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Nusbiarylins, a New Class of Antimicrobial Agents: Rational Design of Bacterial Transcription Inhibitors Targeting the Interaction between the 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 paper, an in-house library consisting of 95 compounds 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, Time-kill kinetics, confocal microscope, ATP production, 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 $\mu\text{g/ml}$ against clinical strains of multidrug-

resistant *Staphylococcus aureus* (MRSA) at a level comparable to some of the marketed antibiotics. Based on the fact that the inhibitors were targeting the NusB protein and with biaryl structure, we named this class of antimicrobial compounds as “nusbiarylins”. Given the improved antimicrobial activity, specific inhibition of target protein-protein interaction and promising pharmacokinetic properties without significant cytotoxicity, nusbiarylins have high potentials to be further developed towards a new class of antimicrobial agent to combat multidrug-resistant bacteria in the near future.

INTRODUCTION

Multidrug-resistant bacteria cause diverse life-threatening infections. *S. aureus* is one of the Gram-positive pathogens leading to a series of conditions ranging from mild skin and soft tissue infections, respiratory infections, to fatal sepsis (1). The antibiotics like β -lactams had a significant and long-term impact in clinics to treat *S. aureus* infections (2). However, antibiotic-resistant *S. aureus* such as methicillin-resistant *S. aureus* (MRSA) strains re-emerged to become problematic causes of severe infections. The rapid infectious diseases like sepsis can be particularly dangerous, which could cause up to 30% mortality rate before the resistant pattern of *S. aureus* is diagnosed and antibiotic susceptibility determined (3). Therefore, discovery of new antimicrobial compounds capable of eliminating MRSA and other drug resistant bacteria is urgently required. Structurally novel antimicrobial agents with distinct target and mechanism will provide invaluable alternative therapeutic choices against the existing resistant pathogens (4).

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 (5). These two proteins are essential and highly conserved transcription factors specifically involved in the bacterial ribosomal RNA (rRNA) transcription. By examine the protein-protein interaction (PPI) interface of the crystal structure of *Escherichia coli* NusB-NusE complex (6), the interactions between NusB E81 and NusE H15, NusB Y18 and NusE D19, NusB E75 and NusE R16, and NusB L22 and NusE V26 (*E. coli* amino acid residue numbering) were chosen to design the pharmacophore model (Figure 1), followed by *in silico* screening (5). One of the short-listed compounds, (*E*)-2-[[3-ethynylphenyl]imino]methyl}-4-nitrophenol 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.

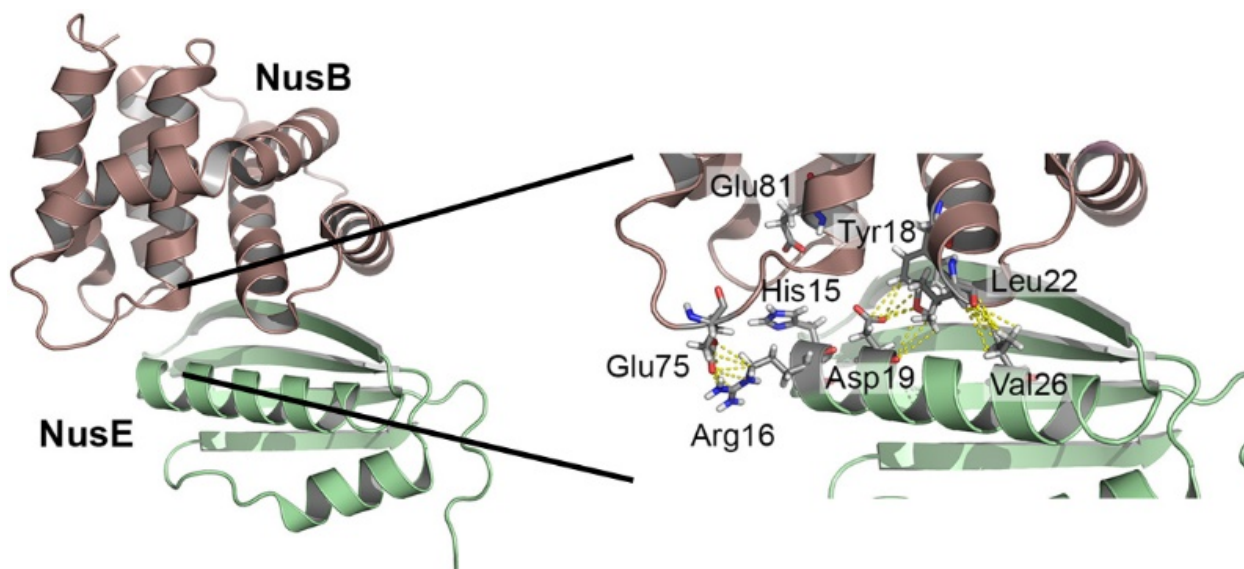


Figure 1. Structure of the *E. coli* NusB-NusE co-crystal complex (left) and conserved interactions across bacterial species at the interface (right)

Chemistry

Derivative Design. The bioactive hit compound, (*E*)-2-[[3-ethynylphenyl]imino]methyl}-4-nitrophenol has a relatively low molecular weight of 263 and a specific conjugated diphenyl imine system. As predicted by the pharmacophore model, the terminal alkyne group of the hit compound exhibited a hydrophobic interaction with the residue chain on Arg76 of NusB, the phenol group of compound bonded to Glu81 of NusB by hydrogen bond, and the nitrate group of compound accepted a hydrogen bond donation from Tyr16 of NusB (Figure 2; left). Note that glutamic acid often exhibits increased pKa due to surrounding amino acid residues forming a hydrophobic pocket and preventing proton loss, while Figure 2 illustrated a similar situation of Glu81 on NusB.

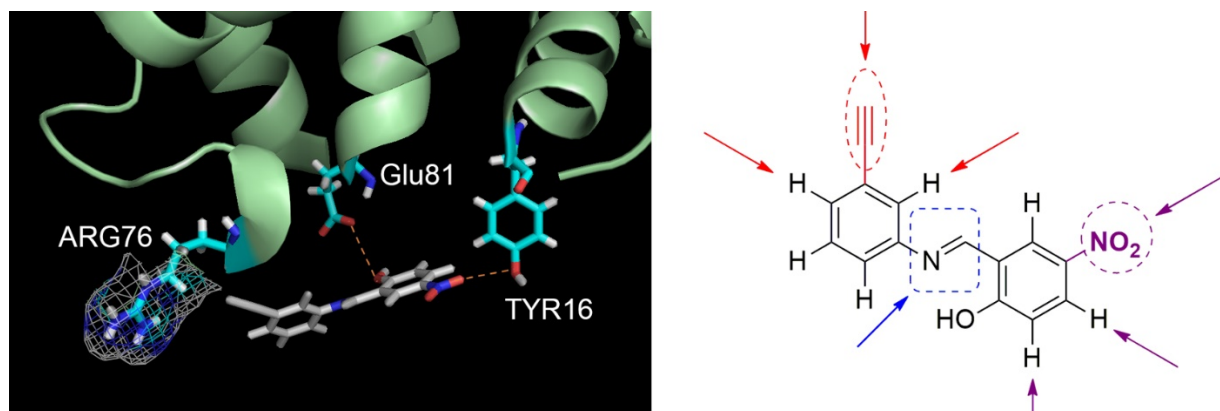


Figure 2. The docking model showing the interaction of hit compound with NusB (left) and the modification plan (right)

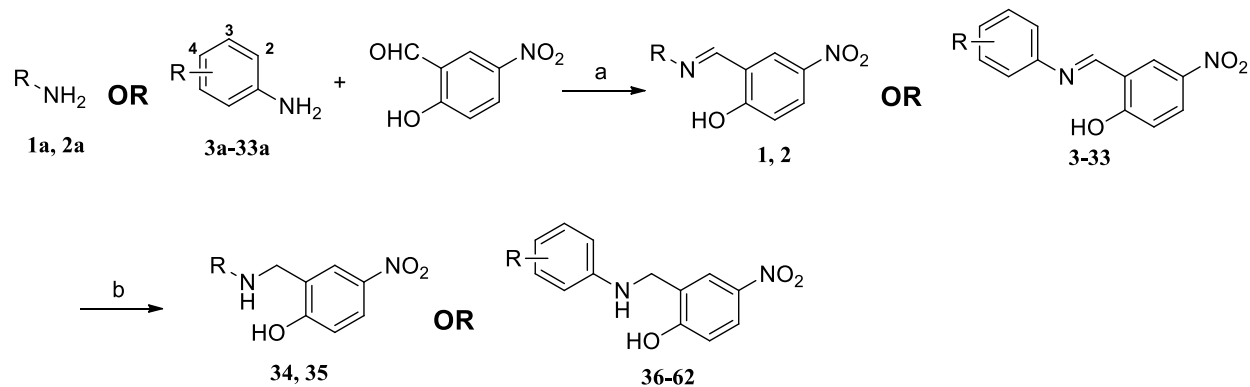
To examine the pharmacophore model and study the SAR, we planned to modify the left and right benzene rings of the hit compound (Figure 2; right). Various substituents such as alkyl, methoxy, hydroxyl, hydroxymethyl, carboxylate, fluoride, chloride, trifluoromethyl have been used to replace ethynyl on the left benzene ring of the hit compound to test the suitability for hydrophobic interaction with Arg76 of NusB.

New analogues with or without the nitro group, hydroxyl of phenol, masked hydroxyl by methoxy, replacement of nitrate by other hydrogen bond acceptors, 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 hit compound with Glu81 and Tyr16 of NusB.

The imine linker of the hit compounds has also been modified 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). Amine, carbon-carbon double bond, amide and carbonyl have

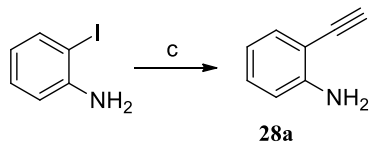
been chosen to replace the imine group of the hit compound regarding the different flexibility and bond lengths of these linkers.

Synthesis. Synthesis of ring A modified derivatives **1-62** is shown in Scheme 1. Reaction of an amine or substituted aniline **1a-33a** with 5-nitrosalicylaldehyde in ethanol at room temperature afforded Schiff base type derivatives **1-33** in 60-93% yields. Reductive amination of the above derivatives using sodium triacetoxyborohydride in dichloromethane gave the corresponding amines **34-62** in 84-94% yields. The intermediate **28a** was not commercially available, so we prepared it by a Sonogashira coupling reaction from 2-iodoaniline in 59% yield (7).



1a, 1, 34	R	12a, 12, 45	R	23a, 23, 55	R
2a, 2, 35	cyclohexyl	13a, 13, 46	4-C(CH ₃) ₃	24a, 24, 56	3-COOCH ₃
3a, 3, 36	1-naphthalenyl	14a, 14, 47	2-OH	25a, 25, 57	4-COOCH ₃
4a, 4, 37	-H	15a, 15	3-OH	26a, 26, 58	2-CF ₃
5a, 5, 38	2-F	16a, 16, 48	4-OH	27a, 27, 59	3-CF ₃
6a, 6, 39	3-F	17a, 17, 49	2-Cl	28a, 28	4-CF ₃
7a, 7, 40	4-F	18a, 18, 50	3-Cl	29a, 29, 60	2-ethynyl
8a, 8, 41	2-CH ₃	19a, 19, 51	4-Cl	30a, 30	3-ethynyl
9a, 9, 42	3-CH ₃	20a, 20, 52	2-OCH ₃	31a, 31, 61	4-ethynyl
10a, 10, 43	4-CH ₃	21a, 21, 53	3-OCH ₃	32a, 32	3-CH ₂ OH
11a, 11, 44	2-C(CH ₃) ₃	22a, 22, 54	4-OCH ₃	33a, 33, 62	4-CH ₂ OH
	3-C(CH ₃) ₃		2-COOCH ₃		3-COOH

Preparation of **28a**

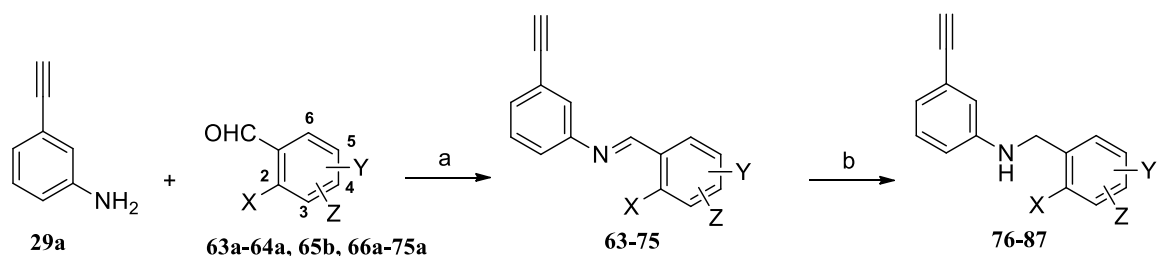


Reagents and conditions: (a) EtOH, rt, overnight, 60-93%; (b) NaBH(OAc)₃, DCM, rt, overnight, 84-94%; (c) PdCl₂(PPh₃)₂, CuI, TMSA, Et₃N, rt., 6h; then K₂CO₃, MeOH, 1h, 59% in two steps.

