **1 (**right).

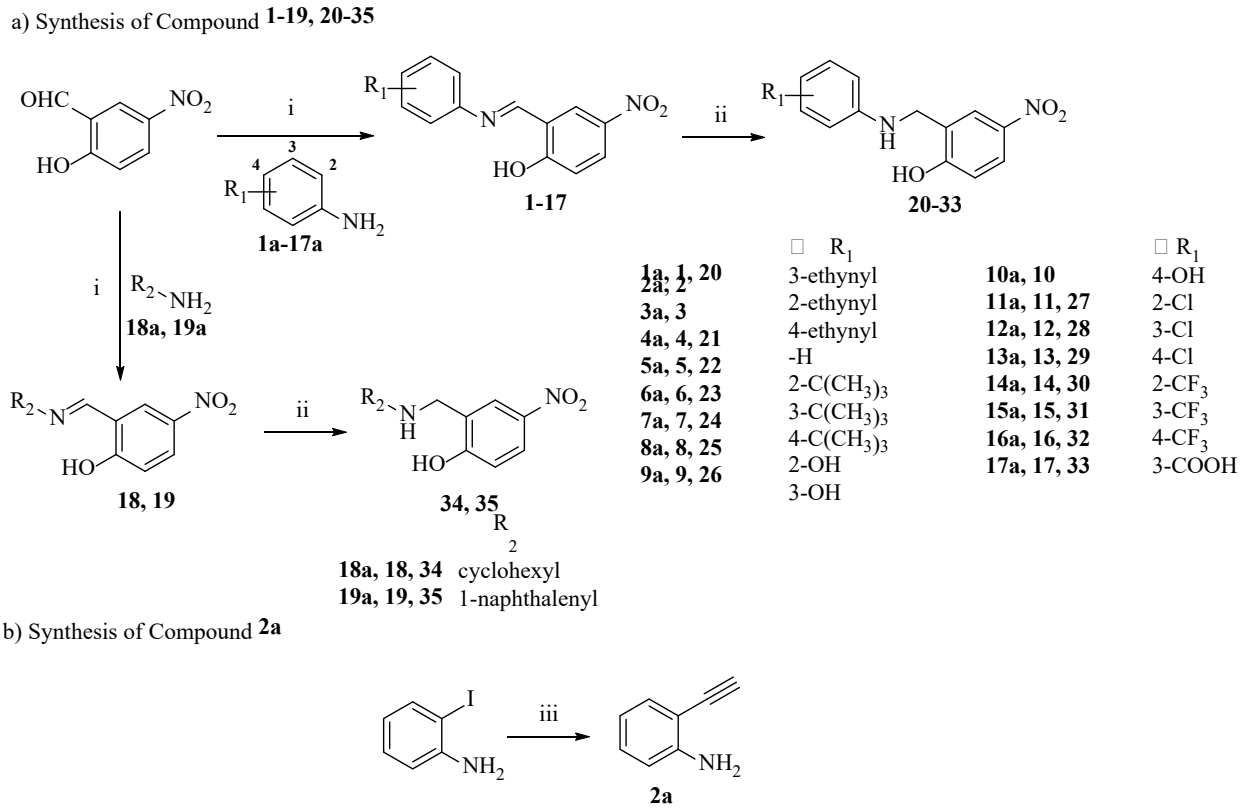
To examine the pharmacophore model and study the SAR, we planned to modify first the left and right benzene rings of the hit compound **1** (Figure 2, right). Various substituents such as alkyl, hydroxyl, chloride, trifluoromethyl have been used to replace ethynyl on the left benzene ring of the hit compound **1** to test the suitability for hydrophobic interaction with Glu75 of NusB.

New analogues with or without the nitro group, hydroxyl of phenol, masked hydroxyl by methoxy, replacement of nitro by other hydrogen bond accepters, electron-withdrawing or –donating groups to alter the pKa of phenol have been tested to confirm the important hydrogen bonds between right benzene ring of **1** with Glu81 and Tyr18 of NusB.

The imine linker of the hit compounds has also been reduced to amine to study how the relative position of two benzene rings of hit compounds affects the NusB-NusE interaction and thus the inhibitory activity against bacteria (Figure 2). As the flexibility of imine and amine linkers are distinct, while bond lengths are also slightly different, we intended to examine whether the entropy penalty played an important role in protein target affinity and antimicrobial activity because of the flexible diaryl system with an amine linker.

2.2 Synthesis

Synthesis of the left ring modified derivatives **1-35** is shown in Scheme 1. Reaction of amines or substituted aniline **1-19a** with 5-nitrosalicylaldehyde in ethanol at room temperature afforded Schiff base type derivatives **1-19** in 60-93% yields. Reductive amination of the above derivatives using sodium triacetoxyborohydride in dichloromethane gave the corresponding amines **20-35** in 86-94% yields. The intermediate **2a** was not commercially available, so we prepared it by a Sonogashira coupling reaction from 2-iodoaniline in 59% yield [6].



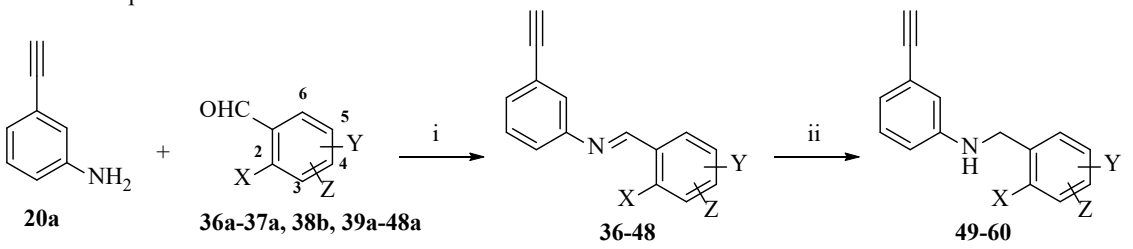
Reagents and conditions: (i) EtOH, rt, overnight, 60-93%; (ii) $NaBH(OAc)_3$, DCM, rt, overnight, 84-94%; (iii) $PdCl_2(PPh_3)_2$, CuI, trimethylsilylacetylene, Et_3N, rt., 6h; then K_2CO_3, MeOH, 1h, 59% in two steps.

Scheme **1**. Synthesis of a) the target compounds **1-19**, **20-35** and b) intermediate **2a**

Synthesis of the right ring modified derivatives **36-60** was performed following the same synthetic method of imine formation and reductive amination as outlined in Scheme 2. Regarding the commercially unavailable substituted benzaldehydes as reaction partners, a modified Casnati-Skattebøl ortho-formylation furnished aldehyde intermediates **36a**, **39a** and **40a** in 52-78% yields [7]. Although **39a** was obtained as a 7:3 mixture of 2-(3-formyl-4-hydroxyphenyl)acetonitrile and the starting material 4-hydroxybenzyl cyanide, as determined by ^{1}H NMR analysis, this mixture could be used directly in the subsequent reaction step, as 4-hydroxybenzyl cyanide would not participate in the imine formation reaction and could be then easily removed through filtration. Nucleophilic aromatic substitution of 2-fluorobenzaldehydes

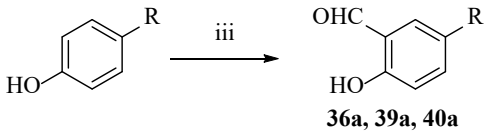
using sodium methoxide in methanol provided benzaldehydes **38a** and **43a** in 85-88% yields. Demethylation of compound **38a** with boron tribromide in dichloromethane provided cyanobenzaldehyde **38b** in 71% yield [8]. In summary, Schiff base type derivatives **36-48** and amines **49-60** were synthesized in 65-87% and 86-94% yields, respectively.

a) Synthesis of compound **36-48, 49-60**



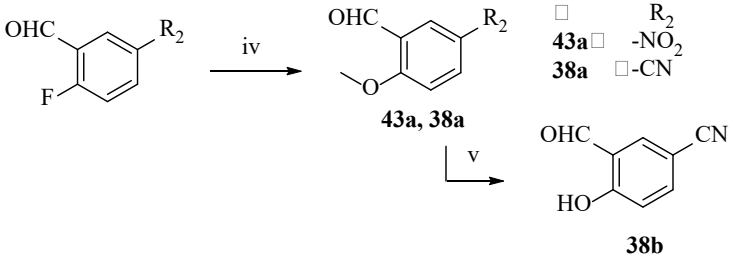
	X	Y	Z		X	Y	Z
36a, 36, 49	-OH	5-$COOCH_3$	-H	**43a, 43, 56**	-OCH_3	5-NO_2	-H
37a, 37, 50	-OH	5-F	-H	**44a, 44, 57**	-OH	4-NO_2	-H
38b, 38, 51	-OH	5-CN	-H	**45a, 45, 58**	-OH	3-NO_2	-H
39a, 39, 52	-OH	5-CH_2CN	-H	**46a, 46**	-H	6-NO_2	3-OH
40a, 40, 53	-OH	5-OCH_3	-H	**47a, 47, 59**	-OH	5-NO_2	3-Br
41a, 41, 54	-OH	-H	-H	**48a, 48, 60**	-OH	5-Cl	3-Cl
42a, 42, 55	-H	5-NO_2	-H				

b) Synthesis of compound **36a**, **39a**, **40a**



	R
36a	-$COOCH_3$
39a	-CH_2CN
40a	-OCH_3

c) Synthesis of compound **38b**, **43a**



	R_2
43a	-NO_2
38a	-CN

Reagents and conditions: (i) EtOH, rt, overnight, 65-87%; (ii) $NaBH(OAc)_3$, DCM, rt, overnight, 86-94%; (iii) paraformaldehyde, $MgCl_2$, Et_3N, THF, reflux, 12h, 52-78%; (iv) CH_3ONa, MeOH, rt, 6h, 85% for **43a**, 88% for **38a**; (v) BBr_3, DCM, rt, 10h, 71%.

Scheme **2**. Synthesis of a) the target compound **36-48**, **49-60**; b) intermediates **36a**, **39a**, **40a**; c) intermediates **38b**, **43a**.

3. Results and Discussion

3.1 Inhibitory activity against the NusB-NusE interaction.

A biochemical inhibitory assay for testing the capacity of disrupting the NusB-NusE PPI has been developed using the luciferase complementation technique [9]. The NusB and NusE proteins were each fused to one of the luciferase complementation fragment and their interaction resulted in reformation of the natural luciferase. Binding of NusB to the inhibitor prevented its interaction with NusE, therefore caused decreased luminescence released from complemented luciferase catalyzed reaction. As the percentage inhibition at a single concentration correlated perfectly with the IC_{50} value [9], we maintained the previous testing conditions of compounds at 125 μM and displayed the percentage of inhibition values in Tables 1 and 2.

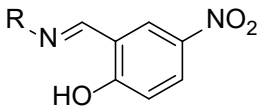
Table 1 includes the testing results of compounds **1**-**19** with modifications of the left ethynyl benzene ring from the hit compound, and the corresponding analogues **20**-**35** containing an amine bond reduced from the imine group. As shown in Table 1, the modifications of the left ethynyl benzene ring were able to affect the inhibitory activity against the NusB-NusE PPI with the percentage inhibition values varied from 16.8% ± 0.7% (compound **5**) to 96.3% ± 0.6% (compound **9**) compared to the hit compound **1** at 85.3% ± 1.5%. Compound **5** contains a *t*-butyl group at 2-position related to imine, which seems too bulky for binding to NusB, and compound 9 has a 3-hydroxyl group as highly preferred. Other derivatives with relatively high inhibitory values includes compound **2** with 2-ethynyl (71.4% ± 1.8%), compound **3** with 4-ethynyl (76.3% ± 3.0%), compound **10** with 4-hydroxyl (89.1% ± 1.1%), and compound **17** with 3-carboxylic acid (78.5% ± 2.6%). In summary, the substitution of the left benzene ring

is necessary as most of the substituted compounds demonstrated improved inhibitory activities compared to compound **4** with an unsubstituted benzene ring (39.2% ± 3.0%). Additionally, our pharmacophore docking model indicated the binding of the substituents to Glu75 of NusB played an important role in the inhibition, and the binding type belongs to the hydrophobic interaction with the residue chain of Glu75. Effectively, the percentage inhibition values suggested that the inhibitory activity could be enhanced regardless hydrophobic or hydrophilic substituents, which correlates the hydrophobic interaction dominate the binding of Glu75 to our compounds using the aliphatic chain. By summarizing the relative positions of substitutions on the left benzene ring to the inhibitory activities, it is suggested that the 3-position was preferred for *t*-butyl, hydroxyl, chloride, ethynyl, and 4-position for CF_3.

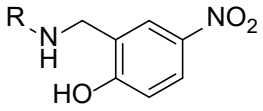
The inhibitory activity of amines **20**-**35** obtained from reduction of imines **1**-**19** were then subjected to the testing. As the molecular conformation of **1**-**19** was released from a quasi-plane to a freely rotatable structure in **20**-**35**, we can draw such a prediction that some compounds may express improved inhibitory activities because of flexible structures promoting better binding affinity with NusB, such as compound **22**. This compound containing a *t*-butyl group at 2-position on the left benzene ring has a significant improvement on percentage inhibition at 72.2% ± 2.7% from its imine analogue **5** at 16.8% ± 0.7% as the imine with the lowest activity in Table 1. By turning the rigid imine to flexible amine, the bulky *t*-butyl group at the 2-position proved to adjust the conformation to accommodate this steric hindrance and displayed appropriate hydrophobic interaction with the amino acid residue of NusB. Another case is compound **25** with an increased percentage inhibition at 81.5% ± 4.0%, compared to the imine analogue **8** at 57.7% ± 2.5%. In the contrast, other compounds display similar or slightly weakened inhibitory activities due to entropy penalty. A special case is amine

compound **21** with an unsubstituted benzene ring, which lost completely the protein affinity to NusB with a percentage inhibition at 4.6% ± 3.4% compared to the imine analogue **4**.

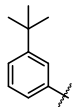
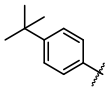
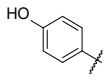
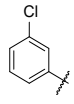
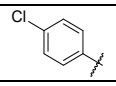
Table 1. Inhibitory and antimicrobial activities of compounds **1-35**

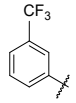
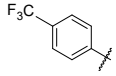
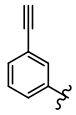
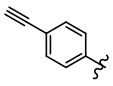
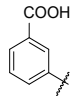


1-19



20-35

Compound	R	MIC	% Inhibition[a]	ClogP
1		4	68.3 ± 2.2	3.87
20		>256	58.2 ± 5.9	3.20
2		4	43.4 ± 3.0	4.22
21		4	47.4 ± 2.8	3.93
3		4	39.2 ± 3.0	3.05
22		16	4.6 ± 3.4	2.75
4		4	16.8 ± 0.7	4.88
23		1	72.2 ± 2.7	4.58
5		4	51.3 ± 1.5	4.88
24		2	39.5 ± 2.7	4.58
6		4	38.8 ± 2.5	4.88
25		2	10.8 ± 5.4	4.58
7		4	57.7 ± 2.5	2.38
26		16	81.5 ± 4.0	2.08
8		16	96.3 ± 0.6	2.38
27		64	66.7 ± 3.5	2.08
9		4	89.1 ± 1.1	2.38
10		2	45.7 ± 2.2	3.93
28		2	31.0 ± 9.2	3.77
11		2	66.5 ± 0.7	3.93
29		4	34.9 ± 5.8	3.77
12		8	50.0 ± 3.8	3.93
30		8	53.6 ± 2.0	3.77
13		8	53.1 ± 2.9	4.23

31		2	61.5 ± 4.0	4.16
14		16	56.2 ± 2.7	4.23
32		4	61.7 ± 3.7	4.16
15		8	68.9 ± 2.1	4.23
33		8	54.1 ± 3.6	4.16
16		4	71.4 ± 1.8	3.32
17		8	85.3 ± 1.5	3.32
34		16	47.7 ± 7.9	3.02
18		4	76.3 ± 3.0	3.32
19		4	78.5 ± 2.6	2.99
35		>256	50.8 ± 3.0	2.84

[a] % Inhibition displayed the percentage of inhibition on the NusB-NusE interaction using the previously described conditions [9]: the compound was used at 125 μM in the NusB-NusE (1:1) solution at 1 μM in PBS buffer.

The imine compounds **36**-**48** with modified right benzene rings and the corresponding amine analogues **49**-**60** have been tested and results were shown in Table 2. In this study, we focused on the modification of hydroxyl and nitro groups. Regarding the hit compound **1** with a percentage inhibition value of 85.3% ± 1.5%, both hydroxyl and nitro groups of **1** seemed necessary as compound **41** without nitro and **42** without hydroxyl showed significant loss of inhibitory activity with respective percentage inhibition values of 25.7% ± 7.9% and 24.7% ± 1.9%, as predicted by our rational design using the pharmacophore model. The transformation from phenol to methyl ether **43** also led to lowered activity (40.4% ± 1.9%). *m*-nitrophnol **44**, or *p*-nitrophenol **46** with relatively changed position to the imine group, exhibited decreased

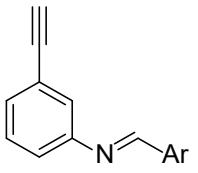
inhibitory activity (46.7% ± 2.5%, 52.2% ± 3.1%). Nitro is a critical group, as it processes the dual effect of serving as a hydrogen bond acceptor and electron-withdrawing group affecting the acidity of *p*-nitrophenol, which is supposed to bind to Glu81 of NusB by the design of hit compound. The replacement of nitro by methoxy in compound **40** kept the hydrogen bonding capability, however, lost the inhibitory activity (29.3% ± 5.6%) as methoxy is an electron-donating group and capable of increasing the pKa of phenol. When nitro was replaced by other hydrogen bond acceptors and electron-withdrawing groups such as carboxylate, fluoride, nitrile, acetonitrile and chloride, the respective compounds **36** (41.4% ± 3.7%), **37** (28.1% ± 2.4%), **38** (61.3% ± 2.0%), **39** (49.9% ± 1.2%) and **48** (54.2% ± 2.8%) displayed noticeably decreased inhibitory activity compared to the hit compound **1**. It is suggested that the pKa of phenol is more important for the inhibitory activity, as *o*-nitrophenol **45** that lost the hydrogen bond at *p*-position of hydroxyl but maintained a similar pKa to *p*-nitrophenol still displayed excellent inhibitory activity (87.7% ± 0.6%). Additionally, an extra *o*-bromo group to *p*-nitrophenol **47** trying to lower further the pKa did not affect the activity.

Amine **49**-**60** demonstrated more favorable changes related to inhibitory activity compared to amine compounds with modifications on the left benzene ring. Amines **49**, **50**, **53**, **55**, **56**, **59** possessed significant improvement over the imine analogues, especially **59** with a measured 99.6% ± 0.1% as the best percentage inhibition so far, indicating that the low pKa of phenol by additional *o*-bromo group is favored, however, affected by the structural rigidity of **47**. When the molecule can freely rotate, the improved binding affinity overpowered the entropy penalty to provide a better inhibitory activity. While amines **51**, **52**, **54**, **57**, **60** with various substituents showed similar activity to the imine analogues, suggesting the binding force are mainly from hydrogen bonds, which were sufficiently robust to maintain the binding to NusB, in contrast to

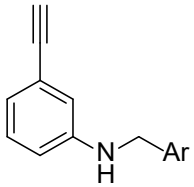
the left benzene ring modifications that were more sensitive to entropy penalty, indicating the Van der Waals interactions. The only dramatic decrease in activity occurred on compound **58** (64.5% ± 1.7%). Despite of maintaining pKa of *o*-nitrophenol, the loss of hydrogen bond between the nitro group and Tyr18 of NusB could not retain the binding of **58** with NusB very firmly as compound **45** by the conformation control as a Schiff base.

We have measured the IC_{50} values of **22**, **27** and **28**, which were 108.1 ± 10.2 μM, 69.2 ± 2.4 μM and 79.6 ± 5.4 μM, respectively. These values were at the same range to the hit compound **1** [4], suggesting similar inhibitory activities targeting the NusB-NusE interaction.

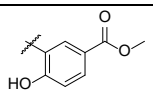
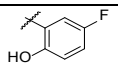
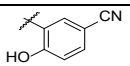
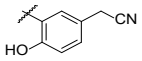
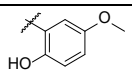
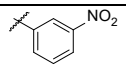
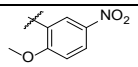
Table 2. Inhibitory and antimicrobial activities of compounds **36-60**

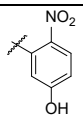
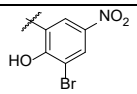
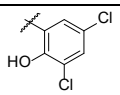


36-48



49-60

Compound	Ar	MIC	%Inhibition[a]	ClogP
36		128	41.4 ± 3.7	3.51
49		32	57.1 ± 0.8	3.15
37		256	28.1 ± 2.4	3.60
50		32	61.8 ± 2.0	3.08
38		32	61.3 ± 2.0	3.03
51		32	64.8 ± 5.4	2.76
39		256	49.9 ± 1.2	2.78
52		64	44.7 ± 1.4	2.06
40		>256	29.3 ± 5.6	3.46
53		64	58.3 ± 0.9	2.74
41		256	25.7 ± 7.9	3.35
54		>256	20.3 ± 1.9	2.64
42		256	24.7 ± 1.9	2.86
55		>256	33.1 ± 2.6	3.10
43		>256	40.4 ± 1.9	3.31
56		>256	56.1 ± 1.4	3.32
44		64	46.7 ± 2.5	3.32

57		8	48.2 ± 1.9	3.02
45		256	87.7 ± 0.6	3.32
58		128	64.5 ± 1.7	3.02
46		256	52.2 ± 3.1	3.32
47		32	74.1 ± 1.6	3.94
59		2	99.6 ± 0.1	3.70
48		32	54.2 ± 2.8	4.59
60		2	50.6 ± 4.9	4.14

[a] % Inhibition displayed the percentage of inhibition on the NusB-NusE interaction using the previously described conditions [9]: the compound was used at 125 μM in the NusB-NusE (1:1) solution at 1 μM in PBS buffer.

3.2 Circular dichroism.

We considered using circular dichroism spectroscopy to detect the influence of representative derivatives on the NusB protein folding, as different secondary structure has a specific circular dichroism signature [10]. Compounds **5** and **22** were chosen as they represent two different linkers: imine and amine that may affect the special position of the two benzene rings and thus the binding affinity to NusB. We first examined the NusB protein at 2.0 μg/mL in neutral phosphate buffer, which displayed a spectrum showing a typical α-helical character (Figure 3), consistent to the multiple α-helices observed by in the X-ray crystallography structure (PDB: 3D3B) [11].

When compounds **5** and **22** were respectively mixed with NusB at 1:1 ratio, the changes on spectra could be observed as a result of the protein-ligand binding. The chiral character of NusB decreased by binding to one of the derivatives. When compounds **5** and **22** were respectively added to NusB, similar changes of the CD spectra were observed, which indicated that the

derivatives induced similar structural changes of NusB. In summary, the CD spectra showed that the two representative compounds were able to cause similar conformational changes of NusB, suggesting they may bind to the same binding site on the protein.

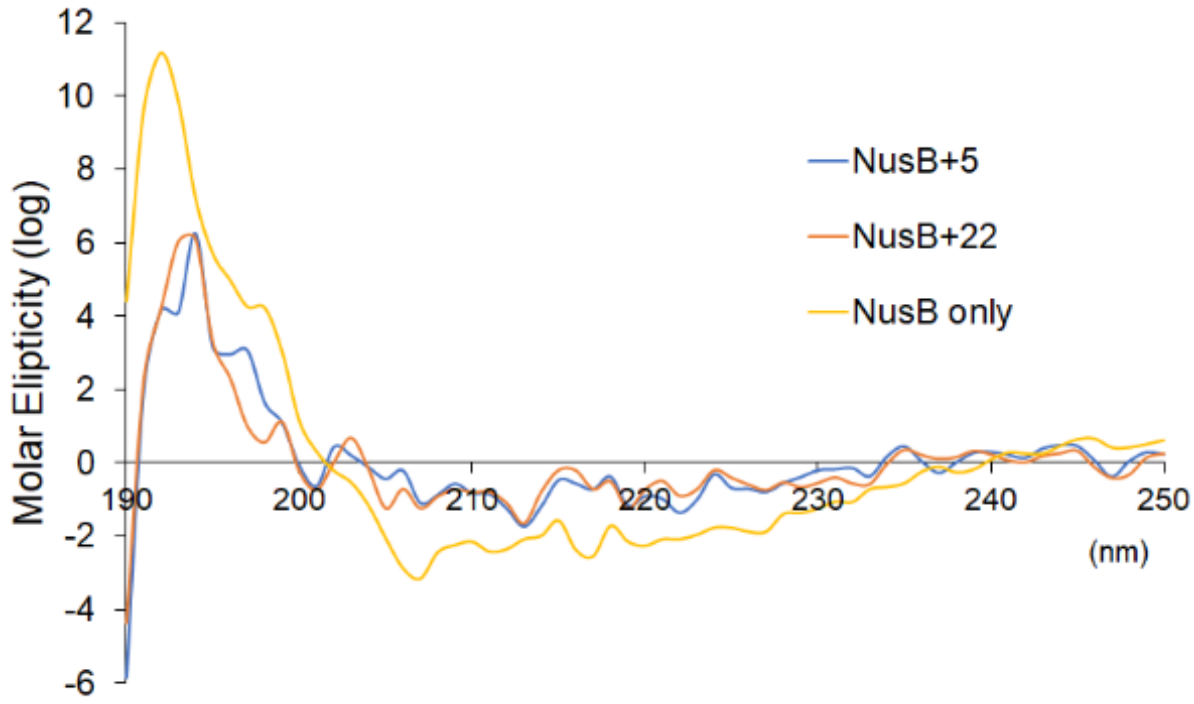


Figure 3. CD spectrum of NusB before and after addition of compounds **5** or **22**

3.3 Antimicrobial activity.

The antimicrobial activity of the compounds was determined by the microdilution method according to the Clinical & Laboratory Standards Institute (CLSI) guidelines against *S. aureus* ATCC 25923 [12].

As shown in Table 1, amongst the compounds **1-19** with imine as the linker, most of the modifications of left benzene ring led to equal or superior antimicrobial activity (MIC 2-8

μg/mL) compared to the hit compound **1**, in which compounds **11** and **12** with chloride displayed the best activity with MIC values of 2 μg/mL. The only compounds demonstrating inferior activity were **9** and **15** with hydroxyl and trifluoromethyl at 3-position. The MIC values of **9** and **15** against *S. aureus* ATCC 25923 were 16 μg/mL, similar with the hit compound regarding the data interpretation in antibiotic susceptibility testing. These results proved again that the binding of left benzene ring to NusB belongs to the hydrophobic interaction. It is worth noting that the *in vitro* inhibitory activity of compounds does not fully correspond to the antimicrobial activity, with an extreme example for comparison between compounds **5** and **9** demonstrating the lowest and highest inhibitory activity, but they displayed reversed antimicrobial activity with MIC values of 4 and 16 μg/mL, respectively. As the cell system is much more complicated than the *in vitro* biochemical assay system, we need to consider the impact of cell permeability, efflux system and metabolic enzymes besides the interaction of two partner proteins. Effectively, the clogP values of compounds **5** and **9** are diverse at 4.88 and 2.38, respectively, which may affect their permeability.

When compounds **20**-**35** with amine as the linker were subjected to the antimicrobial testing, most of the compounds exhibited equal or inferior antimicrobial activity to their imine analogues, except compounds **22**-**24** with *t*-butyl at 2-, 3- and 4-position of left benzene ring, which presented superior antimicrobial activity with MIC values of 1-2 μg/mL. Compound **22** displayed the best antimicrobial activity, with an MIC of 1 μg/mL. In summary, the antimicrobial activity of these series of compounds follows the same trend of their inhibitory activity, which strengthened our hypothesis that the NusB-NusE interaction is the target of these compounds.

As shown in Table 2, compounds **36**-**48** with modifications of the right benzene ring changed nitro and hydroxyl as the hydrogen binding groups, led to dramatic decrease of antimicrobial activity with MIC values of 32-256 μg/mL, in which compound **40** with methoxy replacing nitro, and **43** with methyl ether masking hydroxyl lost their antimicrobial activity at all (>256 μg/mL). As these compounds have imine as the linker, once hydrogen bonding groups were modified, they were not capable of adjusting the rigid structures to accommodate the required binding in a more dynamic cellular environment. The possible interactions of NusB with other proteins that may also competitively change the conformation of NusB. However, when imine was replaced by amine, compounds **49**-**60** with flexible structures were able to improve significantly the antimicrobial activity with MIC values of 2-64 μg/mL. The best antimicrobial activity (2 μg/mL) of these series of compounds were obtained from compound **59** with an additionally bromide at *o*-position to phenol, and compound **60** with dichloride replacing nitro and bromide in compound **59**. This series of compounds again proved the importance of hydrogen bonding groups, as compounds **41**-**43**, **54**-**56** with truncated or masked pharmacophores lost completely the antimicrobial activity regardless imine or amine as linkers.

With 60 derivatives of the hit compound in hand, we can conclude a preliminary SAR of the derivatives on the basis of their antimicrobial activity against *S. aureus* ATCC 25923 (Figure 4).

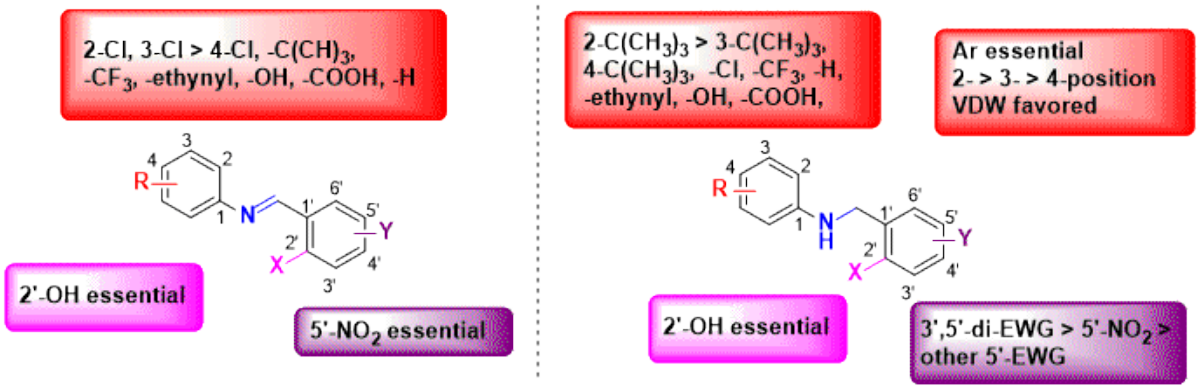


Figure 4. SAR of synthesized derivatives of the hit compounds against *S. aureus* ATCC 25923

On the other hand, we also tried to align the activity to the structure and cell permeability. As shown in Figure 5, we summarized the *in vitro* inhibitory activity of NusB-NusE interaction related to the antimicrobial activity against *S. aureus* 29523 in a bubble chart with the bubble size representing clogP of compounds. It is clear that the MIC values are inversely proportional to the percentage inhibition of compounds, which indicated that the antimicrobial activity and *in vitro* inhibitory activity are in direct proportion. When we plotted the clogP values against the antimicrobial activity with bubble sizes demonstrating percentage inhibition, we could find out that the clogP between 2.5 and 5.0 is also inversely proportional to the MIC values. The statistics of these data suggested that the antimicrobial activity of our inhibitors has a visible relation to the inhibitory activity and cell membrane permeability of compounds.