Scheme 1. Synthesis of target compounds **1-33** and **34-62**

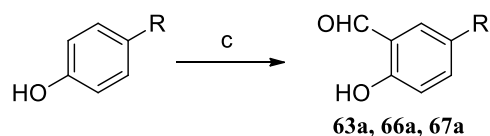
Synthesis of ring B modified derivatives **63-87** was performed following the same synthetic method of imine formation and reductive amination as outlined in Scheme 2. Regarding the commercially unavailable substituted benzaldehydes as reaction partners, a modified Casnati-Skattebøl ortho-formylation furnished aldehyde intermediates **63a**, **66a** and **67a** in 52-78% yields

(8). Although **66a** was obtained as a 7:3 mixture of 2-(3-formyl-4-hydroxyphenyl)acetonitrile and the starting material 4-hydroxybenzyl cyanide, as determined by ¹H 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 CH₃ONa in methanol provided benzaldehydes **65a** and 70a in 85-88% yields. Demethylation of compound **65a** with BBr₃ in dichloromethane provided cyanobenzaldehyde **65b** in 71% yield (9). In summary, Schiff base type derivatives **63-75** and amines **76-87** were synthesized in 65-87% and 86-94% yields, respectively.



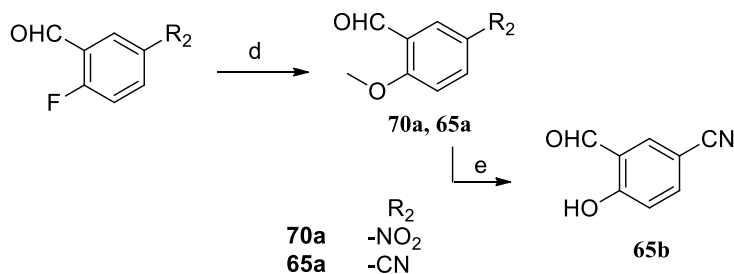
	X	Y	Z		X	Y	Z
63a, 63, 76	-OH	5-COOCH ₃	-H	70a, 70, 83	-OCH ₃	5-NO ₂	-H
64a, 64, 77	-OH	5-F	-H	71a, 71, 84	-OH	4-NO ₂	-H
65b, 65, 78	-OH	5-CN	-H	72a, 72, 85	-OH	3-NO ₂	-H
66a, 66, 79	-OH	5-CH ₂ CN	-H	73a, 73	-H	6-NO ₂	3-OH
67a, 67, 80	-OH	5-OCH ₃	-H	74a, 74, 86	-OH	5-NO ₂	3-Br
68a, 68, 81	-OH	-H	-H	75a, 75, 87	-OH	5-Cl	3-Cl
69a, 69, 82	-H	5-NO ₂	-H				

Preparation of **63a**, **66a**, **67a**



	R
63a	-COOCH ₃
66a	-CH ₂ CN
67a	-OCH ₃

Preparation of **65b**, **70a**

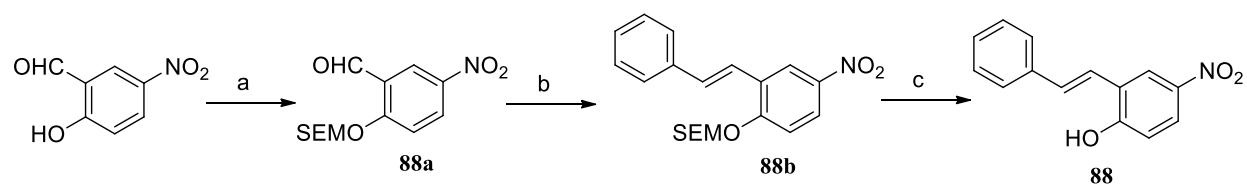


Reagents and conditions: (a) EtOH, rt, overnight, 65-87%; (b) NaBH(OAc)₃, DCM, rt, overnight, 86-94%; (c) paraformaldehyde, MgCl₂, Et₃N, THF, reflux, 12h, 52-78%; (d) CH₃ONa, MeOH, rt, 6h, 85% for **46**, 88% for **82**; (e) BBr₃, DCM, rt, 10h, 71%.

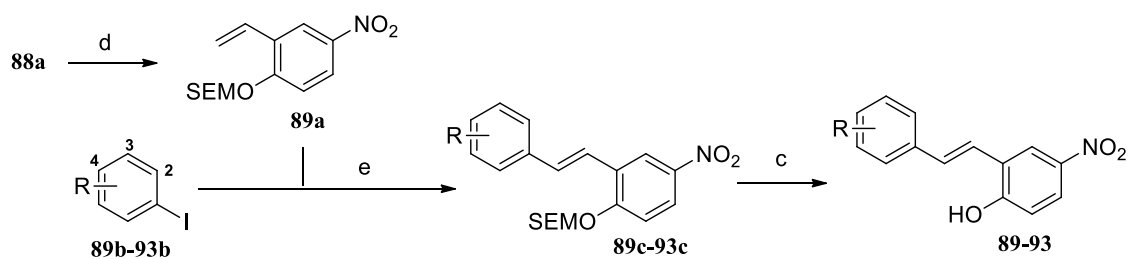
Scheme 2. Synthesis of target compound **63-75** and **76-87**

Synthesis of linker-modified analogues **88-95** is illustrated in **Scheme 3**. An (*E*)-stilbene derivative **88** was obtained through a three-step procedure: Protection of the hydroxyl group of 5-nitrosalicylaldehyde with 2-(trimethylsilyl)ethoxymethyl (SEM) group gave intermediate **88a**, which underwent a Horner-Wadsworth-Emmons reaction with diethyl benzylphosphonate to generate (*E*)-stilbene intermediate **88b** (10). **88b** was treated with 2% H₂SO₄-methanol solution to produce target compound **88**. Alternatively, Wittig reaction between benzaldehyde **88a** with methyltriphenylphosphonium bromide led to olefin **89a** in 62% yield, which was subsequently subjected to Heck reaction in the presence of substituted iodobenzenes **89b-93b** (11), followed by SEM deprotection, to provide (*E*)-stilbene derivatives **89-93** in 58-67% yields. The target compound with an amide linker was obtained by amidation of 5-nitrosalicylic acid with **29a** using triphenyl phosphite as a condensation agent, to provide amide **94** in 74% yield (12). Finally, palladium catalyzed coupling reaction of 5-nitrosalicylaldehyde with iodobenzene in DMF afforded ketone **95** with a carbonyl group as the linker in 74% yield (13).

Preparation of **88**

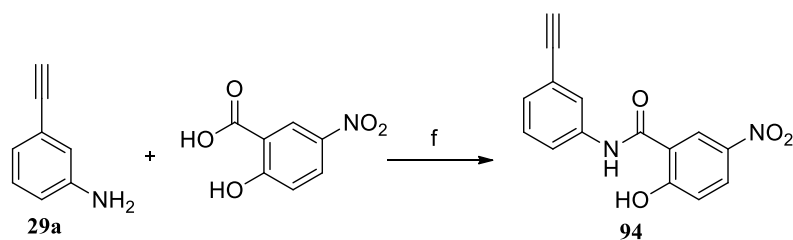


Preparation of **89-93**

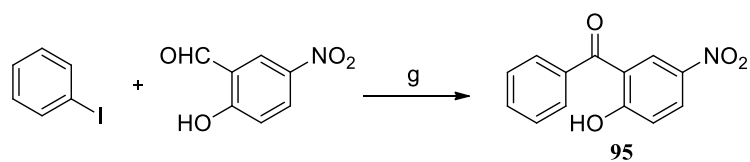


	R
89b, 89c, 89	2-OCH ₃
90b, 90c, 90	3-OCH ₃
91b, 91c, 91	2-CN
92b, 92c, 92	3-COOCH ₃
93b, 93c, 93	4-COOCH ₃

Preparation of **94**



Preparation of **95**



Reagents and conditions: (a) SEMCl, DIPEA, DCM, rt, 3h, 80%; (b) diethyl benzylphosphonate, NaH, THF, rt, overnight; (c) 2% H₂SO₄-MeOH, rt, overnight, 58-67%; (d) methyltriphenylphosphonium bromide, NaH, THF, overnight, 62%; (e) K₃PO₄, Pd(OAc)₂, DMF, 100°C, overnight; (f) triphenyl phosphite, toluene, reflux, 6h, 74%; (g) PdCl₂, LiCl, Na₂CO₃, DMF, 120°C, 6h, 74%

Scheme 3. Synthesis of target compound **88-95**

RESULTS AND DISCUSSION

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 (14). 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 the inhibitor to NusB 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 IC₅₀ value (14), we maintained the previous testing conditions of compounds at 125 μM and displayed the percentage of inhibition values in Tables 1, 2 and 3.

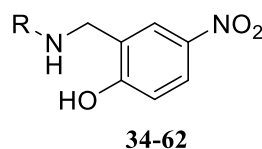
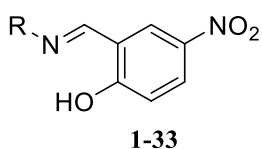
Table 1 included the testing results of compounds **1-33** with modifications of the left ethynyl benzene ring from the hit compound, and the corresponding analogues **34-62** containing a C-N amine bond reduced from the C=N imine group. As shown in Table 1, the modifications of the left ethynyl benzene 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 **10**) to 96.3% ± 0.6% (compound **14**) compared to the hit compound **29** at 85.3% ± 1.5%. Compound **10** contains a *t*-butyl group at 2-position related to imine, which seems too bulky for binding to NusB, and compound **14** has a 3-hydroxyl group as highly preferred. Other derivatives with relatively high inhibitory values includes compound **4** with 2-fluoride (73.4% ± 2.0%), compound **15** with 4-

hydroxyl ($89.1\% \pm 1.1\%$), compound **19** with 2-methoxy ($82.6\% \pm 0.8\%$), compound **22** with 2-carboxylate ($72.5\% \pm 3.3\%$), compound **28** with 2-ethynyl ($71.4\% \pm 1.8\%$), compound **30** with 4-ethynyl ($76.3\% \pm 3.0\%$), compound **31** with 3-hydroxymethyl ($70.6\% \pm 1.8\%$), compound **32** with 4-hydroxymethyl ($72.6\% \pm 2.6\%$), and compound **33** with 3-carboxylic acid ($78.5\% \pm 2.6\%$). In summary, the substitution of the left benzene ring is necessary as most of the substituted compounds demonstrated improved inhibitory activities compared to compound **3** with an unsubstituted benzene ring ($39.2\% \pm 3.0\%$). Additionally, our pharmacophore docking model indicated the binding of the substituents to Arg76 of NusB played an important role in the inhibition, and the binding type belongs to the hydrophobic interaction with the residue chain of Arg76. Effectively, the percentage inhibition values suggested that the inhibitory activity could be enhanced regardless hydrophobic or hydrophilic substituents, which confirmed the hydrophobic interaction dominates the binding of Arg76 to our compounds using the aliphatic chain, despite of its guanidine residue. By summarizing the relative positions of substitutions on the left benzene ring to the inhibitory activities, it is suggested that the 2-position was preferred for fluoride, methoxy, carboxylate, 3-position for *t*-butyl, hydroxyl, chloride, ethynyl, 4-position for CF₃, hydroxymethyl, and equal preference for methyl.


































The inhibitory activity of amines **34-62** obtained from reduction of imines **1-33** were then subjected to the testing. As the molecular conformation of **1-33** was released from a quasi-plane to a freely rotatable structure in **34-62**, 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 **43**. This compound contains a *t*-butyl group at 2-position

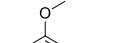
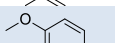
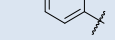
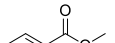
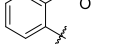
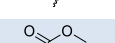
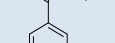
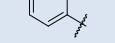

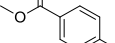
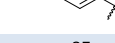
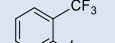
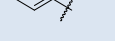
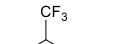
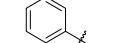

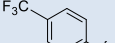
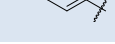
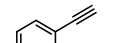
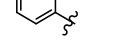

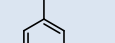
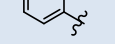
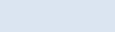
on the left benzene ring has a significant improvement on percentage inhibition at $72.2\% \pm 2.7\%$ from its imine analogue **10** at $16.8\% \pm 0.7\%$ as the imine with the lowest activity in Table 1. By turning the rigid imine to flexible amine, the bulky *t*-butyl group at the 2-position proved 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 **46** with an increased percentage inhibition at $81.5\% \pm 4.0\%$, compared to the imine analogue **13** at $57.7\% \pm 2.5\%$. In the contrast, other compounds may also display weakened inhibitory activities due to entropy penalty. Indeed, the rest of compounds almost demonstrated similar or slightly decreased inhibitory activities. A special case is amine compound **36** with an unsubstituted benzene ring, which lost completely the protein affinity to NusB with a percentage inhibition at $4.6\% \pm 3.4\%$ compared to the imine analogue **3**. The compounds also demonstrated similar dramatic changes are carboxylate-substituted amine compounds **54-56**, with percentage inhibitions from $13.0\% \pm 5.7\%$ to $16.8\% \pm 2.6\%$, dropped from $\geq 50\%$ in the case of imine analogues **22-24**.