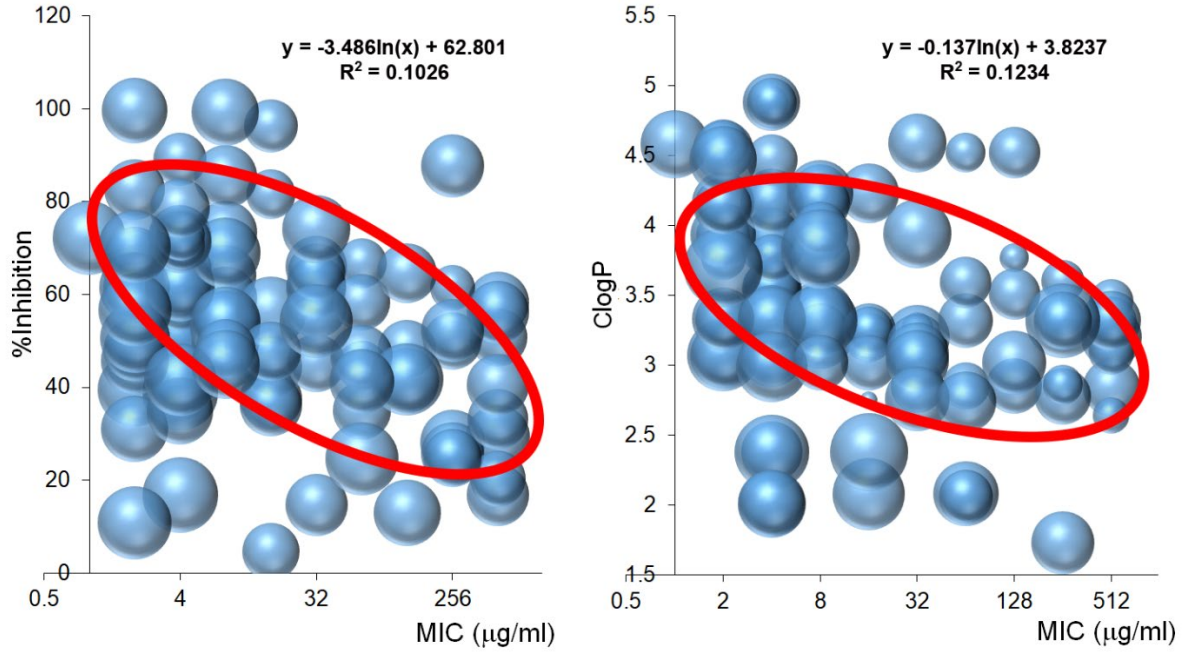


Figure 5. The trend of the inhibitory activity (left y axis) and ClogP (right y axis) are inversely proportional to the antimicrobial activity shown by MIC values (x axis). The size of spheres represents the propotional values of ClogP (left) or inhibitory activity (right).

Both of the values of the coefficient of determination (R^2) of inhibitory activity – MIC and ClogP – MIC were > 0.1, which suggested a certain degree of correlations. While these values were still modest, we need to consider that there are a number of other factors contributing to a compound's antimicrobial activities, such as solubility, membrane permeability, potential bacterial enzyme modification/degradation, and bacterial efflux. Investigations of other possible bacterial targets for our compounds would also provide information on the factors affecting the antimicrobial activity.

We then intended to examine the antibacterial spectrum of this series of molecules targeting the NusB-NusE interaction. The first bacteria set used is top six pathogens amongst the most

recent “WHO priority pathogens list for guiding R&D of new antibiotics” containing three Gram-positive and three Gram-negative bacteria: *Enterococcus faecium*, *S. aureus*, *Streptococcus pneumonia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*. We used the standard strains for antibiotic susceptibility testing and conditions to perform the experiment, and the result was shown in Table 3: the compounds demonstrated broad-spectrum antimicrobial activity against both Gram-positive and -negative bacteria, as the NusB-NusE interaction is essential and conserved in bacteria. Additionally, these compounds displayed specific activities against *S. aureus*, *S. pneumonia*, *E. faecium* and *A. baumannii* over other pathogens. *S. aureus*, *S. pneumonia*, and *E. faecium* are all Gram-positive bacteria, suggesting Gram-positive bacteria may be more susceptible to these compounds. Encouragingly, *A. baumannii* is top one bacterium of the WHO pathogen list that requires new antibiotic development, our compounds may be developed as a specific antimicrobial agent to address this urgent need. In the contrast, other Gram-negative bacteria were less responsive to the compound treatment, probably due to reduced cell permeability and vigorous efflux system, which need further testing in the future.

Table 3. Antimicrobial activity evaluation of compounds **1**-**60** against seven representative bacterial strains including two *S. aureus* strains in the WHO priority pathogens list for guiding R&D of new antibiotics.

Compound	EFAE	SAUR[a]	SAUR[b]	SPNE	ABAU	PAER	ECLO
1	>256	8	16	16	64	>256	>256
2	>256	4	16	32	>256	>256	>256
3	128	4	16	64	>256	>256	>256
4	>256	4	16	32	256	>256	>256
5	>256	4	16	64	>256	>256	>256
6	>256	4	16	256	128	>256	>256
7	>256	4	32	128	>256	>256	>256
8	>256	4	16	64	128	>256	>256
9	>256	16	64	256	>256	>256	>256
10	>256	4	8	64	>256	>256	>256

11	>256	2	16	64	256	>256	>256
12	>256	2	16	64	256	>256	>256
13	256	8	32	64	>256	>256	>256
14	256	8	16	32	>256	>256	>256
15	>256	16	64	32	256	>256	256
16	256	8	16	16	256	>256	>256
17	>256	4	16	16	64	>256	>256
18	>256	4	8	16	256	>256	>256
19	>256	4	16	32	128	>256	>256
20	16	16	16	8	128	>256	>256
21	128	16	32	64	256	>256	>256
22	8	1	4	8	>256	>256	>256
23	32	2	4	8	>256	>256	>256
24	>256	2	4	8	>256	>256	>256
25	256	16	32	64	128	256	256
26	>256	64	128	128	>256	>256	>256
27	32	2	2	8	128	>256	256
28	32	4	4	8	128	>256	256
29	32	8	4	16	>256	256	128
30	8	2	2	8	128	>256	256
31	16	4	4	16	64	>256	256
32	32	8	8	16	32	>256	256
33	>256	>256	>256	>256	>256	>256	>256
34	>256	>256	>256	>256	>256	>256	>256
35	16	4	4	16	256	>256	>256
36	>256	128	>256	128	64	256	>256
37	>256	256	>256	256	256	256	>256
38	256	32	64	128	>256	>256	>256
39	>256	256	>256	>256	64	>256	>256
40	>256	>256	>256	>256	128	>256	>256
41	>256	256	>256	>256	>256	>256	>256
42	>256	256	128	>256	16	>256	>256
43	>256	>256	>256	>256	>256	>256	>256
44	>256	64	256	128	>256	>256	>256
45	256	256	256	256	>256	256	>256
46	>256	256	256	>256	256	>256	>256
47	>256	32	32	128	256	>256	>256
48	256	32	64	8	128	>256	>256
49	32	32	16	8	>256	>256	>256
50	32	32	32	16	128	>256	>256
51	64	32	32	16	>256	>256	>256
52	32	64	32	8	>256	>256	>256
53	64	64	32	8	>256	>256	>256
54	>256	>256	>256	>256	>256	>256	>256
55	>256	>256	>256	>256	>256	>256	>256
56	>256	>256	>256	>256	>256	>256	>256
57	32	8	8	16	64	>256	>256
58	>256	128	16	256	>256	>256	>256
59	64	2	2	32	256	>256	>256
60	32	2	4	256	>256	>256	>256
VAN	1	2	1	0.5	>256	>256	>256
OXA	16	0.5	0.25	2	>256	>256	>256
GEN	16	0.5	0.5	32	32	2	0.5
RIF	2	0.0125	0.0125	0.0625	4	>256	>256

CIP	4	0.5	0.5	0.5	2	0.5	0.0125

EFAE: *Enterococcus faecium* ATCC 19433, SAUR[a]: *S. aureus* ATCC 25923, SAUR[b]: *S. aureus* ATCC 29213, SPNE: *Streptococcus pneumonia* ATCC 49619, ABAU: *Acinetobacter baumannii* ATCC 19606, PAER: *Pseudomonas aeruginosa* ATCC 27853, ECLO: *Enterobacter cloacae* ATCC 13047, VAN: vancomycin, OXA: oxacillin, GEN: gentamicin, RIF: rifampicin, CIP: ciprofloxacin.

A series of compounds were then chosen to test against representative clinically significant Gram-positive pathogens such as *Enterococcus casseliflavus* causing bacteremia, *Staphylococcus epidermidis* able of growing biofilms on plastic devices, most commonly on intravenous catheters, *Staphylococcus saprophyticus* causing urinary tract infections, *Streptococcus pneumoniae* as the major cause of community acquired pneumonia and meningitis, *Streptococcus pyogenes* causing pharyngitis (strep throat), localized skin infection (impetigo), necrotizing fasciitis, and neonatal infections, *Streptococcus agalactiae* causing neonatal infections. As shown in Table 4, most of the compounds displayed antibacterial activity against these Gram-positive pathogens with clinical complexity for treatment, which demonstrated the potentials of our compounds.

Table 4. Antimicrobial activity evaluation of compounds **22**, **23**, **24**, **27**, **28**, **30** and **59** against representative pathogenic Gram-positive bacteria.

Compound	ECAS	SEPI	SSAP	SPNE	SPYO	SAGA
22	16	1	2	2	8	8
23	32	16	1	8	16	16
24	32	4	8	4	16	16
27	32	8	8	8	16	32
28	32	8	16	8	16	32
30	64	2	4	8	16	16
59	64	4	16	32	32	32
CIP	4	0.0625	0.5	0.5	0.5	4

GEN	4	0.125	0.0625	32	8	32
OXA	32	0.25	1	2	0.0625	0.5
VAN	4	1	1	0.5	0.5	0.5

ECAS: *Enterococcus casseliflavus* ATCC 25788, SEPI: *Staphylococcus epidermidis* ATCC 12228, SSAP: *Staphylococcus saprophyticus* ATCC 15305, SPNE: *Streptococcus pneumoniae* ATCC 49619, SPYO: *Streptococcus pyogenes* ATCC 19615, SAGA: *Streptococcus agalactiae* ATCC 12386.

MRSA represents one of the most difficult bacterial strains for treatment due to antibiotic resistance. We also tested the above compounds against a series of globally spread MRSA strains in hospitals and communities: *S. aureus* ST239 [13], *S. aureus* ATCC BAA-43 [14], *S. aureus* ATCC BAA-44 [14], *S. aureus* HA W-231 ST45 [15], CA-MRSA W-47 ST30 [15], CA-MRSA W-45 ST59 [15], CA-MRSA W-46 ST59 [15], *S. aureus* USA300/ST8 [16], *S. aureus* 11B086169 ST22 [17], CA-MRSA W-4 ST338 [17], CA-MRSA W-48 ST217 [18], *S. aureus* HA W-235 ST5 [19]. As shown in Table 5, all of compounds displayed consistent antibacterial activity against these MRSA strains, in which some of them developed resistance to oxacillin, as the first line antibiotic drug used in the USA for MRSA, as well as other antibiotics such as ciprofloxacin and gentamicin. Our compounds were able to maintain stable MIC values against diverse MRSA strains, suggesting the compounds may be developed further to treat MRSA caused infectious diseases.

Table 5. Antimicrobial activity evaluation of compounds **22**, **23**, **24**, **27**, **28**, **30** and **59** against representative globally spread HA- and CA-MRSA strains.

Compound	25923	29213	ST239	BAA-43	BAA-44	ST45	ST30	45ST59	46ST59	USA300	ST22	ST338	ST217	ST5
22	1	4	2	2	2	2	2	2	2	2	2	2	2	2
23	2	4	8	2	8	8	4	8	8	8	8	4	4	4
24	2	4	4	4	4	4	4	4	4	4	4	4	8	4

27	2	2	8	2	8	4	4	8	4	4	4	4	4	4
28	4	4	16	4	8	8	8	8	8	8	4	8	8	8
30	2	2	4	4	4	4	4	4	2	2	4	2	2	2
59	2	2	4	2	16	2	2	2	4	4	4	2	2	2
CIP	1	1	4	2	4	2	0.5	0.25	0.25	4	4	0.5	4	4
GEN	1	1	>256	>256	>256	16	0.25	0.5	0.5	0.5	0.25	0.25	0.5	>256
OXA	1	0.25	>256	>256	>256	4	4	8	8	32	64	0.5	64	>256
VAN	2	1	1	1	1	0.5	0.5	0.5	1	1	0.5	0.5	0.5	0.5

3.4 Time kill assessment

The time kill kinetics were used to demonstrate the *in vitro* reduction of a microbial population by certain antimicrobial(s), and compounds **1** and **27** were tested against *S. aureus* ATCC 25923 (Figure 7). The hit compound **1** started to inhibit bacteria growth at ¼ MIC, and showed bacteriostatic effect at MIC and 4 MIC. **27** demonstrated similar inhibitory effect at the ¼ and 1 MIC, and started to demonstrate slight bactericidal activities at 4 MIC.

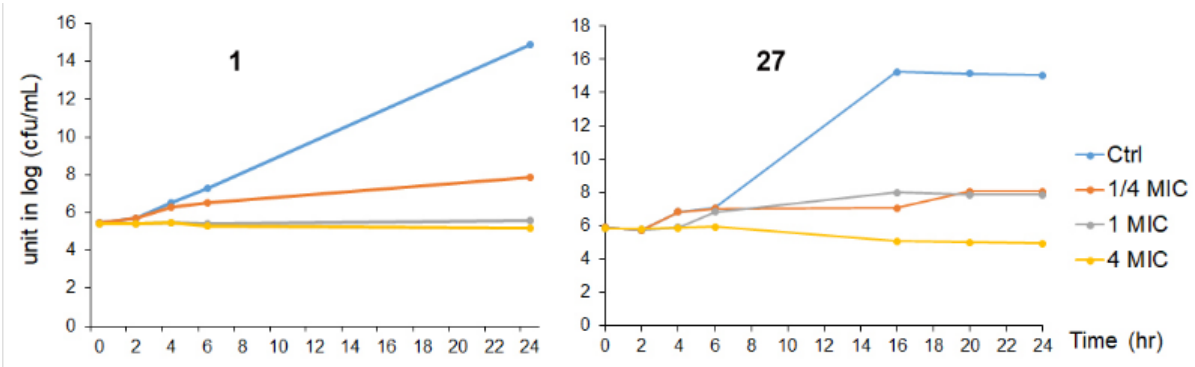


Figure 7. Time-kill kinetics of compounds **1** and **27** at different concentrations against *S. aureus* ATCC 25923

3.5 Cytotoxicity

The compounds within the best range of antimicrobial activity against *S. aureus* ATCC 25923 (MIC 1-2 μg/mL) were subjected to cytotoxicity testing against A549 human lung carcinoma cell line and HaCaT human keratinocytes, as the major *S. aureus* infection sites often occur in lung and skin [4]. As shown in Table 6, compounds **11** and **12** with imine linker did not show significant cytotoxicity (≥200 μM), whereas compounds **22**, **23**, **24**, **27**, **30** with amine linker demonstrated mild cytotoxicity (13-40 μM). The result is understandable as aniline is a known cytotoxic structure. The additional substitution on the right benzene ring of compounds **59** and **60** can respectively reduce the cytotoxicity to about 80 and 110 μM even in the presence of aniline structure. The preliminary therapeutic index were also calculated with 69-119 for imines and 6-12 for amines, which demonstrated very promising safety range. When additional substitutions were installed on the right benzene ring, the therapeutic index of amine compounds could be significantly improved to 25-33. Note that the cell-based cytotoxicity of compounds is not always consistent with *in vivo* toxicity, as the latter is associated with more complexed factors such as whole body pharmacokinetics, organ or tissue distribution and the corresponding specific toxicity. Therefore, the *in vivo* safety needs to be determined using animal models in the future.

Table 6. Cytotoxicity of selective compounds against A549 and HaCaT

Cpd.	MIC[a] (μg/mL)	CC_{50} (μM)		Therapeutic Index[b]	
		A549	HaCaT	A549	HaCaT
11	2	431.69 ± 30.14	346.59 ± 28.93	119	96
12	2	274.16 ± 4.69	249.52 ± 22.73	76	69
22	1	13.42 ± 1.53	15.07 ± 1.81	8	9
23	2	18.32 ± 1.75	20.24 ± 2.42	6	6
24	2	37.23 ± 4.44	38.89 ± 4.18	11	12
27	2	22.52 ± 2.35	20.09 ± 2.05	6	6
30	2	19.98 ± 2.11	18.31 ± 1.97	6	6
59	2	79.14 ± 15.97	72.15 ± 13.31	27	25

60	2	112.18 ± 21.56	108.10 ± 18.46	33	32
DDP[c]	-	7.70 ± 0.58	7.83 ± 0.66	-	-

[a] *S. aureus* ATCC 25923, [b] Calculated by CC_{50} (μg/mL) / ½ MIC (μg/mL), [c] DDP: cisplatin

3.6 Hemolytic property

With the antibacterial activity on hand, we intended to explore the potentials of using these compounds *in vivo*. To initiate the study, we need to confirm that these compounds would not cause hemolysis when injected into blood veins or transported by plasma proteins.

Compounds **27** and **28** were tested for their hemolytic property using human blood cells [20]. As shown in Figure 8, compounds **27** and **28** demonstrated very low haemolytic potential at all tested concentrations (0.1, 1 and 10 μg/mL). The hemolytic values of all tested compounds at all tested concentrations were lower than the suggested nonhemolytic cut-off value.

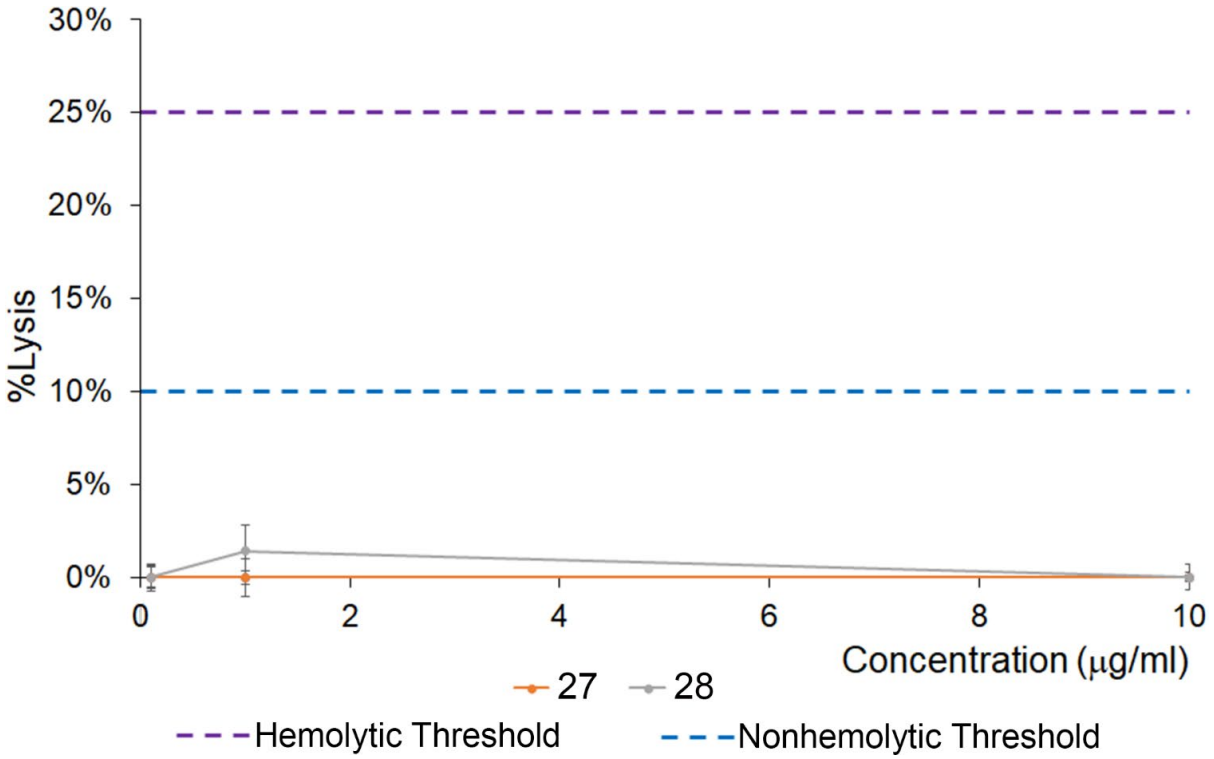


Figure 8. *in vitro* hemolysis activity of compounds **27** and **28**

3.7 Caco-2 cell permeability

If drugs are potentially orally available, the intestinal absorption is critical. The human intestinal epithelial cell line Caco-2 was used to determine the cell permeability of compounds **27** and **28** [20]. As shown in Table 7, compounds **27** and **28** possessed a high apparent permeability, which is larger than 10^{-5} cm/s. Furthermore, the deduced apparent permeability of compounds had a similar magnitude to that of the predicted values.

Table 7. The deduced and predicted apparent permeability of compounds **27** and **28**

Compound	P_{app} Average (n=4) (cm/s)	P_{app} Predicted (cm/s)
27	$1.61 \pm 0.53 \times 10^{-5}$	2.57×10^{-6}
28	$1.49 \pm 0.79 \times 10^{-5}$	7.93×10^{-6}

4. Conclusions

In this study, we described the rational design and synthesis of a new class of diarylimine and -amine compounds based on the pharmacophore model derived from structure-based virtual screening against the bacterial NusB-NusE protein. The entire library of hit compound derivatives were assessed for their inhibition of NusB-NusE interaction, as well as the antimicrobial activities, among which a few compounds demonstrated promising growth inhibitory effect against a panel of bacterial pathogens, including clinical MRSA strains. The cytotoxicity study demonstrated that these compounds are not very toxic to human cell lines, while cell-based pharmacokinetic studies suggested the high druggability of the compounds. These compounds represented a new class of antimicrobial agents against a previously

unidentified protein-protein interaction in bacteria, with a distinct mechanism of action to current antibiotics on the market. Further detailed mechanistic studies will be carried out in the near future.

5. Experimental Section.

5.1 Materials and Chemicals

Sodium Chloride and Phosphate Buffered Saline (tablets) were purchased from Sigma-Aldrich (Saint Louis, Missouri). GR Grade Dimethyl Sulfoxide (DMSO) was purchased from Duksan Pure Chemicals (Ansan City, South Korea) and HPLC Grade Acetonitrile was purchase from Merck KGaA (Darmstadt, Germany). Fresh human whole blood (with CPD as anticoagulant) was obtained from Hong Kong Red Cross and was stored at +4 ± 2 °C before use. For 150 mM NaCl buffer, 4.383 g of NaCl crystals was dissolved in 500 mL of Milli-Q water. 1x PBS was prepared by dissolving 1 PBS tablet into 200 mL of Milli-Q water.

5.2 General procedure (A) for synthesis of Schiff base type derivatives

To a solution of benzaldehydes (1 equiv) in ethanol (10 mL) was added anilines (1 equiv), and the reaction mixture was stirred at room temperature overnight. The solid was collected by filtration, and washed with cold ethanol and hexane.

5.3 General procedure (B) for synthesis of aniline type derivatives

To a solution of Schiff base type derivatives (1 equiv) obtained according general procedure (A) in DCM (10 mL) was added sodium triacetoxyborohydride (3 equiv), and the reaction mixture was stirred at room temperature overnight. The crude reaction mixture was evaporated *in vacuo* and the pure product was obtained after flash chromatography.

5.4 General Procedure (C) for synthesis of salicylaldehydes

To a solution of phenolic derivatives (1 equiv), anhydrous $MgCl_2$ (1.5 equiv) and Et_3N (3.75 equiv) in dry THF (30 mL) was added paraformaldehyde (6.75 equiv) in one portion, and the reaction mixture was stirred at reflux under nitrogen atmosphere overnight. After the reaction mixture was cooled to rt, the reaction was quenched with 1M HCl and the product was extracted with EtOAc (30 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na_2SO_4 and filtered. All volatiles were removed under reduced pressure and the product was isolated by flash chromatography (EtOAc/Hex) on silica gel.

5.4.1 *(E)*-2-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (1)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminophenylacetylene **1a** (135 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (242 mg, 76%), ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.94 (br s, 1H), 9.16 (s, 1H), 8.66 (d, J = 2.8 Hz, 1H), 8.27 (dd, J = 9.3, 2.9 Hz, 1H), 7.59 – 7.44 (m, 4H), 7.13 (d, J = 9.2 Hz, 1H), 4.29 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.8, 162.8, 147.8, 139.8, 131.1, 130.4, 128.9, 128.7, 124.6, 123.4, 123.1, 119.3, 118.6, 83.3, 81.9, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ (M - H^+) 265.0619, found 265.0620, Melting Point: 196-197°C.

5.4.2 *(E)*-2-(((2-ethynylphenyl)imino)methyl)-4-nitrophenol (2)

step 1: To a solution of 2-iodoaniline (300 mg, 1.37 mmol), $PdCl_2(PPh_3)_2$ (29 mg, 0.04 mmol, 0.03 equiv), CuI (13 mg, 0.07 mmol, 0.05 equiv), Et_3N (1 mL) in dry THF (4 mL) was added trimethylsilylacetylene (290 µL, 2.06 mmol, 1.5 equiv) dropwisely *via* syringe under N_2 atmosphere. The resulting mixture was stirred at rt for 6h. After the reaction was completed,

the reaction mixture was filtered through a pad of Celite. The filtrate was evaporated *in vacuo* and the crude product was dissolved in methanol (10 mL), followed by addition of K_2CO_3 (1893 mg, 13.7 mmol, 10 equiv). The mixture was stirred at rt for 1h before extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na_2SO_4 and filtered. All volatiles were evaporated *in vacuo* and the product 2-ethynylaniline (**2a**) was obtained after flash chromatography (hexane : EtOAc = 100: 1) as a yellow liquid (94 mg, 59%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.35 (dd, *J* = 7.6, 1.6 Hz, 1H), 7.17 (td, *J* = 7.8, 1.6 Hz, 1H), 6.79 – 6.64 (m, 2H), 4.27 (s, 2H), 3.41 (s, 1H).

step 2: The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and **2a** (141 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (221 mg, 69%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.40 (s, 1H), 8.83 (s, 1H), 8.44 (d, *J* = 2.9 Hz, 1H), 8.30 (dd, *J* = 9.3, 2.7 Hz, 1H), 7.65 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.51 – 7.47 (m, 1H), 7.43 – 7.30 (m, 2H), 7.14 (d, *J* = 9.2 Hz, 1H), 3.45 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.8, 160.8, 148.3, 140.0, 133.8, 130.1, 128.6, 128.5, 127.8, 118.5, 118.4, 118.1, 117.6, 83.3, 80.3, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ ($M - H^+$) 265.0619, found 265.0618, Melting Point: 169-170°C.

5.4.3 *(E)*-2-(((4-ethynylphenyl)imino)methyl)-4-nitrophenol (3)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-ethynylaniline **3a** (141 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (191 mg, 60%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.17 (s, 1H), 8.73 (s, 1H), 8.43 (d, *J* = 2.9 Hz, 1H), 8.31 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.63 – 7.61 (m, 2H), 7.33 – 7.30 (m, 2H), 7.14 (d, *J* = 9.2 Hz, 1H), 3.20 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.6, 161.2, 147.0, 140.1, 133.5, 128.6, 128.5, 121.9, 121.3,

118.4, 118.1, 82.8, 78.8, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ (M - H^+) 265.0619, found 265.0615, Melting Point: 201-202℃.

5.4.4 *(E)*-4-nitro-2-((phenylimino)methyl)phenol (4)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and aniline **4a** (109 μL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (188 mg, 65%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.49 (s, 1H), 8.74 (s, 1H), 8.42 (d, *J* = 2.8 Hz, 1H), 8.29 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.50 (t, *J* = 7.6 Hz, 2H), 7.40 – 7.35 (m, 3H), 7.12 (d, *J* = 9.1 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.9, 160.6, 146.7, 140.0, 129.7, 128.4, 128.4, 128.1, 121.2, 118.4, 118.1, HRMS (ESI) calcd for $C_{13}H_9N_2O_3$ (M - H^+) 241.0619, found 241.0620, Melting Point: 133-134℃.

5.4.5 *(E)*-2-(((2-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (5)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-tert-butylbenzenamine **5a** (187 μL, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (267 mg, 75%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.18 (s, 1H), 8.55 (s, 1H), 8.43 (d, *J* = 2.9 Hz, 1H), 8.31 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.52 – 7.49 (m, 1H), 7.36 – 7.28 (m, 2H), 7.15 (d, *J* = 9.2 Hz, 1H), 6.96 – 6.94 (m, 1H), 1.45 (s, 8H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.3, 161.1, 147.7, 143.1, 140.1, 128.5, 128.5, 127.6, 127.6, 126.7, 121.1, 118.5, 118.2, 35.3, 30.9, HRMS (ESI) calcd for $C_{17}H_{17}N_2O_3$ (M - H^+) 297.1245, found 297.1247, Melting Point: 152-153℃.

5.4.6 *(E)*-2-(((3-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (6)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-tert-butylbenzenamine **6a** (179 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (244 mg, 68%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.63 (s, 1H), 8.74 (s, 1H), 8.43 (d, *J* = 2.7 Hz, 1H), 8.29 (dd, *J* = 9.2, 2.6 Hz, 1H), 7.43 – 7.41 (m, 2H), 7.36 (d, *J* = 2.3 Hz, 1H), 7.19 – 7.15 (m, 1H), 7.12 (d, *J* = 9.2 Hz, 1H), 1.40 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.1, 160.3, 153.2, 146.5, 139.9, 129.4, 128.4, 128.3, 125.2, 118.8, 118.4, 118.1, 117.7, 34.9, 31.3, HRMS (ESI) calcd for $C_{17}H_{17}N_2O_3$ (M - H^+) 297.1245, found 297.1242, Melting Point: 128-129℃.

5.4.7 *(E)*-2-(((4-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (7)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-tert-butylaniline **7a** (191 μL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (217 mg, 61%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.67 (s, 1H), 8.74 (s, 1H), 8.41 (d, *J* = 2.8 Hz, 1H), 8.28 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.52 – 7.50 (m, 2H), 7.33 – 7.30 (m, 2H), 7.11 (d, *J* = 9.1 Hz, 1H), 1.39 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.1, 159.7, 151.6, 143.8, 139.9, 128.3, 128.2, 126.6, 120.9, 118.3, 118.2, 34.8, 31.3, HRMS (ESI) calcd for $C_{17}H_{17}N_2O_3$ (M - H^+) 297.1245, found 297.1243, Melting Point: 175-176℃.