Table 1. Inhibitory and antimicrobial activities of compounds **1-62**



Cpd.	R	MIC	Percentage Inhibition	ClogP
1		4	68.3 ± 2.2	3.87
34		>256	58.2 ± 5.9	3.20
2		4	43.4 ± 3.0	4.22
35		4	47.4 ± 2.8	3.93
3		4	39.2 ± 3.0	3.05

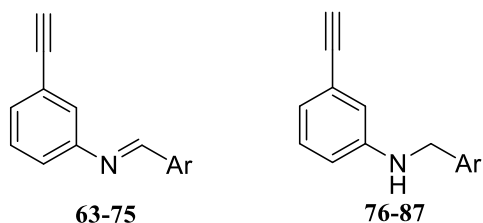
36		16	4.6 ± 3.4	2.75
4		8	73.4 ± 2.0	3.36
37		8	64.0 ± 5.2	3.20
5		4	65.4 ± 3.2	3.36
38		16	37.5 ± 7.1	3.20
6		8	59.2 ± 2.0	3.36
39		32	66.0 ± 2.2	3.20
7		4	53.1 ± 1.3	3.55
40		8	46.1 ± 6.7	3.25
8		4	54.9 ± 2.8	3.55
41		16	44.4 ± 6.2	3.25
9		4	49.2 ± 0.9	3.55
42		16	36.1 ± 6.4	3.25
10		4	16.8 ± 0.7	4.88
43		1	72.2 ± 2.7	4.58
11		4	51.3 ± 1.5	4.88
44		2	39.5 ± 2.7	4.58
12		4	38.8 ± 2.5	4.88
45		2	10.8 ± 5.4	4.58
13		4	57.7 ± 2.5	2.38
46		16	81.5 ± 4.0	2.08
14		16	96.3 ± 0.6	2.38
47		64	66.7 ± 3.5	2.08
15		4	89.1 ± 1.1	2.38
16		2	45.7 ± 2.2	3.93
48		2	31.0 ± 9.2	3.77
17		2	66.5 ± 0.7	3.93
49		4	34.9 ± 5.8	3.77
18		8	50.0 ± 3.8	3.93
50		8	53.6 ± 2.0	3.77
19		2	82.6 ± 0.8	3.07
51		32	45.2 ± 7.9	2.85
20		2	68.0 ± 0.8	3.07

52		64	34.9±10.3	2.85
21		4	61.8±2.8	3.07
53		128	48.2±7.8	2.85
22		4	72.5±3.3	3.33
54		128	13.0±5.7	3.77
23		2	48.1±3.1	3.33
55		32	14.6±10.7	3.27
24		2	61.8±3.3	3.33
56		>256	16.8±2.6	3.27
25		8	53.1±2.9	4.23
57		2	61.5±4.0	4.16
26		16	56.2±2.7	4.23
58		4	61.7±3.7	4.16
27		8	68.9±2.1	4.23
59		8	54.1±3.6	4.16
28		4	71.4±1.8	3.32
29		8	85.3±1.5	3.32
60		16	47.7±7.9	3.02
30		4	76.3±3.0	3.32
31		4	70.6±1.8	2.01
61		256	61.4±4.3	1.73
32		4	72.6±2.6	2.01
33		4	78.5±2.6	2.99
62		>256	50.8±3.0	2.84

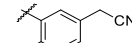
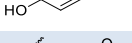
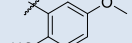
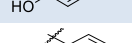
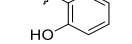
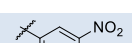
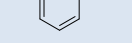
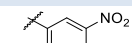
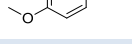
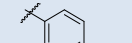
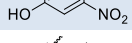
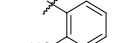
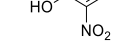
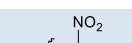
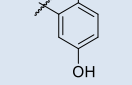
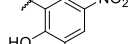
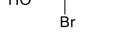
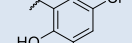
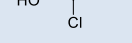
The imine compounds **63-75** with modified right benzene rings and the corresponding amine analogues **76-87** have been tested and results shown in Table 2. In this paper, we focused on the modification of hydroxyl and nitrate groups. Both hydroxyl and nitrate groups are necessary as compound **68** without nitrate and **69** without hydroxyl showed significant loss of inhibitory activity with respective percentage inhibition values of $25.7\% \pm 7.9\%$ and $24.7\% \pm 1.9\%$, as predicted by our rational design using the pharmacophore model. The transformation from phenol to methyl ether **70** also led to lowered activity ($40.4\% \pm 1.9\%$). *m*-nitrophenol **71**, or *p*-nitrophenol **73** with relatively changed position to the imine group, exhibited decreased inhibitory activity ($46.7\% \pm 2.5\%$, $52.2\% \pm 3.1\%$). Nitrate 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 nitrate by methoxy in compound **67** kept the hydrogen bonding capability, however, lost the inhibitory activity ($29.3\% \pm 5.6\%$) as methoxy is an electron-donating group and capable of increasing the pKa of phenol. When nitrate was replaced by other hydrogen bond acceptors and electron-withdrawing groups such as carboxylate, fluoride, nitrile, acetonitrile and chloride, the respective compounds **63** ($41.4\% \pm 3.7\%$), **64** ($28.1\% \pm 2.4\%$), **65** ($61.3\% \pm 2.0\%$), **66** ($49.9\% \pm 1.2\%$) and **75** ($54.2\% \pm 2.8\%$) displayed noticeably decreased inhibitory activity compared to the hit compound **29**. It is suggested that the pKa of phenol is more important for the inhibitory activity as *o*-nitrophenol **72** lost the hydrogen bond at *p*-position of hydroxyl but maintained a similar pKa to *p*-nitrophenol still displayed excellent inhibitory activity ($87.7\% \pm 0.6\%$). Additionally, an extra *o*-bromo group to *p*-nitrophenol **74** trying to lower further the pKa did not affect the activity.

Amine **76-87** demonstrated more favorable changes related to inhibitory activity compared to amine compounds with modifications on the left benzene ring. Amines **76**, **77**, **80**, **82**, **83**, **86** possess significant improvement over the imine analogues, especially **86** with a measured $99.6\% \pm 0.1\%$ 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 stiffness of **74**. When the molecule can freely rotate, the improved binding affinity overpowered the entropy penalty to provide a better inhibitory activity. While amines **78**, **79**, **81**, **84**, **87** showed similar activity to the imines, suggesting the binding force are mainly from hydrogen bonds and not as sensitive as the left benzene ring modifications to the structure flexibility. The only dramatic decrease in activity occurred on compound **85** ($64.5\% \pm 1.7\%$). Despite of maintaining pKa of *o*-nitrophenol, the loss of hydrogen bond between the nitrate group and Tyr16 of NusB could not retain the binding of **85** with NusB very firmly as compound **72** by the conformation control as a Schiff base.

Table 2. Inhibitory and antimicrobial activities of compounds **63-87**



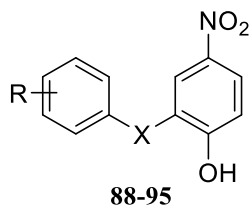
Cpd.	Ar	MIC	Protein	ClogP
63		128	41.4±3.7	3.51
76		32	57.1±0.8	3.15
64		256	28.1±2.4	3.60
77		32	61.8±2.0	3.08
65		32	61.3±2.0	3.03
78		32	64.8±5.4	2.76

66		256	49.9±1.2	2.78
79		64	44.7±1.4	2.06
67		>256	29.3±5.6	3.46
80		64	58.3±0.9	2.74
68		256	25.7 ± 7.9	3.35
81		>256	20.3 ± 1.9	2.64
69		256	24.7 ± 1.9	2.86
82		>256	33.1 ± 2.6	3.10
70		>256	40.4 ± 1.9	3.31
83		>256	56.1 ± 1.4	3.32
71		64	46.7 ± 2.5	3.32
84		8	48.2 ± 1.9	3.02
72		256	87.7 ± 0.6	3.32
85		128	64.5 ± 1.7	3.02
73		256	52.2 ± 3.1	3.32
74		32	74.1 ± 1.6	3.94
86		2	99.6 ± 0.1	3.70
75		32	54.2 ± 2.8	4.59
87		2	50.6 ± 4.9	4.14

Encouraged by the positive result of changing imine to amine giving equal or superior activity, we examined the inhibitory activity of the derivatives with different linkers such as C=C, amide and carbonyl between two benzene rings rather than amine or imine, because amine bond length may be an additional factor to improve the activity of imine (~1.34 Å vs ~1.25 Å). C=C has a bond length of ~1.34 Å, very similar to amine bond length but with a rigid structure as imine, while we also tested amide with a bond length of ~1.32 Å, and a carbonyl maintaining the planar conformation with one nitrogen less to verify the possible negative impact of short bond length.

As shown in Table 3, stilbene **88** with an unsubstituted left benzene ring demonstrated improved inhibitory activity ($56.6\% \pm 6.4\%$) compared to the imine analogue **3** and amine **36**, while ketone **95** with one nitrogen less possess a similar activity to imine **3**. 2-methoxy substituted stilbene **89** showed equal activity to amine **51**, but inferior to imine **19**. 3-methoxy substituted stilbene **90** showed equal activity to imine **20**, significantly better than amine **52**. 2-nitrile **91** displayed very similar activity to 2-methoxy **89**. 3-methyl carboxylate stilbene **92** has an activity between imine **23** and amine **55**. 4-methyl carboxylate stilbene **93** has an activity between imine **23** and amine **55**. Surprisingly, amide **94** displayed excellent activity ($99.2\% \pm 0.2\%$) over amine or imine analogues. In summary, only the amide analogue exhibited significantly increased activity over amine or imine, the C=C and carbonyl replacement led to diverse effects, depending on the substitution on the left benzene ring. Nevertheless, the common property of this series of compounds are obviously the higher ClogP values than imine and amine analogues, which may affect the antimicrobial activity through membrane permeability.

Table 3. Inhibitory and antimicrobial activities of compounds **88-95**



Cpd.	R	X	MIC	Protein	ClogP
88	H	(<i>E</i>)-CH=CH-	2	56.6 ± 6.4	4.54
89	2-OCH ₃	(<i>E</i>)-CH=CH-	4	41.6 ± 4.8	4.47
90	3-OCH ₃	(<i>E</i>)-CH=CH-	2	70.3 ± 4.4	4.47

91	2-CN	(E)-CH=CH-	8	44.9 ± 3.5	3.98
92	3-COOCH ₃	(E)-CH=CH-	64	24.6 ± 6.7	4.52
93	4-COOCH ₃	(E)-CH=CH-	128	41.7 ± 3.7	4.52
94	3-C≡CH	-NHCO-	8	99.2 ± 0.2	3.83
95	H	-CO-	64	41.3 ± 5.3	3.59

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 (15). Compounds **10**, **43**, **90** were chosen as they represent three different linkers: imine, amine and olefin that may affect the special position of 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), consistent to the multiple α -helices observed by in the X-ray crystallography structure (PDB: 3D3B, 16).

When compounds **10**, **43**, **90** were separately mixed with NusB at 1:1 ratio, the obvious 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 **10**, **43**, **90** were respectively added to NusB, similar changes of the CD spectra were observed, especially between 200-250 nm, which indicated that the derivatives induced similar structural changes of NusB. Interestingly, while both compounds **10** and **43** contain a *t*-butyl group at 2-position on the left benzene ring, and differ only from the linker (imine vs amine), the CD spectra of compounds **10** and **43** binding

to NusB were also extremely alike even between 190-200 nm. In comparison, compound **90** with a methoxy group at 3-position of the left benzene ring demonstrated more alterations in this region. In summary, the CD spectra showed that the three representative compounds were able to cause similar conformation changes of NusB, suggesting they may bind to the same binding site on the protein.