5.4.8 *(E)*-2-(((2-hydroxyphenyl)imino)methyl)-4-nitrophenol (8)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-aminophenol **8a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (245 mg, 79%), ^{1}H NMR (400 MHz, DMSO-d_6) δ 15.72 (br s, 1H), 10.41 (br s, 1H), 9.31 (s, 1H), 8.62 (d, *J* = 3.1 Hz, 1H), 8.17 (dd, *J* = 9.4,

3.0 Hz, 1H), 7.60 (d, *J* = 8.1 Hz, 1H), 7.23 (t, *J* = 7.8 Hz, 1H), 7.03 (d, *J* = 8.1 Hz, 1H), 6.97 (t, *J* = 7.7 Hz, 1H), 6.90 (d, *J* = 9.5 Hz, 1H), ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 173.0, 159.7, 151.0, 137.2, 130.9, 130.2, 129.8, 129.1, 121.0, 120.3, 119.2, 117.1, 116.9, HRMS (ESI) calcd for $C_{13}H_9N_2O_4$ (M - H^+) 257.0568, found 257.0565, Melting Point: 269-270℃.

5.4.9 *(E)*-2-(((3-hydroxyphenyl)imino)methyl)-4-nitrophenol (9)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminophenol **9a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (288 mg, 93%), ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 14.53 (s, 1H), 9.79 (s, 1H), 9.14 (s, 1H), 8.68 (d, *J* = 2.9 Hz, 1H), 8.26 (dd, *J* = 9.2, 3.0 Hz, 1H), 7.29 (t, *J* = 8.0 Hz, 1H), 7.09 (d, *J* = 9.2 Hz, 1H), 6.92 (dd, *J* = 7.9, 2.0 Hz, 1H), 6.86 (t, *J* = 2.2 Hz, 1H), 6.79 (dd, *J* = 8.0, 2.3 Hz, 1H), ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 168.1, 161.8, 158.9, 147.6, 139.3, 130.8, 129.2, 128.9, 119.0, 118.7, 115.4, 112.6, 108.5, HRMS (ESI) calcd for $C_{13}H_9N_2O_4$ (M - H^+) 257.0568, found 257.0568, Melting Point: 242-243℃.

5.4.10 *(E)*-2-(((4-hydroxyphenyl)imino)methyl)-4-nitrophenol (10)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-aminophenol **10a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (272 mg, 88%), ^{1}H NMR (400 MHz, DMSO-*d*$_6$) δ 14.95 (br s, 1H), 9.85 (s, 1H), 9.14 (s, 1H), 8.61 (d, *J* = 2.9 Hz, 1H), 8.22 (dd, *J* = 9.1, 2.7 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 2H), 7.06 (d, *J* = 9.2 Hz, 1H), 6.88 (d, *J* = 8.6 Hz, 2H), ^{13}C NMR (101 MHz, DMSO-*d*$_6$) δ 168.2, 158.8, 158.2, 139.1, 137.3, 128.9, 128.3, 123.3, 118.9, 118.7, 116.6, HRMS (ESI) calcd for $C_{13}H_9N_2O_4$ (M - H^+) 257.0568, found 257.0567, Melting Point: 274-275℃.

5.4.11 *(E)*-2-(((2-chlorophenyl)imino)methyl)-4-nitrophenol (11)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-chloroaniline **11a** (126 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (262 mg, 79%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.28 (s, 1H), 8.76 (s, 1H), 8.45 (d, *J* = 2.7 Hz, 1H), 8.32 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.56 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.43 – 7.39 (m, 1H), 7.36 – 7.30 (m, 2H), 7.16 (d, *J* = 9.1 Hz, 1H), 13C NMR (101 MHz, Chloroform-d) δ 166.7, 161.3, 143.8, 140.1, 130.5, 129.9, 128.9, 128.7, 128.5, 128.0, 119.1, 118.5, 118.1, HRMS (ESI) calcd for $C_{13}H_8ClN_2O_3$ (M - H^+) 275.0229, found 275.0227, Melting Point: 188-189°C.

5.4.12 *(E)*-2-(((3-chlorophenyl)imino)methyl)-4-nitrophenol (12)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-chloroaniline **12a** (127 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (267 mg, 81%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.98 (s, 1H), 8.73 (s, 1H), 8.44 (d, *J* = 2.8 Hz, 1H), 8.32 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.43 (t, *J* = 8.2 Hz, 1H), 7.38 – 7.35 (m, 2H), 7.24 (dt, *J* = 8.0, 1.5 Hz, 1H), 7.14 (d, *J* = 9.2 Hz, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.4, 162.9, 149.3, 139.9, 134.4, 131.5, 129.0, 128.5, 127.6, 121.6, 121.1, 119.4, 118.5, HRMS (ESI) calcd for $C_{13}H_8ClN_2O_3$ (M - H^+) 275.0229, found 275.0226, Melting Point: 203-204°C.

5.4.13 *(E)*-2-(((4-chlorophenyl)imino)methyl)-4-nitrophenol (13)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-chloroaniline **13a** (153 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (280 mg, 85%), ^{1}H NMR (400 MHz,

Chloroform-*d*) δ 14.12 (s, 1H), 8.72 (s, 1H), 8.42 (d, *J* = 2.9 Hz, 1H), 8.31 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.47 (d, *J* = 8.3 Hz, 2H), 7.30 (d, *J* = 8.5 Hz, 2H), 7.13 (d, *J* = 9.2 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.5, 161.0, 145.4, 140.2, 133.8, 129.9, 128.6, 128.4, 122.5, 118.3, 118.1, HRMS (ESI) calcd for $C_{13}H_8ClN_2O_3$ (M - H^+) 275.0229, found 275.0226, Melting Point: 225-226℃.

5.4.14 *(E)*-4-nitro-2-(((2-(trifluoromethyl)phenyl)imino)methyl)phenol (14)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-aminobenzotrifluoride **14a** (151 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (228 mg, 61%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.56 (s, 1H), 8.72 (s, 1H), 8.46 (d, *J* = 2.7 Hz, 1H), 8.33 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.80 (d, *J* = 7.7 Hz, 1H), 7.70 (t, *J* = 7.7 Hz, 1H), 7.48 (t, *J* = 7.7 Hz, 1H), 7.32 (d, *J* = 7.9 Hz, 1H), 7.17 (d, *J* = 9.2 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.3, 162.4, 145.6, 140.2, 133.5, 128.9, 128.8, 127.7, 126.8 (q, *J* = 5.1 Hz), 124.4 (q, *J* = 30.3 Hz), 123.7 (q, *J* = 273.7 Hz), 119.3, 118.5, 118.1, HRMS (ESI) calcd for $C_{14}H_8F_3N_2O_3$ (M - H^+) 309.0493, found 309.0492, Melting Point: 161-162℃.

5.4.15 *(E)*-4-nitro-2-(((3-(trifluoromethyl)phenyl)imino)methyl)phenol (15)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminobenzotrifluoride **15a** (150 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (239 mg, 64%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.87 (s, 1H), 8.77 (s, 1H), 8.46 (d, *J* = 2.7 Hz, 1H), 8.33 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.65 – 7.60 (m, 3H), 7.54 – 7.51 (m, 1H), 7.16 (d, *J* = 9.1 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.4, 162.4, 147.7, 140.2, 132.3 (q, *J* = 33.3 Hz), 130.3, 128.8, 128.7,

124.5, 124.5 (q, J = 4.0 Hz), 123.6 (q, J = 272.7 Hz), 118.4, 118.3 (q, J = 4.0 Hz),118.0, HRMS (ESI) calcd for $C_{14}H_8F_3N_2O_3$ (M - H^+) 309.0493, found 309.0491, Melting Point: 141-142℃.

5.4.16 *(E)*-4-nitro-2-(((4-(trifluoromethyl)phenyl)imino)methyl)phenol (16)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-aminobenzotrifluoride **16a** (151 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (224 mg, 60%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.86 (s, 1H), 8.75 (s, 1H), 8.46 (d, J = 2.8 Hz, 1H), 8.33 (dd, J = 9.2, 2.7 Hz, 1H), 7.76 (d, J = 8.2 Hz, 2H), 7.43 (d, J = 8.2 Hz, 2H), 7.16 (d, J = 9.2 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.5, 162.8, 150.1, 140.2, 129.8 (q, J = 33.3 Hz), 128.9, 128.7, 126.9 (q, J = 4.0 Hz), 123.9 (q, J = 273.7 Hz), 121.6, 118.4, 117.9, HRMS (ESI) calcd for $C_{14}H_8F_3N_2O_3$ (M - H^+) 309.0493, found 309.0490, Melting Point: 169-170℃.

5.4.17 *(E)*-3-((2-hydroxy-5-nitrobenzylidene)amino)benzoic acid (17)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminobenzoic acid **17a** (165 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (309 mg, 90%), ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.98 (br s, 1H), 13.23 (br s, 1H), 9.24 (s, 1H), 8.74 (d, J = 2.9 Hz, 1H), 8.30 (dd, J = 9.3, 3.0 Hz, 1H), 8.01 – 7.92 (m, 2H), 7.74 – 7.61 (m, 2H), 7.16 (d, J = 9.1 Hz, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 167.3, 166.8, 162.8, 147.7, 139.8, 132.7, 130.3, 129.0, 128.7, 128.6, 126.7, 122.1, 119.3, 118.6, HRMS (ESI) calcd for $C_{14}H_9N_2O_5$ (M - H^+) 285.0517, found 271.0513, Melting Point: 330-331℃.

5.4.18 *(E)*-2-((cyclohexylimino)methyl)-4-nitrophenol (18)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and cyclohexylamine **18a** (137 μL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (183 mg, 62%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 15.18 (s, 1H), 8.34 (s, 1H), 8.24 (d, *J* = 2.9 Hz, 1H), 8.18 (dd, *J* = 9.3, 2.9 Hz, 1H), 6.91 (d, *J* = 9.3 Hz, 1H), 3.50 – 3.43 (m, 1H), 1.99 – 1.85 (m, 4H), 1.73 – 1.55 (m, 3H), 1.50 – 1.30 (m, 3H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 172.7, 162.0, 137.5, 129.2, 128.6, 120.5, 115.5, 64.3, 33.6, 25.1, 24.1, HRMS (ESI) calcd for $C_{13}H_{15}N_2O_3$ (M - H^+) 247.1088, found 247.1089, Melting Point: 139-140℃.

5.4.19 *(E)*-2-((naphthalen-1-ylimino)methyl)-4-nitrophenol (19)

The title compound was prepared according to general procedure (**A**) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 1-aminonaphthalene **19a** (172 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (299 mg, 85%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.55 (s, 1H), 8.82 (s, 1H), 8.48 – 8.47 (m, 1H), 8.34 (dd, *J* = 9.2, 2.4 Hz, 1H), 8.23 – 8.21 (m, 1H), 8.03 – 7.90 (m, 1H), 7.88 (d, *J* = 8.3 Hz, 1H), 7.63 – 7.60 (m, 2H), 7.56 (t, *J* = 8.0 Hz, 1H), 7.27 (d, *J* = 7.9 Hz, 1H), 7.20 (d, *J* = 9.2 Hz, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.7, 161.6, 144.5, 140.1, 134.0, 128.5, 128.4, 128.2, 128.1, 128.0, 127.0, 125.9, 122.7, 118.5, 118.3, 114.4, HRMS (ESI) calcd for $C_{17}H_{11}N_2O_3$ (M - H^+) 291.0775, found 291.0773, Melting Point: 160-161℃.

5.4.20 2-(((3-ethynylphenyl)amino)methyl)-4-nitrophenol (20)

The title compound was prepared according to general procedure (**B**) using **1** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (hexane : EtOAc = 15: 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.58 (br s,

1H), 8.16 – 8.14 (m, 2H), 7.24 (t, *J* = 7.9 Hz, 1H), 7.13 (d, *J* = 7.8 Hz, 1H), 7.00 – 6.95 (m, 2H), 6.86 (d, *J* = 8.2 Hz, 1H), 4.54 (s, 2H), 4.16 (s, 1H), 3.09 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.81, 146.12, 140.93, 129.59, 125.66, 125.53, 124.63, 123.34, 122.81, 119.54, 117.14, 116.90, 83.21, 77.59, 48.46, HRMS (ESI) calcd for $C_{15}H_{11}N_2O_3$ (M - H^+) 267.0775, found 267.0773, Melting point: 134-135°C.

5.4.21 4-nitro-2-((phenylamino)methyl)phenol (21)

The title compound was prepared according to general procedure (**B**) using **4** (100 mg, 0.41 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.13 (br s, 1H), 8.17 – 8.15 (m, 2H), 7.32 – 7.28 (m, 2H), 7.02 (t, *J* = 7.4 Hz, 1H), 6.95 (d, *J* = 8.7 Hz, 1H), 6.90 (d, *J* = 8.0 Hz, 2H), 4.57 (s, 2H), 4.15 (br s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.3, 146.0, 140.8, 129.6, 125.4, 124.5, 122.9, 122.1, 117.1, 116.6, 49.1, HRMS (ESI) calcd for $C_{13}H_{11}N_2O_3$ (M - H^+) 243.0775, found 243.0775, Melting Point: 102-103°C.

5.4.22 2-(((2-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (22)

The title compound was prepared according to general procedure (**B**) using **5** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.29 (br s, 1H), 8.19 – 8.15 (m, 2H), 7.38 (dd, *J* = 8.0, 1.5 Hz, 1H), 7.20 (td, *J* = 7.6, 1.5 Hz, 1H), 7.01 – 6.95 (m, 3H), 4.60 (s, 2H), 4.43 (br s, 1H), 1.48 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.3, 144.9, 140.8, 137.4, 127.6, 126.9, 125.4, 124.5, 123.2, 121.9, 117.1, 116.8, 50.0, 34.4, 30.7, HRMS (ESI) calcd for $C_{17}H_{19}N_2O_3$ (M - H^+) 299.1401, found 299.1401, Melting Point: 135-136°C.

5.4.23 2-(((3-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (23)

The title compound was prepared according to general procedure (**B**) using **6** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.30 (br s, 1H), 8.17 – 8.15 (m, 2H), 7.24 (t, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 7.9 Hz, 1H), 6.97 – 6.92 (m, 2H), 6.72 (d, *J* = 8.1 Hz, 1H), 4.57 (s, 2H), 4.08 (s, 1H), 1.30 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.4, 152.9, 145.8, 140.7, 129.3, 125.4, 124.5, 123.1, 119.4, 117.1, 114.3, 113.4, 49.4, 34.7, 31.2, HRMS (ESI) calcd for $C_{17}H_{19}N_2O_3$ (M - H^+) 299.1401, found 299.1399, Melting Point: 112-113℃.

5.4.24 2-(((4-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (24)

The title compound was prepared according to general procedure (**B**) using **7** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.44 (br s, 1H), 8.17 – 8.12 (m, 2H), 7.34 – 7.28 (m, 2H), 6.94 (d, *J* = 8.8 Hz, 1H), 6.87 – 6.84 (m, 2H), 4.55 (s, 2H), 4.07 (br s, 1H), 1.31 (s, 9H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.6, 145.2, 143.5, 140.7, 126.4, 125.4, 124.5, 123.0, 117.1, 116.5, 49.5, 34.2, 31.4, HRMS (ESI) calcd for $C_{17}H_{19}N_2O_3$ (M - H^+) 299.1401, found 299.1404, Melting Point: 158-159℃.

5.4.25 2-(((2-hydroxyphenyl)amino)methyl)-4-nitrophenol (25)

The title compound was prepared according to general procedure (**B**) using **8** (100 mg, 0.39 mmol), yielding the pure product as a red solid (90 mg, 89%) after flash chromatography (DCM : MeOH = 100 : 1), ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.17 (br s, 1H), 9.35 (br s, 1H), 8.06 (d, *J* = 2.9 Hz, 1H), 8.02 (dd, *J* = 8.9, 3.0 Hz, 1H), 6.99 (d, *J* = 8.9 Hz, 1H), 6.68 (d, *J* =

7.7 Hz, 1H), 6.55 (t, *J* = 7.6 Hz, 1H), 6.41 (t, *J* = 7.6 Hz, 1H), 6.31 (d, *J* = 7.8 Hz, 1H), 4.29 (s, 2H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.1, 144.7, 140.1, 137.3, 128.4, 124.6, 124.2, 120.1, 116.6, 115.5, 114.1, 110.3, 41.7, HRMS (ESI) calcd for $C_{13}H_{11}N_2O_4$ (M - H^+) 259.0724, found 259.0723, Melting Point: 136-137°C.

5.4.26 2-(((3-hydroxyphenyl)amino)methyl)-4-nitrophenol (26)

The title compound was prepared according to general procedure (**B**) using **9** (100 mg, 0.39 mmol), yielding the pure product as a red solid (87 mg, 86%) after flash chromatography (DCM : MeOH = 100 : 1), ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.29 (br s, 1H), 8.93 (s, 1H), 8.06 – 8.02 (m, 2H), 7.00 (d, *J* = 8.9 Hz, 1H), 6.83 (t, *J* = 7.8 Hz, 1H), 6.04 – 5.96 (m, 3H), 4.19 (s, 2H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.3, 158.7, 150.2, 139.9, 130.1, 128.3, 124.6, 124.0, 115.6, 104.3, 104.1, 99.5, 41.4, HRMS (ESI) calcd for $C_{13}H_{11}N_2O_4$ (M - H^+) 259.0724, found 259.0723, Melting Point: 142-143°C.

5.4.27 2-(((2-chlorophenyl)amino)methyl)-4-nitrophenol (27)

The title compound was prepared according to general procedure (**B**) using **11** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.27 (br s, 1H), 8.18 – 8.16 (m, 2H), 7.38 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.21 (td, *J* = 7.8, 1.6 Hz, 1H), 6.97 (d, *J* = 9.8 Hz, 1H), 6.95 – 6.88 (m, 2H), 4.69 (d, *J* = 5.9 Hz, 1H), 4.59 (d, *J* = 4.9 Hz, 2H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.5, 142.6, 141.0, 129.6, 128.1, 125.5, 124.7, 123.2, 122.3, 121.6, 117.1, 114.8, 47.7, HRMS (ESI) calcd for $C_{13}H_{10}ClN_2O_3$ (M - H^+) 277.0385, found 277.0384, Melting Point: 104-105°C.

5.4.28 2-(((3-chlorophenyl)amino)methyl)-4-nitrophenol (28)

The title compound was prepared according to general procedure (**B**) using **12** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.27 (s, 1H), 8.17 (d, *J* = 8.5 Hz, 2H), 7.20 (t, *J* = 8.0 Hz, 1H), 6.98 – 6.95 (m, 2H), 6.87 (d, *J* = 2.2 Hz, 1H), 6.74 (dd, *J* = 8.1, 2.4 Hz, 1H), 4.53 (d, *J* = 5.1 Hz, 2H), 4.17 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.5, 147.5, 141.0, 135.3, 130.6, 125.5, 124.7, 122.9, 121.6, 117.1, 116.1, 114.2, 48.1, HRMS (ESI) calcd for $C_{13}H_{10}ClN_2O_3$ (M - H^+) 277.0385, found 277.0387, Melting Point: 116-117℃.

5.4.29 2-(((4-chlorophenyl)amino)methyl)-4-nitrophenol (29)

The title compound was prepared according to general procedure (**B**) using **13** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (Hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.67 (br s, 1H), 8.17 – 8.14 (m, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 6.96 (d, *J* = 8.6 Hz, 1H), 6.81 (d, *J* = 8.4 Hz, 2H), 4.53 (s, 2H), 4.16 (br s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.9, 144.7, 140.9, 129.5, 126.9, 125.5, 124.6, 122.8, 117.5, 117.1, 48.7, HRMS (ESI) calcd for $C_{13}H_{10}ClN_2O_3$ (M - H^+) 277.0385, found 277.0386, Melting Point: 123-124℃.

5.4.30 4-nitro-2-(((2-(trifluoromethyl)phenyl)amino)methyl)phenol (30)

The title compound was prepared according to general procedure (**B**) using **14** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.81 (br s, 1H), 8.20 (d, *J* = 2.8 Hz, 1H), 8.16 (dd, *J* = 8.9, 2.8 Hz, 1H), 7.60 (d, *J* = 7.8 Hz, 1H), 7.47 (t, *J* = 7.9 Hz, 1H), 7.04 (t, *J* = 7.6 Hz, 1H), 7.06 – 6.95 (m, 2H), 4.71 (br s, 1H), 4.61 (d, *J* = 5.1

Hz, 2H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.1, 143.9, 141.1, 133.4, 126.9 (q, *J* = 6.1 Hz), 125.5, 124.9, 124.7 (q, *J* = 273.7 Hz), 123.2, 120.1, 117.3 (q, *J* = 30.3 Hz), 117.0, 114.8, 46.5, HRMS (ESI) calcd for $C_{14}H_{10}F_3N_2O_3$ (M - H^+) 311.0649, found 311.0651, Melting Point: 114-115℃.

5.4.31 4-nitro-2-(((3-(trifluoromethyl)phenyl)amino)methyl)phenol (31)

The title compound was prepared according to general procedure (**B**) using **15** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.17 – 8.15 (m, 2H), 7.39 (t, *J* = 8.0 Hz, 1H), 7.22 (d, *J* = 7.7 Hz, 1H), 7.10 (s, 1H), 7.02 – 6.96 (m, 2H), 4.57 (s, 2H), 4.36 (br s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.3, 146.9, 140.9, 131.8 (q, *J* = 32.3 Hz), 130.1, 125.5, 124.8, 123.9 (q, *J* = 273.7 Hz), 123.3, 118.6, 117.7 (q, *J* = 4.0 Hz), 116.9, 112.3 (q, *J* = 4.0 Hz), 47.4, HRMS (ESI) calcd for $C_{14}H_{10}F_3N_2O_3$ (M - H^+) 311.0649, found 311.0650, Melting Point: 104-105℃.

5.4.32 4-nitro-2-(((4-(trifluoromethyl)phenyl)amino)methyl)phenol (32)

The title compound was prepared according to general procedure (**B**) using **16** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (89 mg, 88%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.68 (s, 1H), 8.19 – 8.15 (m, 2H), 7.52 (d, *J* = 8.3 Hz, 2H), 6.97 (d, *J* = 8.6 Hz, 1H), 6.89 (d, *J* = 8.3 Hz, 2H), 4.57 (d, *J* = 4.6 Hz, 2H), 4.42 (br s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.1, 151.8, 140.1, 127.1, 126.8 (d, *J* = 4.0 Hz), 125.7 (q, *J* = 271.7 Hz), 124.9, 124.1, 116.2 (q, *J* = 31.3 Hz), 115.7, 112.0, 40.9, HRMS (ESI) calcd for $C_{14}H_{10}F_3N_2O_3$ (M - H^+) 311.0649, found 311.0654, Melting Point: 151-152℃.

5.4.33 3-((2-hydroxy-5-nitrobenzyl)amino)benzoic acid (33)

The title compound was prepared according to general procedure (**B**) using **17** (100 mg, 0.35 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (DCM : MeOH = 50 : 1), ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.65 (br s, 1H), 11.41 (br s, 1H), 8.45 – 7.86 (m, 2H), 7.20 – 7.13 (m, 3H), 7.02 (d, J = 8.8 Hz, 1H), 6.79 (d, J = 7.7 Hz, 1H), 6.58 (br s, 1H), 4.28 (s, 2H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 168.2, 162.1, 148.9, 140.1, 132.0, 129.6, 127.6, 124.8, 124.0, 117.6, 116.8, 115.6, 113.1, 41.2, HRMS (ESI) calcd for $C_{14}H_{11}N_2O_5$ (M - H^+) 287.0673, found 287.0673, Melting Point: 190-191℃.

5.4.34 2-((cyclohexylamino)methyl)-4-nitrophenol (34)

The title compound was prepared according to general procedure (**B**) using **18** (100 mg, 0.40 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (Hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-d) δ 8.08 (d, J = 9.0 Hz, 1H), 7.94 (s, 1H), 6.83 (d, J = 9.1 Hz, 1H), 6.27 (br s, 2H), 4.13 (s, 2H), 2.59 – 2.54 (m, 1H), 2.08 – 1.99 (m, 2H), 1.79 – 1.64 (m, 3H), 1.34 – 1.12 (m, 5H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 176.1, 131.8, 127.1, 127.0, 120.8, 118.5, 55.4, 46.7, 29.9, 25.4, 24.4, HRMS (ESI) calcd for $C_{13}H_{17}N_2O_3$ (M - H^+) 249.1245, found 249.1245, Melting Point: 177-178℃.

5.4.35 2-((naphthalen-1-ylamino)methyl)-4-nitrophenol (35)

The title compound was prepared according to general procedure (**B**) using **19** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-d) δ 10.00 (br s, 1H), 8.22 (s, 1H), 8.16 (d, J = 9.2 Hz, 1H), 7.95 – 7.88 (m, 2H), 7.57 – 7.53 (m, 3H), 7.40 (t, J = 7.9 Hz, 1H), 6.97 – 6.93 (m, 2H), 4.70 – 4.62 (m, 3H), ^{13}C NMR (101 MHz, Chloroform-d)

δ 163.2, 140.9, 140.8, 134.2, 129.0, 126.4, 126.2, 126.0, 125.5, 125.4, 124.9, 122.9, 122.4, 119.8, 117.2, 110.0, 48.4, HRMS (ESI) calcd for $C_{17}H_3N_2O_3$ (M - H^+) 293.0932, found 293.0929, Melting Point: 144-145°C.

5.4.36 methyl *(E)*-3-(((3-ethynylphenyl)imino)methyl)-4-hydroxybenzoate (36)

step 1: methyl 3-formyl-4-hydroxybenzoate (**36a**). The title compound was prepared according to general procedure (**C**) using methyl 4-hydroxybenzoate (500 mg, 3.29 mmol), yielding the pure product as a white solid (310 mg, 52%) after flash chromatography (hexane : EtOAc = 30: 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 11.42 (s, 1H), 9.98 (s, 1H), 8.35 (d, *J* = 2.2 Hz, 1H), 8.22 (dd, *J* = 8.8, 2.2 Hz, 1H), 7.06 (d, *J* = 8.7 Hz, 1H), 3.96 (s, 3H).

step 2: The title compound was prepared according to general procedure (**A**) using **36a** (200 mg, 1.11 mmol) and 3-aminophenylacetylene **17a** (125 µL, 1.11 mmol, 1 equiv), yielding the pure product as a white solid (257 mg, 83%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.68 (s, 1H), 8.70 (s, 1H), 8.19 (d, *J* = 2.2 Hz, 1H), 8.09 (dd, *J* = 8.7, 2.2 Hz, 1H), 7.47 – 7.41 (m, 3H), 7.32 – 7.29 (m, 1H), 7.08 (d, *J* = 8.7 Hz, 1H), 3.94 (s, 3H), 3.16 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.2, 165.1, 162.7, 147.9, 134.8, 134.6, 130.9, 129.6, 124.6, 123.5, 122.0, 121.4, 118.5, 117.6, 82.8, 78.1, 52.1, HRMS (ESI) calcd for $C_{17}H_{12}NO_3$ (M - H^+) 278.0823, found 278.0822, Melting Point: 153-154°C.

5.4.37 *(E)*-2-(((3-ethynylphenyl)imino)methyl)-4-fluorophenol (37)

The title compound was prepared according to general procedure (**A**) using 5-fluorosalicylaldehyde **37a** (300 mg, 2.14 mmol) and 3-aminophenylacetylene **17a** (241 µL, 2.14 mmol, 1 equiv), yielding the pure product as a red solid (346 mg, 68%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.80 (s, 1H), 8.58 (s, 1H), 7.47 – 7.40 (m, 3H), 7.31 – 7.30 (m, 1H),

7.17 – 7.11 (m, 2H), 7.01 (dd, *J* = 8.9, 4.5 Hz, 1H), 3.16 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.3 (d, *J* = 2.9 Hz), 157.3 (d, *J* = 1.6 Hz), 155.6 (d, *J* = 237.3 Hz), 148.2, 130.8, 129.5, 124.5, 123.4, 122.1, 120.6 (d, *J* = 23.4 Hz), 118.7 (d, *J* = 7.2 Hz), 118.5 (d, *J* = 7.4 Hz), 117.2 (d, *J* = 23.2 Hz), 82.8, 78.1, HRMS (ESI) calcd for $C_{15}H_9FNO$ (M - H^+) 238.0674, found 238.0672, Melting Point: 113-114°C.

5.4.38 *(E)*-3-(((3-ethynylphenyl)imino)methyl)-4-hydroxybenzonitrile (38)

step 1: 5-cyano-2-methoxybenzaldehyde (**38a**). To a solution of 5-cyano-2-fluorobenzaldehyde (500 mg, 3.35 mmol) in MeOH (5 mL) was added CH_3ONa (362 mg, 6.71 mmol, 2 equiv). The mixture was stirred at rt for 3h. After the reaction was completed, the product was extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na_2SO_4 and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 30: 1) as a white solid (478 mg, 88%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.44 (s, 1H), 8.13 (s, 1H), 7.84 (d, *J* = 8.8 Hz, 1H), 7.12 (d, *J* = 8.9 Hz, 1H), 4.04 (s, 3H).

step 2: 3-formyl-4-hydroxybenzonitrile (**38b**). To a solution of compound **38a** (478 mg, 2.97 mmol) in dry DCM (10 mL) was added BBr_3 (8.9 mL, 1 M in DCM, 3 equiv) dropwisely via syringe under N_2 atmosphere. The resulting mixture was stirred at rt for 10h before the reaction mixture was quenched with MeOH, and the product was extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na_2SO_4 and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 15: 1) as a white solid (309 mg, 71%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 11.48 (s, 1H), 9.95 (s, 1H), 7.96 (d, *J* = 2.1 Hz, 1H), 7.80 (dd, *J* = 8.8, 2.1 Hz, 1H), 7.13 (d, *J* = 8.8 Hz, 1H).

step 3: The title compound was prepared according to general procedure (**A**) using **38b** (200 mg, 1.36 mmol) and 3-aminophenylacetylene **17a** (153 µL, 1.36 mmol, 1 equiv), yielding the pure product as a yellow solid (287 mg, 86%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.86 (s, 1H), 8.65 (s, 1H), 7.76 (d, *J* = 2.0 Hz, 1H), 7.66 (dd, *J* = 8.6, 2.1 Hz, 1H), 7.50 – 7.42 (m, 3H), 7.31 (d, *J* = 7.8 Hz, 1H), 7.13 (d, *J* = 8.6 Hz, 1H), 3.17 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.3, 163.0, 148.2, 137.3, 137.0, 131.0, 130.4, 124.6, 123.4, 123.1, 120.4, 119.1, 118.8, 102.0, 83.3, 81.9, HRMS (ESI) calcd for $C_{16}H_9N_2O$ (M - H^+) 245.0720, found 245.0719, Melting Point: 222-223℃.

5.4.39 *(E)*-2-(3-(((3-ethynylphenyl)imino)methyl)-4-hydroxyphenyl)acetonitrile (39)

step 1: 2-(3-formyl-4-hydroxyphenyl)acetonitrile (**39a**). The title compound was prepared according to general procedure (**C**) using 4-hydroxybenzyl cyanide (500 mg, 3.76 mmol), yielding the mixture (product : starting material = 7:3) as a white solid (486 mg) after flash chromatography (hexane : EtOAc = 30: 1), which was used directly in the next step.

step 2: The title compound was prepared according to general procedure (**A**) using crude **39a** obtained in step 1 (486 mg) and 3-aminophenylacetylene **17a** (251 µL, 2.23 mmol), yielding the pure product as a white solid (348 mg, 36% in two steps), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.12 (s, 1H), 8.63 (s, 1H), 7.46 – 7.39 (m, 4H), 7.34 – 7.28 (m, 2H), 7.06 (d, *J* = 8.5 Hz, 1H), 3.75 (s, 2H), 3.16 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.6, 160.9, 148.2, 132.8, 131.5, 130.8, 129.6, 124.6, 123.5, 122.0, 120.4, 119.2, 118.3, 117.8, 82.8, 78.1, 22.8, HRMS (ESI) calcd for $C_{17}H_{11}N_2O$ (M - H^+) 259.0877, found 259.0876, Melting Point: 119-120℃.