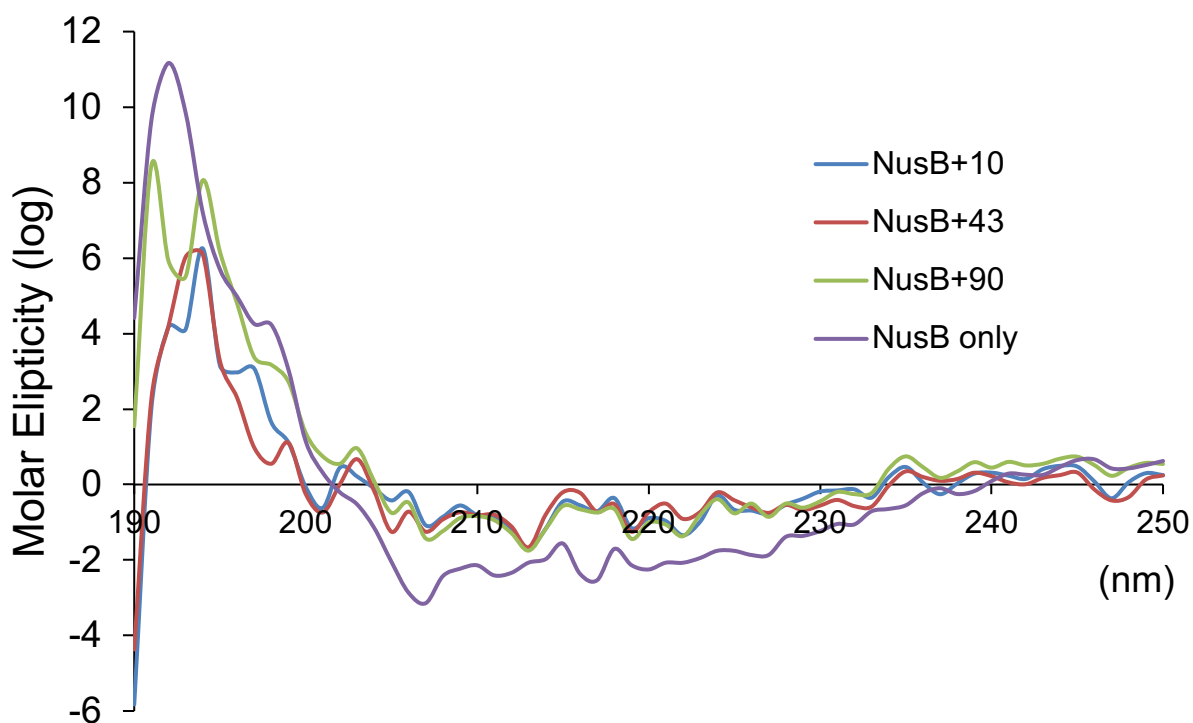


Figure 3. CD spectrum of NusB before and after addition of compounds

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.

As shown in Table 1, amongst the compounds **1-33** 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 **29**, in which compounds **16** and **17** with chloride, **19** and **20** with methoxy respectively at 2- and 3-position, **23** and **24** with methyl carboxylate respectively at 3- and 4- position displayed the best activity with MIC values of 2 µg/ml. The only compounds demonstrated inferior activity were **14** and **26** with hydroxyl and trifluoromethyl at 3-position. The MIC values of **14** and **26** 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 correspond directly to the antimicrobial activity, with an extreme example for comparison between compounds **10** and **14** 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 **10** and **14** are 4.88 and 2.38, respectively, which may affect their permeability to the bacterial cytosol.

When compounds **34-62** 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 **43-45** with t-butyl at 2-, 3- and 4-position of left benzene ring, which presented superior antimicrobial activity with MIC values of 1-2 $\mu\text{g/ml}$. Compound **43** displayed the best antimicrobial activity so far, with an MIC of 1 $\mu\text{g/ml}$. Statistically, 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 **63-75** with modifications of the right benzene ring changed nitrate and hydroxyl as the hydrogen binding groups, led to dramatic decrease of antimicrobial activity with MIC values of 32-256 $\mu\text{g/ml}$, in which compound **67** with methoxy replacing nitrate, and **70** with methyl ether masking hydroxyl lost their antimicrobial activity at all ($>256 \mu\text{g/ml}$). 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 with possible interactions of NusB with other proteins that may also competitively change the conformation of NusB. However, when imine was replaced by amine, compounds **76-87** with flexible structures were able to improve significantly the antimicrobial activity with MIC values of 2-64 $\mu\text{g/ml}$. The best antimicrobial activity (2 $\mu\text{g/ml}$) of these series of compounds were obtained from compound **86** with an additionally bromide at *o*-position to phenol, and compound **87** with dichloride replacing nitrate and bromide in compound **86**. This series of compounds again proved the importance of hydrogen bonding groups, as compounds **68-**

70, **81-83** with truncated or masked pharmacophores lost completed the antimicrobial activity regardless imine or amine as linkers.

Compounds with linkers other than imine and amine were also tested as shown in Table 3. Compound **88-90** with C=C presented similar antimicrobial activities to imine analogues **3**, **19-20** with MIC values of 2-4 µg/ml, while nitrile **91** had slightly inferior activity with MIC of 8 µg/ml. Compounds **92-93** with methyl carboxylate at 3- and 4-position seemed to be incompatible with the C=C linker causing reduced activity compared to imine analogues **23-24**. The amide linker did not show any difference to imine by comparing compound **94** and **29**. While a short carbonyl linker in compound **95** led to decreased activity in contrast to compounds **3** and **88**.

With 94 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).

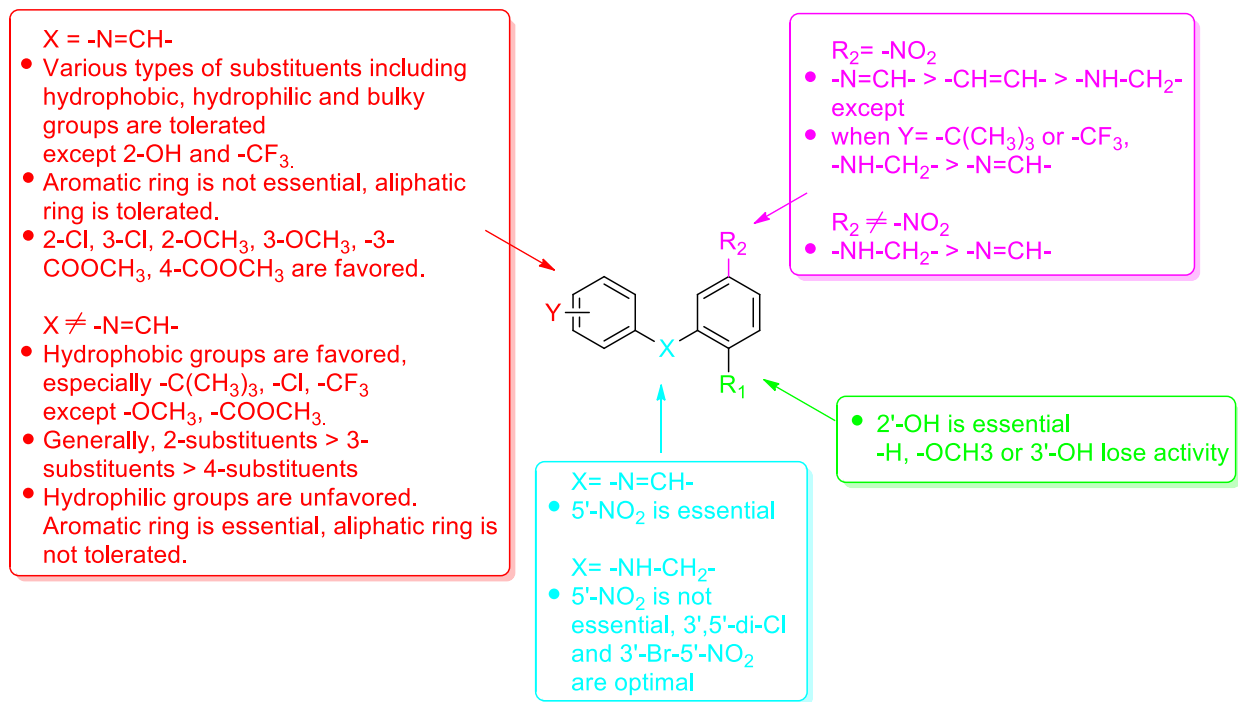


Figure 4. SAR of synthesized derivatives of the hit compounds against *S. aureus* ATCC 25923

On the other hand, we also tried to align the activity to the structure and hydrophilicity. As shown in Figure 4, 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 obviously 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 are mainly determined by the inhibitory activity and cell membrane permeability of compounds.

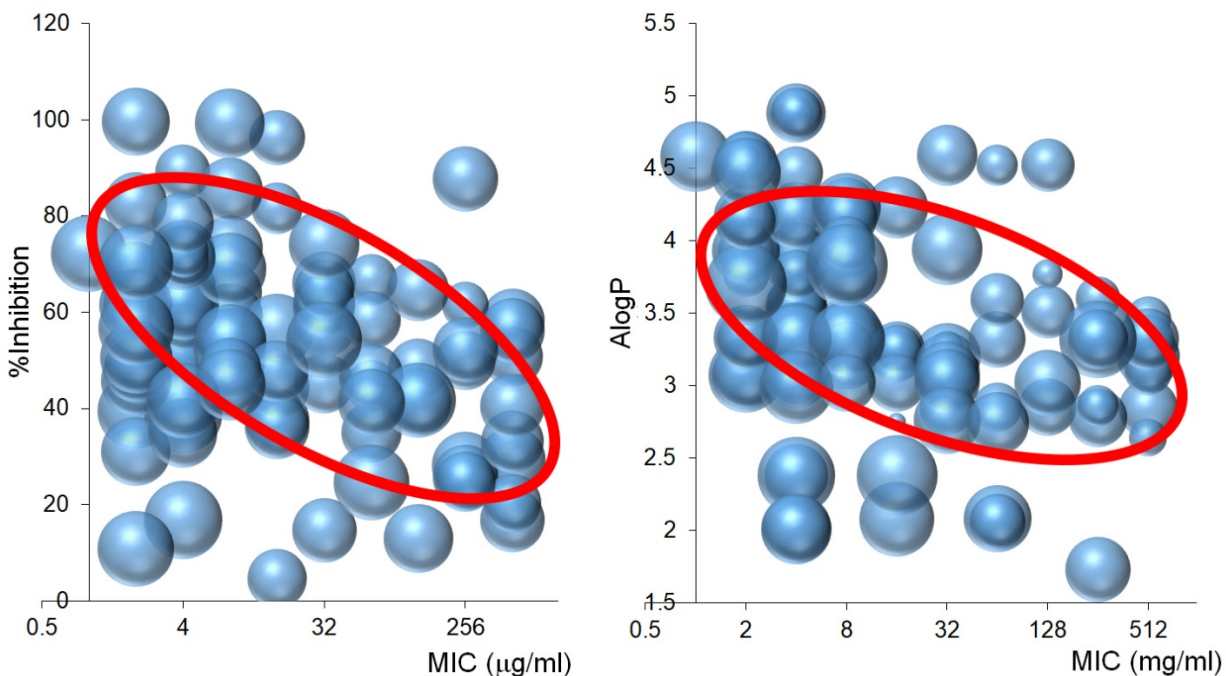


Figure 5. The trend of the inhibitory activity (left y axis) and clogP (right y axis) are inversely proportional to antimicrobial activity showing by MIC values (x axis).

With regard to this proportion between antimicrobial activity and inhibitory activity or clogP, we also noticed that the structural modifications of compounds focus on the substitutions on benzene rings. We decided to conclude the quantitative structure–activity relationship (QSAR) based on the data in hand. 37 amine compounds (**36-62**, **76-87**) were used to calculate QSAR as the MIC values are more diverse for greater QSAR demonstration. These compounds were divided into a training set (ca. 80% of the dataset) for QSAR modeling and a test set (ca. 20% of the dataset) for external QSAR model validation. The calculation was performed employing DS2016 and the equation is shown below:

$$pMIC = 7.4965 + 1.0929 \times ALogP - 0.22991 \times Llm - 0.39452 \times Llp - 0.77528 \times Lro - 0.51672 \times pKa$$

Based on the QSAR equation, we calculated the MIC values using the test set of compounds and plotted against the observed MIC values obtained in antimicrobial activity testing experiment. As shown in Figure 6, the two set of MIC values are highly correlated with $R^2 > 0.8$, which indicated that the antimicrobial activity of compounds could be improved by rational design of compound structures following the QSAR equation.

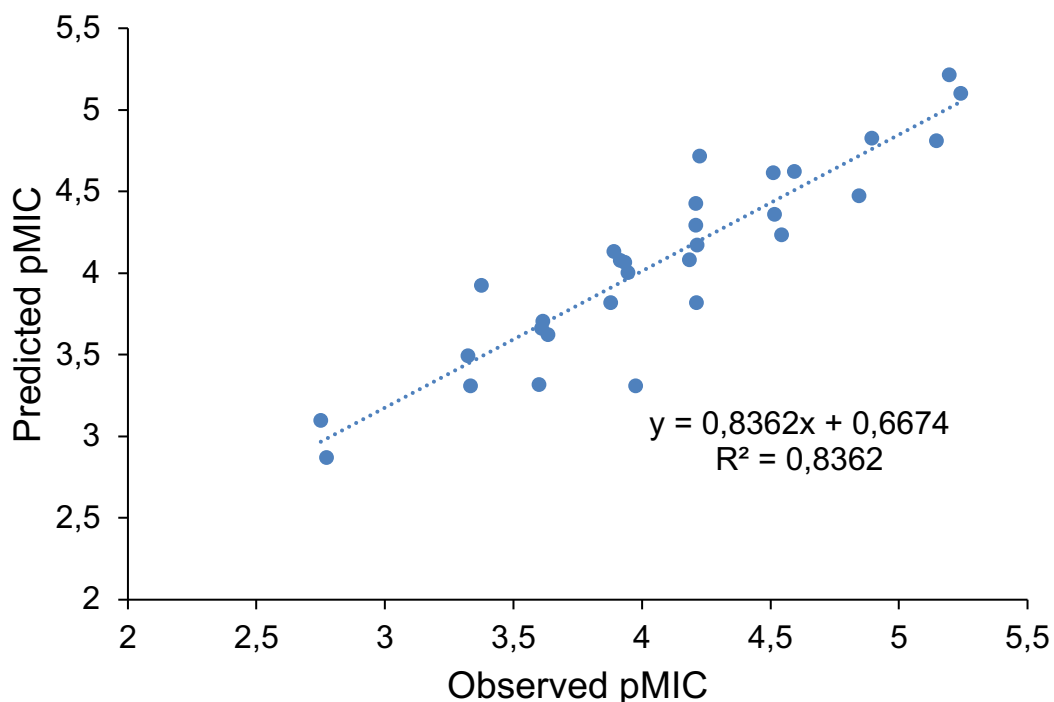
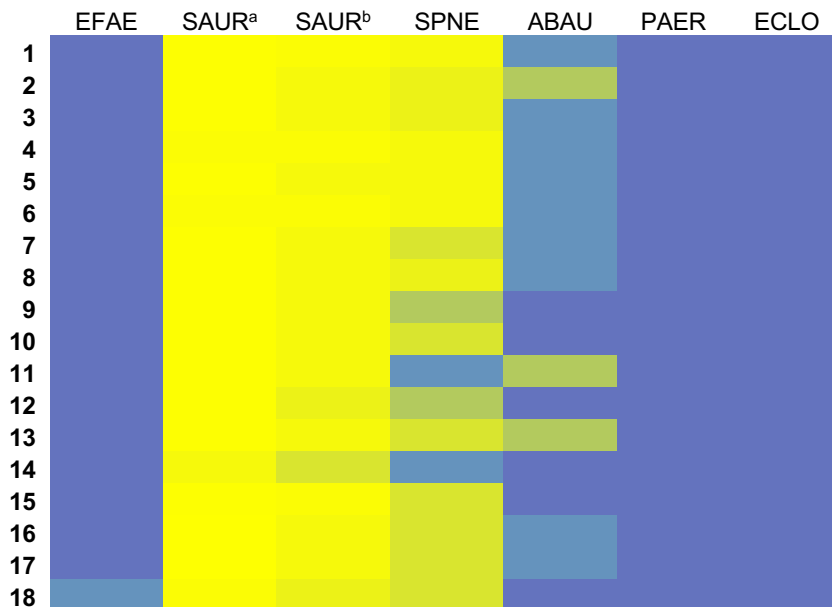


Figure 6. Correlation of predicted pMIC calculated from the QSAR equation to observed pMIC

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 five 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 as a heat map. As shown in Figure 7, 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.



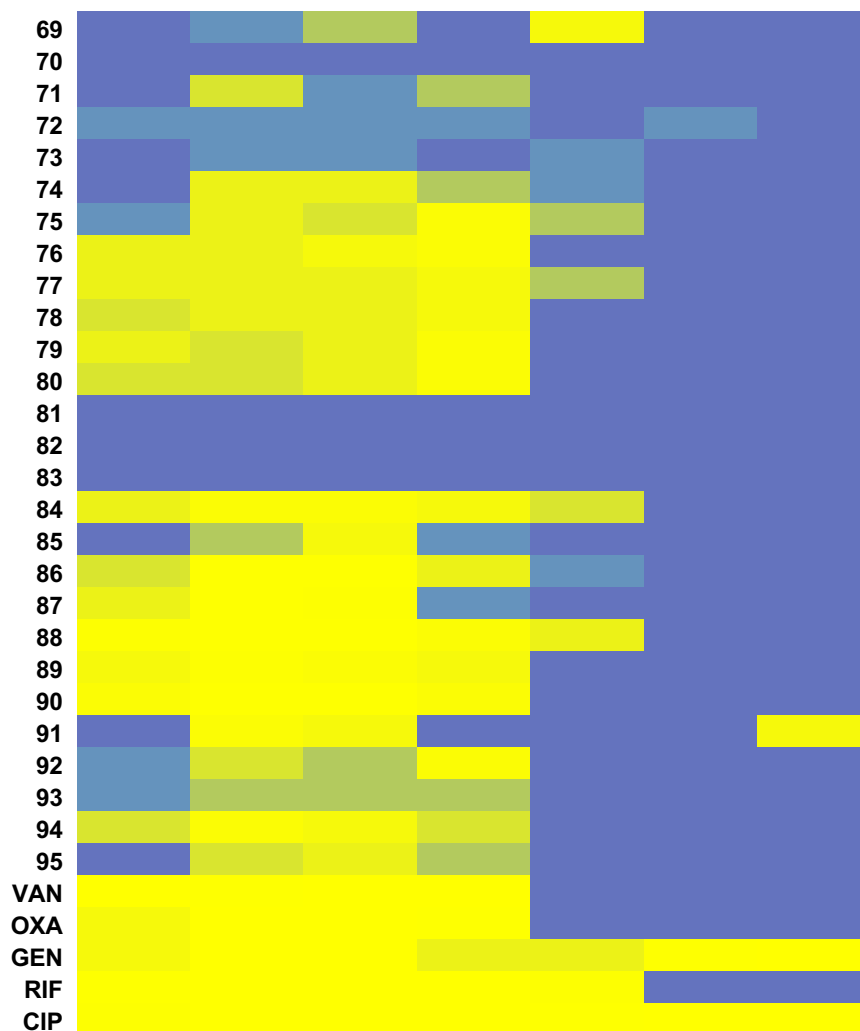


Figure 7. Antimicrobial activity evaluation of compounds against six representative bacteria in the WHO priority pathogens list for guiding R&D of new antibiotics. EFAE: *Enterococcus faecium* ATCC 19433, SAUR^a: *S. aureus* ATCC 25923, SAUR^b: *S. aureus* ATCC 29213, SPNE: *Streptococcus pneumonia* 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 Figure 8, most of the compounds displayed antibacterial activity against these Gram-positive pathogens with clinical complexity for treatment, which demonstrated the potentials of our compounds.

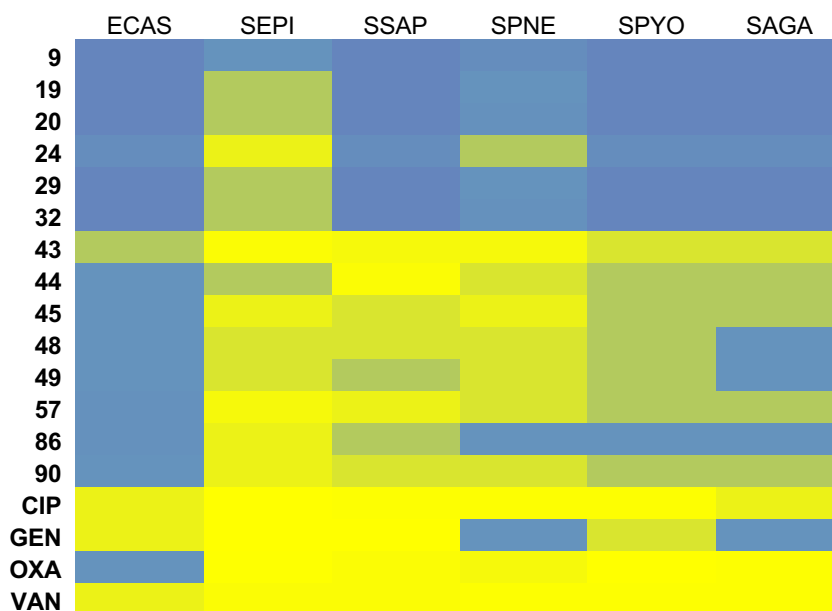


Figure 8. Antimicrobial activity evaluation of compounds against representative pathogenic Gram-positive bacteria. ECAS: *Enterococcus casseliflavus* ATCC 25788, SEPI: *Staphylococcus epidermidis* ATCC 12228, SSAP: *Staphylococcus saprophyticus* ATCC 15305, SPNE:

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 (17), *S. aureus* ATCC BAA-43 (18), *S. aureus* ATCC BAA-44 (18), *S. aureus* HA W-231 ST45 (19), CA-MRSA W-47 ST30 (19), CA-MRSA W-45 ST59 (19), CA-MRSA W-46 ST59 (19), *S. aureus* USA300/ST8 (20), *S. aureus* 11B086169 ST22 (21), CA-MRSA W-4 ST338 (22), CA-MRSA W-48 ST217 (22), *S. aureus* HA W-235 ST5 (23). As shown in Figure 9, 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.

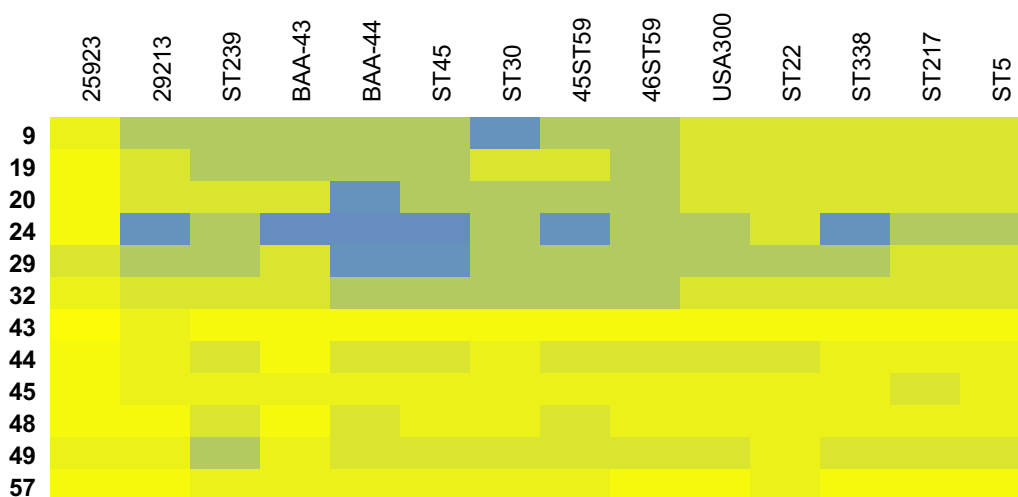




Figure 9. Antimicrobial activity evaluation of compounds against representative globally spread HA- and CA-MRSA.

Time kill assay.

The time kill kinetics were used to demonstrate the *in vitro* reduction of a microbial population by certain antimicrobial(s), and compounds **29**, **44** and **48** were tested against *S. aureus* ATCC 25923 (Figure 10). The hit compound **29** started to inhibit bacteria growth at $\frac{1}{4}$ MIC, and showed bacterial static effect at MIC and 4 MIC. **44** and **48** demonstrated similar inhibitory effect at the $\frac{1}{4}$ and 1 MIC, and started to demonstrate slight bactericidal activities at 4 MIC. When we used higher levels of **44** in this experiment, *S. aureus* could be efficiently eradicated in 2h, 16h, and 20h using 32, 16 and 8 MIC, respectively, which indicated the compounds were able to kill bacteria at higher concentration in a very short time.