5.4.40 *(E)*-2-(((3-ethynylphenyl)imino)methyl)-4-methoxyphenol (40)

step 1: 2-hydroxy-5-methoxybenzaldehyde (**40a**). The title compound was prepared according to general procedure (**C**) using 4-methoxyphenol (300 mg, 2.42 mmol), yielding the pure product as a white solid (286 mg, 78%) after flash chromatography (hexane : EtOAc = 30: 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.7 (s, 1H), 9.9 (s, 1H), 7.2 (dd, *J* = 9.1, 3.1 Hz, 1H), 7.0 (d, *J* = 3.1 Hz, 1H), 7.0 (d, *J* = 9.0 Hz, 1H), 3.8 (s, 4H).

step 2: The title compound was prepared according to general procedure (**A**) using **40a** (300 mg, 1.97 mmol) and 3-aminophenylacetylene **17a** (222 µL, 1.97 mmol, 1 equiv), yielding the pure product as a white solid (295 mg, 65%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 12.58 (s, 1H), 8.60 (s, 1H), 7.45 – 7.38 (m, 3H), 7.31 – 7.28 (m, 1H), 7.06 – 6.98 (m, 2H), 6.92 (d, *J* = 3.0 Hz, 1H), 3.83 (s, 3H), 3.15 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.4, 154.8, 152.4, 149.3, 130.4, 130.3, 124.5, 123.3, 123.0, 121.3, 119.6, 118.0, 115.6, 83.5, 81.7, 56.0, HRMS (ESI) calcd for $C_{16}H_{12}NO_2$ (M - H^+) 250.0874, found 250.0875, Melting Point: 116-117°C.

5.4.41 *(E)*-2-(((3-ethynylphenyl)imino)methyl)phenol (41)

The title compound was prepared according to general procedure (**A**) using salicylaldehyde **41a** (250 mg, 2.05 mmol) and 3-aminophenylacetylene **17a** (230 µL, 2.05 mmol, 1 equiv), yielding the pure product as a white solid (303 mg, 67%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.03 (s, 1H), 8.64 (s, 1H), 7.45 – 7.39 (m, 5H), 7.31 – 7.28 (m, 1H), 7.06 (dd, *J* = 8.7, 1.1 Hz, 1H), 6.98 (td, *J* = 7.4, 1.1 Hz, 1H), 3.15 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.5, 161.2, 148.6, 133.5, 132.5, 130.4, 129.5, 124.5, 123.3, 122.1, 119.2, 119.1, 117.3, 83.0, 77.9, HRMS (ESI) calcd for $C_{15}H_{10}NO$ (M - H^+) 220.0768, found 220.0772, Melting Point: 116-117°C.

5.4.42 *(E)*-N-(3-ethynylphenyl)-1-(3-nitrophenyl)methanimine (42)

The title compound was prepared according to general procedure (**A**) using 3-nitro benzaldehyde **42a** (250 mg, 1.65 mmol) and 3-aminophenylacetylene **17a** (186 µL, 1.65 mmol, 1 equiv), yielding the pure product as a yellow solid (284 mg, 69%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.77 (t, *J* = 2.0 Hz, 1H), 8.56 (s, 1H), 8.37 (ddd, *J* = 8.2, 2.3, 1.1 Hz, 1H), 8.27 (dt, *J* = 7.7, 1.4 Hz, 1H), 7.70 (t, *J* = 7.9 Hz, 1H), 7.45 – 7.38 (m, 3H), 7.27 (dt, *J* = 7.3, 1.9 Hz, 1H), 3.14 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 158.0, 150.9, 148.7, 137.6, 134.2, 130.4, 129.9, 129.4, 125.8, 124.2, 123.6, 123.2, 121.9, 83.1, 77.8, HRMS (ESI) calcd for $C_{15}H_{11}N_2O_2$ (M + H^+) 251.0815, found 251.0818, Melting Point: 126-127°C.

5.4.43 *(E)*-N-(3-ethynylphenyl)-1-(2-methoxy-5-nitrophenyl)methanimine (43)

step 1: 2-methoxy-5-nitrobenzaldehyde (**43a**). To a solution of 2-fluoro-5-nitrobenzaldehyde (300 mg, 1.77 mmol) in MeOH (5 mL) was added CH_3ONa (144 mg, 2.67 mmol, 1.5 equiv). The mixture was stirred at rt for 3h before additional CH_3ONa (48 mg, 0.89 mmol, 0.5 equiv) was added, and resulting mixture was allowed to stirred at rt for another 1h. After the reaction was completed, the product was extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na_2SO_4 and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 20: 1) as a pale yellow solid (273 mg, 85%),

step 2: The title compound was prepared according to general procedure (**A**) using **43a** (200 mg, 1.10 mmol) and 3-aminophenylacetylene **17a** (124 µL, 1.10 mmol, 1 equiv), yielding the pure product as a yellow solid (265 mg, 86%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.06 (d, *J* = 2.9 Hz, 1H), 8.88 (s, 1H), 8.36 (dd, *J* = 9.1, 2.9 Hz, 1H), 7.43 – 7.36 (m, *J* = 4.5 Hz,

3H), 7.27 – 7.26 (m, 1H), 7.08 (d, *J* = 9.2 Hz, 1H), 4.06 (s, 3H), 3.13 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.5, 154.5, 151.6, 141.8, 130.1, 129.2, 127.9, 125.1, 124.2, 123.6, 123.0, 122.3, 111.3, 83.3, 77.5, 56.5, HRMS (ESI) calcd for $C_{16}H_{13}N_2O_3$ (M + H^+) 281.0921, found 281.0922, Melting Point: 156-157°C.

5.4.44 *(E)*-2-(((3-ethynylphenyl)imino)methyl)-5-nitrophenol (44)

The title compound was prepared according to general procedure (**A**) using 4-nitrosalicylaldehyde **44a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **17a** (135 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (278 mg, 87%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 13.44 (s, 1H), 8.74 (s, 1H), 7.89 (d, *J* = 2.2 Hz, 1H), 7.81 (dd, *J* = 8.4, 2.2 Hz, 1H), 7.60 (d, *J* = 8.4 Hz, 1H), 7.51 – 7.43 (m, 3H), 7.35 (dt, *J* = 7.8, 1.7 Hz, 1H), 3.18 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.2, 160.5, 150.3, 148.9, 133.2, 131.1, 130.4, 125.4, 124.7, 123.4, 123.2, 114.2, 111.9, 83.3, 81.9, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ (M - H^+) 265.0619, found 265.0620, Melting Point: 204-205°C.

5.4.45 *(E)*-2-(((3-ethynylphenyl)imino)methyl)-6-nitrophenol (45)

The title compound was prepared according to general procedure (**A**) using 3-nitrosalicylaldehyde **45a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **17a** (135 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (235 mg, 74%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 14.92 (s, 1H), 8.76 (s, 1H), 8.16 (dd, *J* = 8.1, 1.7 Hz, 1H), 7.76 (dd, *J* = 7.6, 1.7 Hz, 1H), 7.50 – 7.42 (m, 3H), 7.34 (dt, *J* = 7.8, 1.7 Hz, 1H), 7.06 (t, *J* = 7.9 Hz, 1H), 3.17 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.2, 156.6, 146.5, 137.8, 137.5, 131.5, 129.7, 129.6, 124.3, 123.7, 122.1, 121.5, 118.1, 82.5, 78.5, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ (M - H^+) 265.0619, found 265.0616, Melting Point: 140-141°C.

5.4.46 *(E)*-3-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (46)

The title compound was prepared according to general procedure (**A**) using 5-hydroxy-2-nitrobenzaldehyde **46a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **17a** (135 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (261 mg, 82%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.07 (s, 1H), 8.16 (d, *J* = 8.8 Hz, 1H), 7.61 (d, *J* = 2.9 Hz, 1H), 7.47 – 7.44 (m, 3H), 7.32 (dt, *J* = 7.8, 1.8 Hz, 1H), 7.03 (dd, *J* = 8.8, 2.8 Hz, 1H), 3.15 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 163.0, 159.4, 151.6, 141.3, 134.0, 130.3, 128.2, 124.3, 123.2, 122.7, 118.4, 115.4, 83.5, 81.7, HRMS (ESI) calcd for $C_{15}H_9N_2O_3$ (M - H^+) 265.0619, found 265.0621, Melting Point: 168-169°C,

5.4.47 *(E)*-2-bromo-6-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (47)

The title compound was prepared according to general procedure (**A**) using 3-bromo-2-hydroxy-5-nitrobenzaldehyde **47a** (150mg, 0.61 mmol) and 3-aminophenylacetylene **17a** (69 µL, 0.61 mmol, 1 equiv), yielding the pure product as a yellow solid (182 mg, 86%), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 15.52 (s, 1H), 8.73 (s, 1H), 8.61 (d, *J* = 2.7 Hz, 1H), 8.40 (d, *J* = 2.7 Hz, 1H), 7.55 – 7.46 (m, 3H), 7.37 (dt, *J* = 7.9, 1.8 Hz, 1H), 3.20 (s, 1H), ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.3, 163.4, 141.3, 136.5, 132.0, 131.9, 131.7, 130.7, 124.0, 123.7, 122.6, 115.8, 115.1, 82.9, 82.6, HRMS (ESI) calcd for $C_{15}H_8BrN_2O_3$ (M - H^+) 342.9724, found 342.9727, Melting Point: 221-222°C.

5.4.48 *(E)*-2,4-dichloro-6-(((3-ethynylphenyl)imino)methyl)phenol (48)

The title compound was prepared according to general procedure (**A**) using 3,5-dichlorosalicylaldehyde **48a** (200 mg, 1.05 mmol) and 3-aminophenylacetylene **17a** (118 µL, 1.05 mmol, 1 equiv), yielding the pure product as a yellow solid (224 mg, 74%), ^{1}H NMR (400

MHz, Chloroform-*d*) δ 13.96 (s, 1H), 8.58 (s, 1H), 7.51 – 7.41 (m, 4H), 7.34 – 7.28 (m, 2H), 3.17 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 161.2, 155.9, 147.1, 133.0, 131.4, 129.9, 129.7, 124.4, 123.6, 123.6, 122.9, 122.1, 120.1, 82.6, 78.4, HRMS (ESI) calcd for $C_{15}H_8Cl_2N_2O$ (M - H^+) 287.9988, found 287.9987, Melting Point: 122-123℃.

5.4.49 methyl 3-(((3-ethynylphenyl)amino)methyl)-4-hydroxybenzoate (49)

The title compound was prepared according to general procedure (**B**) using **36** (100 mg, 0.36 mmol), yielding the pure product as a white solid (90 mg, 89%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.92 (br s, 1H), 7.95 – 7.91 (m, 2H), 7.21 (t, *J* = 7.8 Hz, 1H), 7.10 – 7.08 (m, 1H), 6.99 (t, *J* = 1.9 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 1H), 6.85 (dd, *J* = 8.0, 2.4 Hz, 1H), 4.47 (s, 2H), 4.09 (br s, 1H), 3.91 (s, 3H), 3.07 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.88, 161.00, 146.72, 131.28, 130.64, 129.44, 124.97, 123.13, 122.40, 122.09, 119.23, 116.62, 116.60, 83.44, 77.29, 51.96, 48.24, HRMS (ESI) calcd for $C_{17}H_{14}NO_3$ (M - H^+) 280.0979, found 280.0974, Melting point: 94-95℃.

5.4.50 2-(((3-ethynylphenyl)amino)methyl)-4-fluorophenol (50)

The title compound was prepared according to general procedure (B) using **37** (100 mg, 0.41 mmol), yielding the pure product as a red solid (90 mg, 89%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.77 (s, 1H), 7.21 (t, *J* = 7.9 Hz, 1H), 7.07 (d, *J* = 7.6 Hz, 1H), 6.97 – 6.90 (m, 3H), 6.86 – 6.81 (m, 2H), 4.39 (s, 2H), 4.02 (br s, 1H), 3.07 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 156.6 (d, *J* = 237.9 Hz), 152.3, 146.9, 129.4, 124.6, 123.8 (d, *J* = 7.0 Hz), 123.1, 118.8, 117.4 (d, *J* = 7.9 Hz), 116.4, 115.5 (d,

J = 22.8 Hz), 115.1 (d, *J* = 23.5 Hz), 83.5, 77.2, 47.8, HRMS (ESI) calcd for $C_{15}H_{11}FNO$ (M - H^+) 240.0830, found 240.0829, Melting Point: 78-79℃.

5.4.51 3-(((3-ethynylphenyl)amino)methyl)-4-hydroxybenzonitrile (51)

The title compound was prepared according to general procedure (**B**) using **38** (100 mg, 0.41 mmol), yielding the pure product as a white solid (94 mg, 93%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 9.11 (br s, 1H), 7.54 (dd, *J* = 8.4, 2.1 Hz, 1H), 7.50 (d, *J* = 2.0 Hz, 1H), 7.23 (t, *J* = 7.9 Hz, 1H), 7.11 (d, *J* = 7.6 Hz, 1H), 6.98 – 6.94 (m, 2H), 6.83 (dd, *J* = 8.1, 2.5 Hz, 1H), 4.46 (s, 2H), 3.09 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 160.7, 146.3, 133.6, 132.6, 129.5, 125.2, 123.9, 123.2, 119.2, 117.6, 116.7, 103.3, 83.3, 77.5, 47.9, HRMS (ESI) calcd for $C_{16}H_{11}N_2O$ (M - H^+) 247.0877, found 247.0876, Melting Point: 110-111℃.

5.4.52 2-(3-(((3-ethynylphenyl)amino)methyl)-4-hydroxyphenyl)acetonitrile (52)

The title compound was prepared according to general procedure (**B**) using **39** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.25 – 7.12 (m, 3H), 7.07 (d, *J* = 7.6 Hz, 1H), 6.97 – 6.97 (m, 1H), 6.90 (d, *J* = 8.1 Hz, 1H), 6.83 (dd, *J* = 8.1, 2.4 Hz, 1H), 4.42 (s, 2H), 3.70 (s, 2H), 3.08 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 156.2, 146.9, 129.4, 128.8, 128.3, 124.6, 123.6, 123.1, 121.3, 118.9, 118.3, 117.3, 116.4, 83.5, 77.2, 47.9, 22.9, HRMS (ESI) calcd for $C_{17}H_{13}N_2O$ (M - H^+) 261.1033, found 261.1032, Melting Point: 113-114℃.

5.4.53 2-(((3-ethynylphenyl)amino)methyl)-4-methoxyphenol (53)

The title compound was prepared according to general procedure (**B**) using **40** (100 mg, 0.39 mmol), yielding the pure product as a white solid (92 mg, 91%) after flash chromatography (hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.42 (br s, 1H), 7.20 (t, *J* = 7.9 Hz, 1H), 7.06 – 7.03 (m, 1H), 6.96 (t, *J* = 1.9 Hz, 1H), 6.85 – 6.76 (m, 4H), 4.36 (s, 2H), 4.02 (br s, 1H), 3.79 (s, 3H), 3.07 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 153.3, 150.0, 147.2, 129.3, 124.2, 123.7, 123.0, 118.7, 117.1, 116.2, 114.6, 114.1, 83.7, 55.8, 48.0, HRMS (ESI) calcd for $C_{16}H_{14}NO_2$ (M - H^+) 252.1030, found 252.1023, Melting Point: 92-93℃.