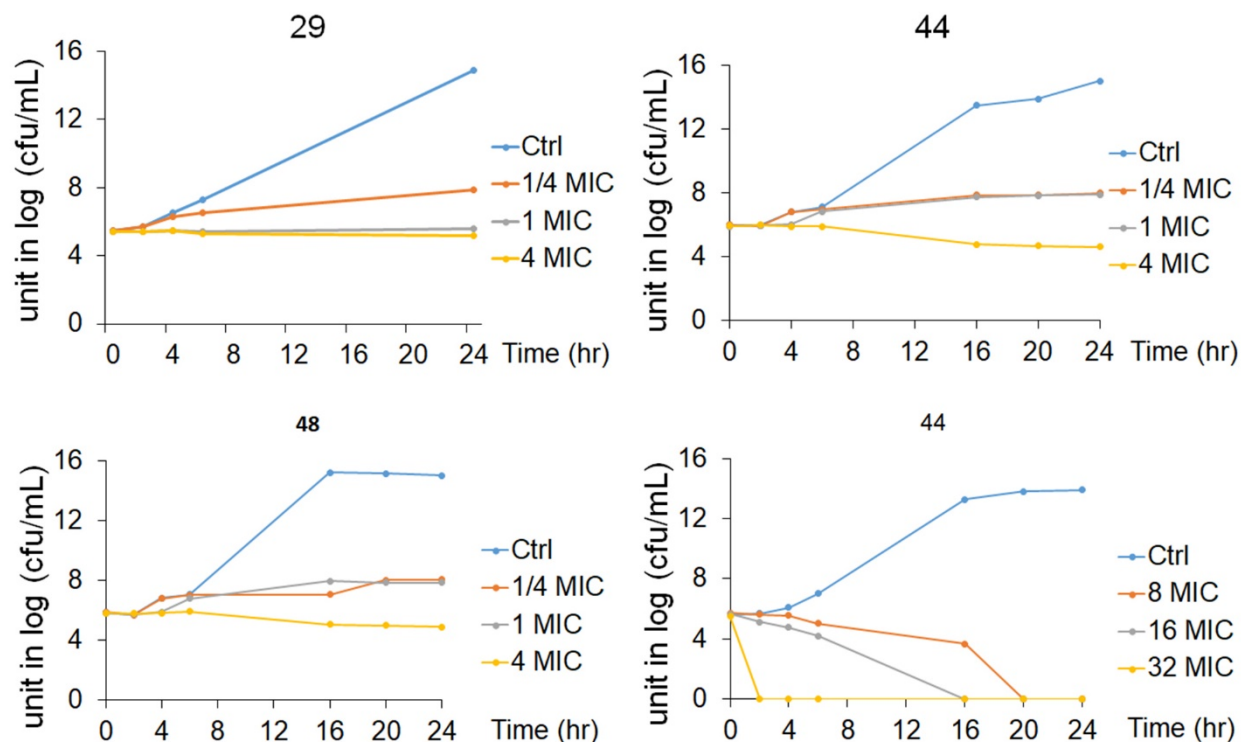


Figure 10. Time-kill kinetics of compounds **29**, **44**, **48** at different concentrations against *S. aureus* ATCC 25923

Confocal microscopy.

The subcellular localization of certain proteins can be observed by fluorescence microscopy as shown in our previous work (24). In this paper, we used BS61, a *Bacillus subtilis* strain that contains a *gfp* fusion to the *nusB* gene on the bacterial chromosome (25). Using confocal microscopy, we can clearly notice the fluorescence of NusB in the control group localizing at the sub-nucleoid regions called transcription foci as shown previously (Figure 11) (25). When the cells were treated with compound **44** at only 1/8 MIC for 15 min, we could already identify the diffusion

of NusB fluorescence into the cytosol, although its localization at the nucleoid regions could still be observed. When **44** was used at $\frac{1}{4}$ MIC, the localization of NusB fluorescence at the nucleoid could barely be seen with the signal diffused into the cytosolic regions, which indicated the disruption of normal NusB function. The result is consistent with the time-kill assay, in which inhibition of bacterial growth by **44** was observed as low as $\frac{1}{4}$ MIC. When **44** was used at $\frac{1}{2}$ or 1 MIC, no localization of fluorescent signal at the nucleoid could be detected, suggesting the function of NusB were completely halted.

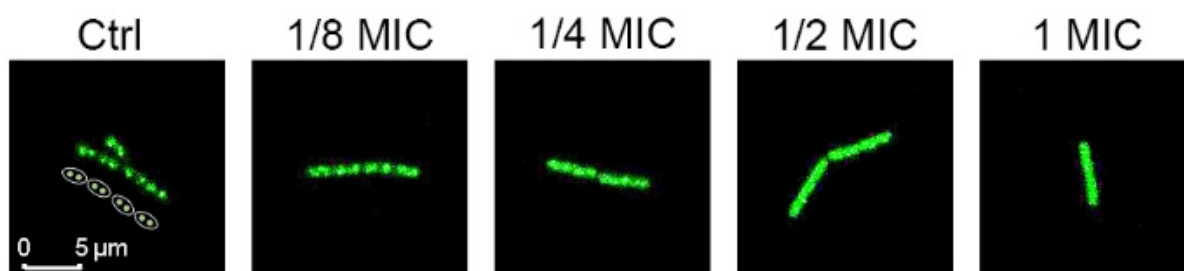


Figure 11. Confocal microscope of *B. subtilis* NusB treated with compound **44** at different concentrations

ATP production

It was reported that inhibition of macromolecular synthesis such as transcription were able to decrease bacterial central metabolism, and cellular respiration (26). That is why rifampicin is supposed to be bacteriostatic by targeting RNAP to inhibit transcription, which disrupt ATP production (26). We have examined compound **44** on its effect on the ATP production of *S. aureus* ATCC 29523 as shown in the Figure 12. When **44** was used at $\frac{1}{4}$ or 1 MIC, detectable reduction

of ATP production occurred as compared to the untreated cells. As existing proteins were still capable of carrying normal cellular function even when transcription is inhibited, treatment of **44** at 4 MIC completely obstructed cell respiration within 2 hours.

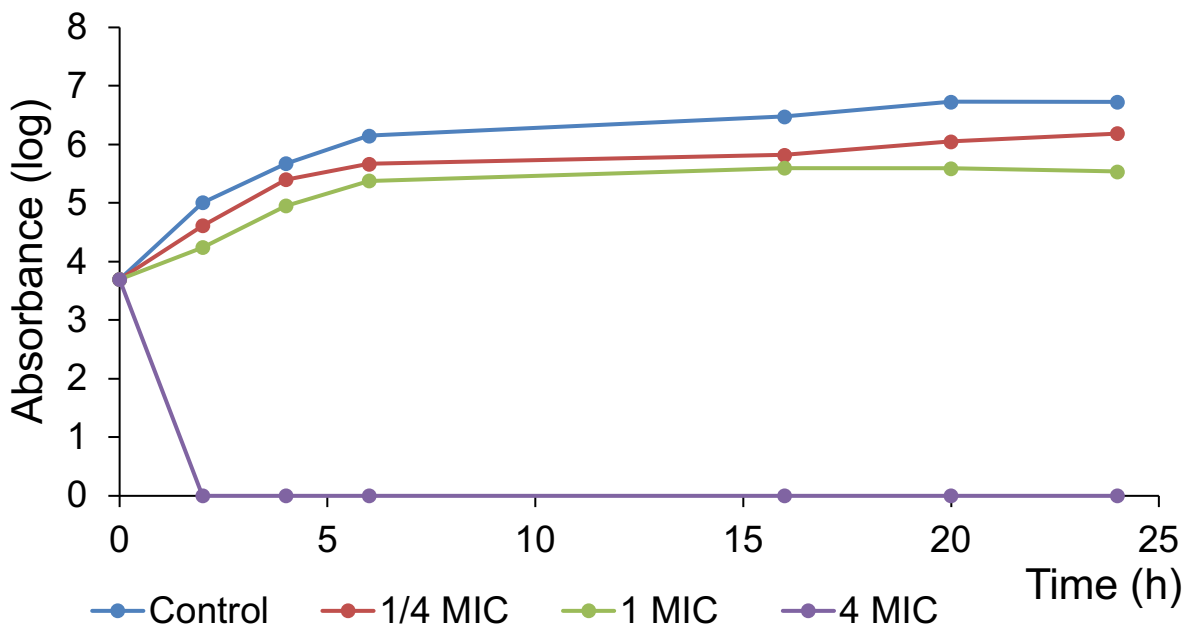


Figure 12. ATP production of *S. aureus* ATCC 29523 under treatment of compound **44**

Cytotoxicity.

The compounds within the best range of antimicrobial activity against *S. aureus* ATCC 25923 (MIC 1-2 $\mu\text{g/ml}$) 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 (5). As shown in Table 4, compounds **16**, **17**, **19**, **20**, **23**, **24** with imine linker did not show significant cytotoxicity ($\geq 200 \mu\text{M}$), whereas compounds **43**, **44**, **45**, **48**, **57** 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 **86** and **87** can respectively reduce the cytotoxicity to about 80 and 110 μM even in the presence of aniline structure, while compound **90** with C=C linker showed similar cytotoxicity to compounds with amine linker. The preliminary therapeutic index were also calculated with 53-153 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 4. Cytotoxicity of selective compounds against A549 and HaCaT

Cpd.	MIC ^a ($\mu\text{g/ml}$)	CC ₅₀ (μM)		Therapeutic Index ^b	
		A549	HaCaT	A549	HaCaT
16	2	431.69 \pm 30.14	346.59 \pm 28.93	119	96
17	2	274.16 \pm 4.69	249.52 \pm 22.73	76	69
19	2	454.40 \pm 27.45	438.94 \pm 24.81	124	120
20	2	232.10 \pm 9.46	195.82 \pm 23.08	63	53
23	2	446.51 \pm 28.56	391.59 \pm 25.80	134	118
24	2	510.42 \pm 29.40	381.19 \pm 24.41	153	114
43	1	13.42 \pm 1.53	15.07 \pm 1.81	8	9
44	2	18.32 \pm 1.75	20.24 \pm 2.42	6	6
45	2	37.23 \pm 4.44	38.89 \pm 4.18	11	12
48	2	22.52 \pm 2.35	20.09 \pm 2.05	6	6
57	2	19.98 \pm 2.11	18.31 \pm 1.97	6	6
86	2	79.14 \pm 15.97	72.15 \pm 13.31	27	25
87	2	112.18 \pm 21.56	108.10 \pm 18.46	33	32
90	2	34.58 \pm 4.06	30.51 \pm 3.43	9	8

DDP ^c	-	7.70 ± 0.58	7.83 ± 0.66	-	-
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^a *S. aureus* ATCC 25923; ^b Calculated by $CC_{50} (\mu\text{g/ml}) / \frac{1}{2} \text{MIC} (\mu\text{g/ml})$; ^c DDP: cisplatin

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 **44**, **48** and **49** were tested for their hemolytic property using human blood cells. As shown in Figure 13, compounds **44**, **48** and **49** demonstrated very low haemolytic potential at all tested concentrations (0.1, 1 and 10 $\mu\text{g/mL}$). The hemolytic values of all tested compounds at all tested concentrations were lower than the suggested nonhemolytic cut-off value.

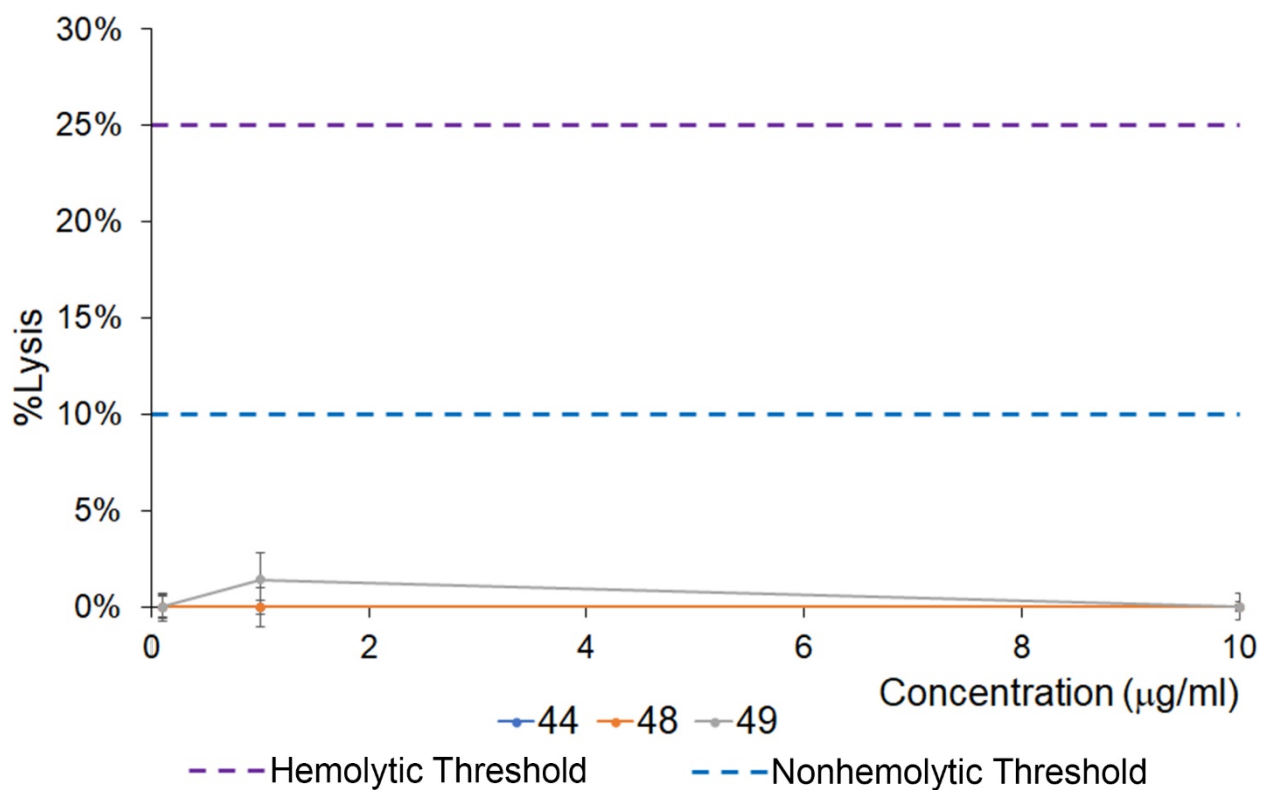


Figure 13. *in vitro* hemolysis activity of compounds **44**, **48** and **49**

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 **44**, **48** and **49** (27). As shown in Table 5, compounds **44**, **48** and **49** possessed a high apparent permeability, which is larger than 10^{-5} cm s⁻¹. Furthermore, the deduced apparent permeability of compounds had a similar magnitude to that of the predicted values.

Table 5. The deduced and predicted apparent permeability of compounds **44**, **48** and **49**

Compound	P _{app} average (n=4) (cm/s)	P _{app} Predicted (cm/s)
44	$1.87 \pm 0.47 \times 10^{-5}$	8.09×10^{-6}
48	$1.61 \pm 0.53 \times 10^{-5}$	2.57×10^{-6}
49	$1.49 \pm 0.79 \times 10^{-5}$	7.93×10^{-6}

CONCLUSIONS

In this paper, we described the rational design and synthesis of a new class of diphenyl 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 mechanism of the compounds were studied at both cellular and molecular levels, and 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. We named this class of antimicrobial compounds as “nusbiarylins” based on their target (NusB) and the core biaryl structure. The nusbiarylins represented a new class of antimicrobial agent against a previously unidentified protein-protein interaction in bacteria, with a distinct mechanism of action to current antibiotics on the market. We believe the subsequent investigation and development on the nusbiarylin compounds will make its contribution to the new era of antibiotic discovery.

EXPERIMENTAL SECTION.

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 $+4 \pm 2$ °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.

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.

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.

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 (30mL 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.

General Procedure (D) for synthesis of (E)-stilbenes

step 1: Hydroxyl group-protected (E)-stilbenes. To a mixture of compound **89a** (1.2 equiv), K_3PO_4 (1.4 equiv) and $\text{Pd}(\text{OAc})_2$ (0.03 equiv) in a sealed tube was added iodobenzenes (1 equiv)

and anhydrous DMF (3 mL) in glovebox. The resulting mixture was stirred at 100°C overnight. After the reaction mixture was cooled to rt, the product was extracted with EtOAc (30mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were removed under reduced pressure and the crude product containing 2-5% isomer was isolated by flash chromatography (EtOAc/Hex) on silica gel.

step 2: To a solution of crude product obtained above in MeOH (10 mL) was added H₂SO₄ (100 µL). The mixture was stirred at rt overnight. After the reaction was completed, the product was extracted with EtOAc (30mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were removed under reduced pressure and the title product was isolated by flash chromatography (EtOAc/Hex) on silica gel.

(E)-2-((cyclohexylimino)methyl)-4-nitrophenol (1)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and cyclohexylamine **1a** (137 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (183 mg, 62%); ¹H 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); ¹³C 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 C₁₃H₁₅N₂O₃ (M - H⁺) 247.1088, found 247.1089; Melting Point: 139-140°C.

(E)-2-((naphthalen-1-ylimino)methyl)-4-nitrophenol (2)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 1-aminonaphthalene **2a** (172 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (299 mg, 85%); ¹H 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); ¹³C 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 C₁₇H₁₁N₂O₃ (M - H⁺) 291.0775, found 291.0773; Melting Point: 160-161°C.

(E)-4-nitro-2-((phenylimino)methyl)phenol (3)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and aniline **3a** (109 μL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (188 mg, 65%); ¹H 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); ¹³C 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 C₁₃H₉N₂O₃ (M - H⁺) 241.0619, found 241.0620; Melting Point: 133-134°C.

(E)-2-(((2-fluorophenyl)imino)methyl)-4-nitrophenol (4)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-fluoroaniline **4a** (116 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (201 mg, 65%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.25 (s, 1H), 8.85 (s, 1H), 8.43 (d, $J = 2.8$ Hz, 1H), 8.31 (dd, $J = 9.2, 2.8$ Hz, 1H), 7.40 – 7.32 (m, 2H), 7.28 – 7.23 (m, 2H), 7.15 (d, $J = 9.2$ Hz, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.7, 162.4 (d, $J = 3.0$ Hz), 155.9 (d, $J = 253.5$ Hz), 140.0, 134.6 (d, $J = 9.1$ Hz), 129.2 (d, $J = 8.1$ Hz), 128.5 (d, $J = 10.1$ Hz), 124.9 (d, $J = 4.0$ Hz), 121.3, 118.4, 118.2, 116.9 (d, $J = 19.2$ Hz); HRMS (ESI) calcd for $\text{C}_{13}\text{H}_8\text{FN}_2\text{O}_3$ (M - H^+) 259.0524, found 259.0523; Melting Point: 166-167°C.

(E)-2-(((3-fluorophenyl)imino)methyl)-4-nitrophenol (5)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-fluoroaniline **5a** (115 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (230 mg, 74%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.03 (s, 1H), 8.73 (s, 1H), 8.43 (d, $J = 2.8$ Hz, 1H), 8.31 (dd, $J = 9.1, 2.7$ Hz, 1H), 7.49 – 7.43 (m, 1H), 7.15 – 7.06 (m, 4H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.5, 163.4 (d, $J = 249.5$ Hz), 161.8, 148.7 (d, $J = 8.9$ Hz), 140.2, 131.0 (d, $J = 9.1$ Hz), 128.6 (d, $J = 10.1$ Hz), 118.4, 118.0, 117.1 (d, $J = 3.0$ Hz), 114.9 (d, $J = 21.2$ Hz), 108.6 (d, $J = 23.2$ Hz); HRMS (ESI) calcd for $\text{C}_{13}\text{H}_8\text{FN}_2\text{O}_3$ (M - H^+) 259.0524, found 259.0828; Melting Point: 172-173°C.

(E)-2-(((4-fluorophenyl)imino)methyl)-4-nitrophenol (6)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-fluoroaniline **6a** (114 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (195 mg, 63%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.24 (s, 1H), 8.71 (s, 1H), 8.42 (d, $J = 2.8$ Hz, 1H), 8.30 (dd, $J = 9.2, 2.8$ Hz, 1H), 7.37 – 7.34 (m, 2H), 7.22 – 7.14 (m, 2H), 7.13 (d, $J = 9.2$ Hz, 1H); ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 166.7, 161.8, 161.8 (d, $J = 245.4$ Hz), 143.7 (d, $J = 3.0$ Hz), 139.7, 128.8, 128.6, 123.9 (d, $J = 9.1$ Hz), 119.2, 118.6, 116.8 (d, $J = 23.2$ Hz); HRMS (ESI) calcd for C₁₃H₈FN₂O₃ (M - H⁺) 259.0524, found 259.0522; Melting Point: 196-197°C.

(E)-4-nitro-2-((o-tolylimino)methyl)phenol (7)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-methylaniline **7a** (128 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (215 mg, 70%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.74 (s, 1H), 8.70 (s, 1H), 8.44 (d, $J = 2.8$ Hz, 1H), 8.30 (dd, $J = 9.2, 2.8$ Hz, 1H), 7.36 – 7.28 (m, 3H), 7.21 – 7.18 (m, 1H), 7.13 (d, $J = 9.1$ Hz, 1H), 2.46 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.2, 160.2, 145.6, 139.9, 132.6, 131.1, 128.4, 128.3, 128.1, 127.3, 118.4, 118.2, 117.6, 18.2; HRMS (ESI) calcd for C₁₄H₁₁N₂O₃ (M - H⁺) 255.0775, found 255.0771; Melting Point: 169-170°C.

(E)-4-nitro-2-((m-tolylimino)methyl)phenol (8)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-methylaniline **8a** (128 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (230 mg, 75%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.58 (s, 1H), 8.73 (s, 1H), 8.41 (d, $J = 2.9$ Hz, 1H), 8.28 (dd, $J = 9.2, 2.8$ Hz, 1H), 7.37 (t, $J = 7.5$ Hz, 1H), 7.21 – 7.15 (m, 3H), 7.11 (d, $J = 9.3$ Hz, 1H), 2.45 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.1, 160.3, 146.6, 139.9, 139.7, 129.5, 128.9, 128.4, 128.3, 121.9, 118.4, 118.2, 118.1, 21.4; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{11}\text{N}_2\text{O}_3$ (M - H^+) 255.0775, found 255.0772; Melting Point: 137-138°C.

(E)-4-nitro-2-((p-tolylimino)methyl)phenol (9)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-methylaniline **9a** (129 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (248 mg, 81%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.65 (s, 1H), 8.73 (s, 1H), 8.40 (d, $J = 2.7$ Hz, 1H), 8.28 (dd, $J = 9.2, 2.8$ Hz, 1H), 7.31 – 7.26 (m, 4H), 7.11 (d, $J = 9.1$ Hz, 1H), 2.43 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 167.0, 159.5, 143.9, 139.9, 138.4, 130.3, 128.2, 128.2, 121.1, 118.3, 118.2, 21.1; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{11}\text{N}_2\text{O}_3$ (M - H^+) 255.0775, found 255.0772; Melting Point: 160-161°C.

(E)-2-(((2-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (10)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-tert-butylbenzenamine **10a** (187 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (267 mg, 75%); ^1H 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 $\text{C}_{17}\text{H}_{17}\text{N}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 297.1245, found 297.1247; Melting Point: 152-153°C.

(E)-2-(((3-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (11)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-tert-butylbenzenamine **11a** (179 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (244 mg, 68%); ^1H 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 $\text{C}_{17}\text{H}_{17}\text{N}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 297.1245, found 297.1242; Melting Point: 128-129°C.

(E)-2-(((4-(tert-butyl)phenyl)imino)methyl)-4-nitrophenol (12)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-tert-butylaniline **12a** (191 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (217 mg, 61%); ^1H 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 $\text{C}_{17}\text{H}_{17}\text{N}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 297.1245, found 297.1243; Melting Point: 175-176°C.

(E)-2-(((2-hydroxyphenyl)imino)methyl)-4-nitrophenol (13)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-aminophenol **13a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (245 mg, 79%); ^1H 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 $\text{C}_{13}\text{H}_9\text{N}_2\text{O}_4$ ($\text{M} - \text{H}^+$) 257.0568, found 257.0565; Melting Point: 269-270°C.

(E)-2-(((3-hydroxyphenyl)imino)methyl)-4-nitrophenol (14)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminophenol **14a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (288 mg, 93%); ¹H 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); ¹³C 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 C₁₃H₉N₂O₄ (M - H⁺) 257.0568, found 257.0568; Melting Point: 242-243°C.

(E)-2-(((4-hydroxyphenyl)imino)methyl)-4-nitrophenol (15)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-aminophenol **15a** (131 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (272 mg, 88%); ¹H 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); ¹³C 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 C₁₃H₉N₂O₄ (M - H⁺) 257.0568, found 257.0567; Melting Point: 274-275°C.

(E)-2-(((2-chlorophenyl)imino)methyl)-4-nitrophenol (16)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-chloroaniline **16a** (126 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (262 mg, 79%); ^1H 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); ^{13}C 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 $\text{C}_{13}\text{H}_8\text{ClN}_2\text{O}_3$ (M - H^+) 275.0229, found 275.0227; Melting Point: 188-189°C.

(E)-2-(((3-chlorophenyl)imino)methyl)-4-nitrophenol (17)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-chloroaniline **17a** (127 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (267 mg, 81%); ^1H 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 $\text{C}_{13}\text{H}_8\text{ClN}_2\text{O}_3$ (M - H^+) 275.0229, found 275.0226; Melting Point: 203-204°C.

(E)-2-(((4-chlorophenyl)imino)methyl)-4-nitrophenol (18)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-chloroaniline **18a** (153 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (280 mg, 85%); ¹H 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); ¹³C 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 C₁₃H₈ClN₂O₃ (M - H⁺) 275.0229, found 275.0226; Melting Point: 225-226°C.