5.4.54 2-(((3-ethynylphenyl)amino)methyl)phenol (54)

The title compound was prepared according to general procedure (**B**) using **41** (100 mg, 0.44 mmol), yielding the pure product as a white solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.88 (s, 1H), 7.26 – 7.18 (m, 3H), 7.06 (d, *J* = 7.6 Hz, 1H), 6.99 (t, *J* = 1.9 Hz, 1H), 6.93 – 6.90 (m, 2H), 6.84 (dd, *J* = 8.1, 2.5 Hz, 1H), 4.42 (d, *J* = 5.6 Hz, 2H), 4.00 (d, *J* = 7.0 Hz, 1H), 3.07 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 156.4, 147.2, 129.4, 129.4, 128.9, 124.4, 123.0, 122.7, 120.3, 118.8, 116.6, 116.4, 83.6, 77.1, 48.1, HRMS (ESI) calcd for $C_{15}H_{12}NO$ (M - H^+) 222.0924, found 222.0923, Melting Point: 125-126℃.

5.4.55 3-ethynyl-N-(3-nitrobenzyl)aniline (55)

The title compound was prepared according to general procedure (**B**) using **42** (100 mg, 0.40 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 20 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.26 – 8.25 (m, 1H), 8.16 (dd, *J* = 8.2, 2.2 Hz, 1H), 7.72 (d, *J* = 7.6 Hz, 1H), 7.54 (t, *J* = 7.9 Hz, 1H), 7.14

(t, *J* = 7.9 Hz, 1H), 6.91 (d, *J* = 7.6 Hz, 1H), 6.74 (t, *J* = 1.9 Hz, 1H), 6.62 (dd, *J* = 8.3, 2.5 Hz, 1H), 4.48 (d, *J* = 5.9 Hz, 2H), 4.29 (t, *J* = 6.1 Hz, 1H), 3.03 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 148.6, 147.2, 141.5, 133.2, 129.7, 129.4, 122.9, 122.4, 122.2, 122.0, 116.0, 113.9, 84.0, 76.7, 47.3, HRMS (ESI) calcd for $C_{15}H_{13}N_2O_2$ ($M + H^+$) 253.0972, found 253.0974, Melting Point: 62-63°C.

5.4.56 3-ethynyl-N-(2-methoxy-5-nitrobenzyl)aniline (56)

The title compound was prepared according to general procedure (**B**) using **43** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (Hexane : EtOAc = 20 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.21 – 8.17 (m, 2H), 7.13 (t, *J* = 7.8 Hz, 1H), 6.96 (d, *J* = 8.7 Hz, 1H), 6.89 (d, *J* = 7.5 Hz, 1H), 6.74 (s, 1H), 6.64 (dd, *J* = 8.2, 2.5 Hz, 1H), 4.39 (s, 2H), 4.26 (s, 1H), 4.01 (s, 3H), 3.02 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.1, 147.4, 141.5, 129.3, 128.6, 124.8, 123.8, 122.8, 121.9, 116.0, 114.0, 110.0, 84.2, 76.5, 56.2, 42.7, HRMS (ESI) calcd for $C_{16}H_{15}N_2O_3$ ($M + H^+$) 283.1077, found 283.1080, Melting Point: 83-84°C.

5.4.57 2-(((3-ethynylphenyl)amino)methyl)-5-nitrophenol (57)

The title compound was prepared according to general procedure (**B**) using **44** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.86 (br s, 1H), 7.77 (dd, *J* = 8.3, 2.3 Hz, 1H), 7.73 (d, *J* = 2.3 Hz, 1H), 7.34 (d, *J* = 8.3 Hz, 1H), 7.21 (d, *J* = 7.8 Hz, 1H), 7.11 (d, *J* = 7.7 Hz, 1H), 6.97 (t, *J* = 1.9 Hz, 1H), 6.83 (dd, *J* = 8.3, 2.4 Hz, 1H), 4.54 (d, *J* = 5.0 Hz, 2H), 4.14 (d, *J* = 6.3 Hz, 1H), 3.08 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 157.2, 148.6, 146.3, 130.0, 129.5, 128.9, 125.2, 123.3, 119.1, 116.6, 115.2,

111.8, 83.3, 77.5, 48.1, HRMS (ESI) calcd for $C_{15}H_{11}N_2O_3$ (M - H^+) 267.0775, found 267.0773, Melting Point: 117-118℃.

5.4.58 2-(((3-ethynylphenyl)amino)methyl)-6-nitrophenol (58)

The title compound was prepared according to general procedure (**B**) using **45** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 11.0 (s, 1H), 8.1 – 8.0 (m, 1H), 7.6 (d, *J* = 7.4 Hz, 1H), 7.1 (t, *J* = 7.9 Hz, 1H), 7.0 (t, *J* = 8.0 Hz, 1H), 6.9 (d, *J* = 7.5 Hz, 1H), 6.8 – 6.8 (m, 1H), 6.6 (dd, *J* = 8.1, 2.6 Hz, 1H), 4.5 (d, *J* = 5.8 Hz, 2H), 4.3 (s, 1H), 3.0 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 153.1, 147.3, 136.0, 133.7, 129.9, 129.3, 123.9, 122.9, 121.9, 119.7, 116.1, 113.9, 84.0, 76.5, 42.6, HRMS (ESI) calcd for $C_{15}H_{12}N_2O_3$ (M - H^+) 267.0775, found 267.0770, Melting Point: 82-83℃.

5.4.59 2-bromo-6-(((3-ethynylphenyl)amino)methyl)-4-nitrophenol (59)

The title compound was prepared according to general procedure (**B**) using **47** (100 mg, 0.29 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 10 : 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.93 (s, 1H), 8.42 (s, 1H), 8.14 (s, 1H), 7.21 (t, *J* = 8.0 Hz, 1H), 7.06 (d, *J* = 7.7 Hz, 1H), 6.90 (s, 1H), 6.77 (d, *J* = 8.2 Hz, 1H), 4.54 (s, 2H), 4.26 (s, 1H), 3.07 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 157.9, 146.1, 141.1, 129.6, 127.8, 125.1, 124.7, 123.4, 123.3, 118.4, 115.9, 110.6, 83.4, 46.7, HRMS (ESI) calcd for $C_{15}H_{10}BrN_2O_3$ (M - H^+) 344.9880, found 344.9878, Melting Point: 94-95℃.

5.4.60 2,4-dichloro-6-(((3-ethynylphenyl)amino)methyl)phenol (60)

The title compound was prepared according to general procedure (**B**) using **48** (100 mg, 0.36 mmol) and sodium triacetoxyborohydride (228 mg, 1.08 mmol, 3 equiv), yielding the pure product as a white solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15: 1), ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.30 (d, *J* = 2.5 Hz, 1H), 7.22 (br s, 1H), 7.20 – 7.16 (m, 2H), 6.99 (d, *J* = 7.6 Hz, 1H), 6.85 (t, *J* = 1.9 Hz, 1H), 6.72 (dd, *J* = 8.1, 2.4 Hz, 1H), 4.40 (s, 2H), 4.17 (br s, 1H), 3.06 (s, 1H), ^{13}C NMR (101 MHz, Chloroform-*d*) δ 149.37, 146.89, 129.40, 128.03, 127.11, 126.72, 125.14, 123.50, 122.99, 121.12, 117.59, 115.19, 83.78, 76.96, 45.43, HRMS (ESI) calcd for $C_{15}H_{10}Cl_2NO$ (M - H^+) 290.0145, found 290.0141. Melting point: 105-106℃.

5.5 Determination of inhibitory activity against the NusB-NusE interaction. For inhibitor testing, chemical compounds were dissolved to 10 mg/mL in DMSO as the stock solution. 40 μL N-LgBiT-NusB (2.5 μM in PBS) was added to 96-well plates, and then mixed with 20 μL compound at 500 μM in PBS. The mixture was incubated for 10 minutes at 37°C. 40 μL C-SmBiT-NusE (2.5 μM in PBS) was then added to each well, followed by incubation for 10 minutes at 37°C. After the final incubation step, equal volume of Promega Nano-Glo® Luciferase Assay Substrate was added to the reaction mixture. Luminescence emitted was measured using a Victor X3 Multilabel plate reader. Experiment was performed in triplicate. Technical repeats were taken to ensure consistent results were obtained. For IC_{50} measurement, 2-fold dilution of compound was performed starting from 3 mM.

5.6 Determination of minimum inhibitory concentration (MIC). The antimicrobial activity of the compounds was determined by broth microdilution according to the Clinical & Laboratory Standards Institute (CLSI) guidelines (28). The test medium was cation-adjusted Mueller-Hinton broth (MH). Serial two-fold dilutions were performed for the tested chemicals

starting from 256 μg/mL to 0.5 μg/mL, and the bacterial cell inoculum was adjusted to approximately 5×10^5 CFU/mL. Results were taken after 20 h of incubation at 37 °C. The MIC was defined as the lowest concentration of antibiotic with no visible growth. Experiments were performed in duplicates.

5.7 Time kill kinetics. *S. aureus* cells were suspended to ~1.5×10^6 CFU/mL at log phase in MH broth with compounds at various concentrations. As an untreated control, bacteria were incubated in MH broth without compounds. The cultures were grown at 37°C with shaking at 200 rpm, and 10 μL sample were taken at defined time points (0, 2, 4, 6, 16, 20, 24hours) for each treatment group, followed by 10 fold serial dilution. 5 μL sample were taken from each dilution and spotted on blood agar plate. After incubation at 37°C overnight, the number of viable bacteria in each sample was counted and expressed as CFU/mL. Experiment was performed in triplicate. Technical repeats were taken to ensure consistent results were obtained.

5.8 Protein overproduction and purification. Purification of the His-tagged proteins have been described previously [21]. *E. coli* BL21 (DE3) pLysS transformed with pCU190 and pCU194 (Table 1) was grown in 800 mL auto-induction media (AIM-LB base w/o trace elements, FORMEDIUM UK) supplemented with 0.5% (v/v) glycerol at room temperature for 24 h. Cells were pelleted by centrifugation at 5000 g for 5 min and lysed in 5 mL/g cells lysis buffer (20 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, 0.5 mg/mL lysozyme, pH 8.0). After sonication and centrifugation at 8000 g for 45 min, the supernatant was filtered and passed through a 1 mL His-trap FF column (GE) pre-equilibrated with the lysis buffer without lysozyme. The column was then washed with 10 CV of wash buffer (20 mM NaH_2PO_4, 500 mM NaCl, 40 mM imidazole, pH 8.0) and protein eluted with elution buffer (20 mM NaH_2PO_4,

500 mM NaCl, 200 mM imidazole, pH 8.0). Fractions containing target proteins were pooled and dialyzed into PBS + 30% (v/v) glycerol, pH 8.0 and stored at −80 °C.

5.9 Binding inhibition assay. For inhibitor testing, chemical compounds were dissolved to 10 mg/mL in DMSO. 40 μL N-LgBiT-NusB (2.5 μM in PBS) was added to 96-well plates, and then mixed with 20 μL compound. The mixture was incubated for 10 minutes at 37°C. 40 μL C-SmBiT-NusE (2.5 μM in PBS) was then added to each well, followed by incubation for 10 minutes at 37°C. The final concentration of the compounds was at 125 μM. After the final incubation step, equal volume of Promega Nano-Glo® Luciferase Assay Substrate was added to the reaction mixture. Luminescence emitted was measured using a Victor X3 Multilabel plate reader. Experiment was performed in triplicate. Technical repeats were taken to ensure consistent results were obtained.

5.10 Cytotoxicity assay. Human cell lines A549 lung carcinoma and HaCaT immortalized keratinocytes were used in this study. The cells will be seed at 2.5 x 10^5 per well. After 24 hr incubation, the tested compounds will be added in a 2-fold serial dilution ranging from 1.562 μg/mL to 50 μg/mL. The plates will be incubated at 37°C. At 48 hr and 72 hr after adding the compound, the MTT assay will be performed as described previously [26]. 5-fluorouracil was used as the positive control, and DMSO as the negative control.

5.11 Hemolysis assay

To separate erythrocytes from human whole blood, 35 mL of human whole blood was centrifuged at 500 x g, +4 ˚C for 5 mins. After centrifugation, the upper layers of plasma and buffy coat were aspirated by micropipette. 150 mM NaCl solution was added to the packed erythrocytes and filled up to the original volume mark, and the solution was gently shaken to

resuspend the erythrocytes. The centrifugation process was repeated with the same condition and after centrifugation, the upper layers were aspirated and discarded, followed by the resuspension of erythrocytes using PBS (pH 7.4). A 40x-diluted erythrocyte suspension was prepared by adding 1 mL of the final suspension into 39 mL of PBS (pH 7.4). Compounds with a final concentration of 0.1, 1 and 10 μg/mL were incubated at 37°C with 2% blood solution in PBS (pH 7.4) for 45 min. 1600 μL of the 40x-diluted erythrocyte suspension was added to 400 μL of the drug solution in PBS (with 0.5% DMSO). The mixture was vortexed gently for 10 seconds and inverted several times to ensure a thorough mixing. The prepared mixture was transferred to 96-well microplates for incubation (200 μL per well, n=6). Positive control was Triton X-100, a known hemolytic agent, with a final concentration of 1% v/v in PBS. Negative control is the vehicle which is PBS (pH 7.4). After incubation, the reactions was terminated by centrifuging the samples 10 min at 500 × g, 4°C, to pellet the remaining erythrocytes and erythrocyte ghosts. Then, 100 μL of the supernatant from each well was transferred to a new 96-well plate, and the absorbance of the supernatant was measured at 540 nm, which is the hemoglobin absorbance peak, with CLARIOstar microplate reader (BMG Labtech, Offenburg, Germany). % of lysis was calculated with the following equation:

$$\%\ of\ lysis = \frac{A_{sample} - A_{-ve\ control}}{A_{+ve\ control} - A_{-ve\ control}}$$

5.12 Caco-2 Permeability Study

Caco-2 cells were cultured at 37°C in Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/mL penicillin, and 100 μg/mL streptomycin in an atmosphere of 5% CO2 and 90% relative humidity. The cells were passaged after 90% confluence using trypsin-EDTA and plated at a 1:5 ratio in 75-cm^2 flasks. The cells (passage number: 45) were seeded at a density of 60,000 cells/cm^2 on polycarbonate

membranes of Transwells (12 mm inner diameter, 0.4 μm pore size, 1 cm^2, Corning, NY, USA). Medium was changed the day after seeding and every second day thereafter (AP volume 0.5 mL and BL volume 1.5 mL). The Caco-2 cell monolayers were used 21 to 28 days after seeding. Transepithelial electrical resistance (TEER) was measured to ensure monolayer integrity. Cell monolayers with TEER values less than 165 Ω cm^2 were discarded in transport experiments. Transport studies involved only apical (AP) to basolateral (BL) direction. Cell monolayers were preincubated with transport buffer solution (PBS+) for 20 min at 37°C. To study absorptive transport, the donor (AP) compartment buffer was replaced with 0.5 mL of transport buffer containing 10 μg/mL of compounds. The pH in both AP and BL compartments was maintained at 7.4 for all transport studies. Concentration of MC4 compounds in the receiver (BL) compartment was monitored as a function of time in the linear region of transport and under sink condition (which is the receiver concentration is < 10% of the donor concentration). At each sampling time point, 100 μL of sample was drawn from the receiver compartment, and a same volume of blank buffer was added into receiver compartment. Concentration of compounds in sample solution was determined using LC-MS/MS.

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ABBREVIATIONS

MRSA, methicillin-resistant *Staphylococcus aureus*, PPI, protein-protein interaction, MIC, minimum inhibition concentration, SAR, structure-activity relationship, CD, circular dichroism, QSAR, quantitative structure–activity relationship, rRNA, ribosomal RNA, MIC, minimum inhibition concentration, DCM, dichloromethane, THF, tetrahydrofuran, DMSO, dimethyl sulfoxide.

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