(E)-2-(((2-methoxyphenyl)imino)methyl)-4-nitrophenol (19)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-methoxyaniline **19a** (135 μL, 1.20 mmol, 1 equiv), yielding the pure product as a red solid (259 mg, 79%); ¹H NMR (400 MHz, Chloroform-*d*) δ 15.41 (s, 1H), 8.84 (s, 1H), 8.40 (d, *J* = 2.8 Hz, 1H), 8.26 (dd, *J* = 9.3, 2.8 Hz, 1H), 7.37 – 7.32 (m, 2H), 7.09 – 7.04 (m, 3H), 3.97 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 168.9, 159.4, 153.0, 139.2, 133.9, 129.3, 128.5, 128.3, 121.1, 119.7, 119.0, 117.8, 112.1, 55.9; HRMS (ESI) calcd for C₁₄H₁₁N₂O₄ (M - H⁺) 271.0724, found 271.0719; Melting Point: 186-187°C.

(E)-2-(((3-methoxyphenyl)imino)methyl)-4-nitrophenol (20)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-methoxyaniline **20a** (136 μL, 1.20 mmol, 1

equiv), yielding the pure product as a yellow solid (264 mg, 81%); ¹H NMR (400 MHz, Chloroform-*d*) δ 14.43 (s, 1H), 8.73 (s, 1H), 8.42 (d, *J* = 2.7 Hz, 1H), 8.29 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.39 (t, *J* = 8.1 Hz, 1H), 7.12 (d, *J* = 9.2 Hz, 1H), 6.96 – 6.92 (m, 2H), 6.88 (t, *J* = 2.2 Hz, 1H), 3.89 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 166.9, 160.8, 160.7, 148.0, 140.0, 130.5, 128.4, 128.4, 118.4, 118.1, 113.8, 113.3, 107.1, 55.5; HRMS (ESI) calcd for C₁₄H₁₁N₂O₄ (M - H⁺) 271.0724, found 271.0721; Melting Point: 167-168°C.

(*E*)-2-(((4-methoxyphenyl)imino)methyl)-4-nitrophenol (21)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-methoxyaniline **21a** (148 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (268 mg, 82%); ¹H NMR (400 MHz, Chloroform-*d*) δ 14.70 (s, 1H), 8.71 (s, 1H), 8.39 (d, *J* = 2.8 Hz, 1H), 8.26 (dd, *J* = 9.1, 2.8 Hz, 1H), 7.38 – 7.34 (m, 2H), 7.10 (d, *J* = 9.2 Hz, 1H), 7.03 – 6.99 (m, 2H), 3.89 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 166.9, 159.8, 158.0, 139.9, 139.4, 128.0, 127.9, 122.5, 118.3, 118.2, 114.9, 55.6; HRMS (ESI) calcd for C₁₄H₁₁N₂O₄ (M - H⁺) 271.0724, found 271.0727; Melting Point: 169-170°C.

methyl (*E*)-2-((2-hydroxy-5-nitrobenzylidene)amino)benzoate (22)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and methyl 2-aminobenzoate **22a** (155 μL, 1.20 mmol,

1 equiv), yielding the pure product as a red solid (285 mg, 79%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.29 (s, 1H), 8.66 (s, 1H), 8.43 (d, $J = 2.7$ Hz, 1H), 8.30 (dd, $J = 9.2, 2.8$ Hz, 1H), 8.09 (dd, $J = 7.9, 1.6$ Hz, 1H), 7.66 (td, $J = 7.7, 1.6$ Hz, 1H), 7.44 (td, $J = 7.6, 1.1$ Hz, 1H), 7.26 (dd, $J = 8.0, 1.1$ Hz, 1H), 7.14 (d, $J = 9.3$ Hz, 1H), 3.96 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 166.9, 166.3, 161.0, 147.3, 139.8, 133.7, 131.7, 128.6, 128.6, 127.4, 124.4, 119.1, 118.7, 118.3, 52.4; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_5$ ($\text{M} - \text{H}^+$) 299.0673, found 299.0672; Melting Point: 176-177°C.

methyl (*E*)-3-((2-hydroxy-5-nitrobenzylidene)amino)benzoate (23)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and methyl 3-aminobenzoate **23a** (181 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (307 mg, 85%); ^1H NMR (400 MHz, Chloroform-*d*) δ 14.12 (s, 1H), 8.80 (s, 1H), 8.45 (d, $J = 2.8$ Hz, 1H), 8.32 (dd, $J = 9.1, 2.8$ Hz, 1H), 8.07 – 8.02 (m, 2H), 7.60 – 7.53 (m, 2H), 7.15 (d, $J = 9.2$ Hz, 1H), 3.99 (s, 3H); ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 166.6, 166.2, 162.8, 147.9, 139.8, 131.4, 130.5, 129.0, 128.6, 128.4, 127.0, 122.1, 119.4, 118.5, 52.8; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_5$ ($\text{M} - \text{H}^+$) 299.0673, found 299.0669; Melting Point: 201-202°C.

methyl (*E*)-4-((2-hydroxy-5-nitrobenzylidene)amino)benzoate (24)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and methyl 4-aminobenzoate **24a** (181 mg, 1.20 mmol,

1 equiv), yielding the pure product as a yellow solid (294 mg, 82%); ¹H NMR (400 MHz, Chloroform-*d*) δ 13.99 (s, 1H), 8.76 (s, 1H), 8.45 (d, *J* = 2.8 Hz, 1H), 8.32 (dd, *J* = 9.2, 2.7 Hz, 1H), 8.18 – 8.16 (m, 2H), 7.40 – 7.38 (m, 2H), 7.15 (d, *J* = 9.1 Hz, 1H), 3.98 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 166.6, 166.3, 162.4, 150.8, 140.2, 131.3, 129.5, 128.9, 128.7, 121.2, 118.5, 118.0, 52.3; HRMS (ESI) calcd for C₁₅H₁₁N₂O₅ (M - H⁺) 299.0673, found 299.0672; Melting Point: 206-207°C.

(*E*)-4-nitro-2-(((2-(trifluoromethyl)phenyl)imino)methyl)phenol (25)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 2-aminobenzotrifluoride **25a** (151 μL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (228 mg, 61%); ¹H 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); ¹³C 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 C₁₄H₈F₃N₂O₃ (M - H⁺) 309.0493, found 309.0492; Melting Point: 161-162°C.

(*E*)-4-nitro-2-(((3-(trifluoromethyl)phenyl)imino)methyl)phenol (26)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminobenzotrifluoride **26a** (150 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (239 mg, 64%); ^1H 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 $\text{C}_{14}\text{H}_8\text{F}_3\text{N}_2\text{O}_3$ (M - H^+) 309.0493, found 309.0491; Melting Point: 141-142°C.

(E)-4-nitro-2-(((4-(trifluoromethyl)phenyl)imino)methyl)phenol (27)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-aminobenzotrifluoride **27a** (151 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (224 mg, 60%); ^1H 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 $\text{C}_{14}\text{H}_8\text{F}_3\text{N}_2\text{O}_3$ (M - H^+) 309.0493, found 309.0490; Melting Point: 169-170°C.

(E)-2-(((2-ethynylphenyl)imino)methyl)-4-nitrophenol (28)

step 1: 2-ethynylaniline (**28a**). To a solution of 2-iodoaniline (300 mg, 1.37 mmol), PdCl₂(PPh₃)₂ (29 mg, 0.04 mmol, 0.03 equiv), CuI (13 mg, 0.07 mmol, 0.05 equiv), Et₃N (1 mL) in dry THF (4 mL) was added trimethylsilylacetylene (290 μL, 2.06 mmol, 1.5 equiv) dropwisely via syringe under N₂ 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 K₂CO₃ (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 Na₂SO₄ and filtered. All volatiles were evaporated in vacuo and the product was obtained after flash chromatography (hexane : EtOAc = 100: 1) as a yellow liquid (94 mg, 59%); ¹H 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 **28a** (141 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (221 mg, 69%); ¹H 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); ¹³C 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 C₁₅H₉N₂O₃ (M - H⁺) 265.0619, found 265.0618; Melting Point: 169-170°C.

(E)-2-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (29)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminophenylacetylene **29a** (135 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (242 mg, 76%); ^1H 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 $\text{C}_{15}\text{H}_9\text{N}_2\text{O}_3$ (M - H^+) 265.0619, found 265.0620; Melting Point: 196-197°C.

(E)-2-(((4-ethynylphenyl)imino)methyl)-4-nitrophenol (30)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-ethynylaniline **30a** (141 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (191 mg, 60%); ^1H 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 $\text{C}_{15}\text{H}_9\text{N}_2\text{O}_3$ (M - H^+) 265.0619, found 265.0615; Melting Point: 201-202°C;

(E)-2-(((3-(hydroxymethyl)phenyl)imino)methyl)-4-nitrophenol (31)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminobenzyl alcohol **31a** (148 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (277 mg, 85%); ¹H NMR (400 MHz, DMSO-*d*₆) δ 14.52 (br s, 1H), 9.21 (s, 1H), 8.71 (d, *J* = 2.9 Hz, 1H), 8.26 (dd, *J* = 9.3, 2.9 Hz, 1H), 7.48 – 7.44 (m, 2H), 7.38 – 7.31 (m, 2H), 7.11 (d, *J* = 9.1 Hz, 1H), 5.34 (t, *J* = 5.8 Hz, 1H), 4.58 (d, *J* = 3.9 Hz, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.9, 161.8, 146.5, 144.8, 139.4, 129.8, 129.0, 128.9, 126.2, 120.1, 119.7, 119.0, 63.0; HRMS (ESI) calcd for C₁₄H₁₁N₂O₄ (M - H⁺) 271.0724, found 271.0721; Melting Point: 170-171°C.

(E)-2-(((4-(hydroxymethyl)phenyl)imino)methyl)-4-nitrophenol (32)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 4-aminobenzyl alcohol **32a** (148 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (293 mg, 90%); ¹H NMR (400 MHz, DMSO-*d*₆) δ 14.58 (br s, 1H), 9.20 (s, 1H), 8.66 (d, *J* = 3.0 Hz, 1H), 8.25 (dd, *J* = 9.3, 2.9 Hz, 1H), 7.48 – 7.43 (m, 4H), 7.10 (d, *J* = 9.3 Hz, 1H), 5.29 (br s, 1H), 4.55 (s, 2H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 167.8, 161.5, 145.1, 142.9, 139.4, 129.0, 128.8, 128.0, 121.6, 118.9, 118.9, 62.9; HRMS (ESI) calcd for C₁₄H₁₁N₂O₄ (M - H⁺) 271.0724, found 271.0722; Melting Point: 184-185°C.

(E)-3-((2-hydroxy-5-nitrobenzylidene)amino)benzoic acid (33)

The title compound was prepared according to general procedure (A) using 5-nitrosalicylaldehyde (200 mg, 1.20 mmol) and 3-aminobenzoic acid **33a** (165 mg, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (309 mg, 90%); ¹H 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); ¹³C 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 C₁₄H₉N₂O₅ (M - H⁺) 285.0517, found 271.0513; Melting Point: 330-331°C.

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

The title compound was prepared according to general procedure (B) using **1** (100 mg, 0.40 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₃H₁₇N₂O₃ (M - H⁺) 249.1245, found 249.1245; Melting Point: 177-178°C.

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

The title compound was prepared according to general procedure (B) using **2** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (Hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₇H₃N₂O₃ (M - H⁺) 293.0932, found 293.0929; Melting Point: 144-145°C.

4-nitro-2-((phenylamino)methyl)phenol (36)

The title compound was prepared according to general procedure (B) using **3** (100 mg, 0.41 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₃H₁₁N₂O₃ (M - H⁺) 243.0775, found 243.0775; Melting Point: 102-103°C.

2-(((2-fluorophenyl)amino)methyl)-4-nitrophenol (37)

The title compound was prepared according to general procedure (B) using **4** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography

(Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 9.57 (br s, 1H), 8.17 – 8.15 (m, 2H), 7.12 – 7.05 (m, 2H), 6.98 – 6.89 (m, 3H), 4.57 (s, 2H), 4.42 (s, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 162.8, 153.1 (d, $J = 242.4$ Hz), 140.9, 134.6 (d, $J = 12.1$ Hz), 125.5, 124.9 (d, $J = 4.0$ Hz), 124.7, 123.1, 121.7 (d, $J = 8.1$ Hz), 117.1, 116.1 (d, $J = 3.0$ Hz), 115.2 (d, $J = 19.2$ Hz), 48.3; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{10}\text{FN}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 261.0681, found 261.0683; Melting Point: 107-108°C.

2-(((3-fluorophenyl)amino)methyl)-4-nitrophenol (38)

The title compound was prepared according to general procedure (B) using **5** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 9.37 (br s, 1H), 8.16 – 8.14 (m, 2H), 7.22 (q, $J = 7.7$ Hz, 1H), 6.97 – 6.95 (m, 1H), 6.69 – 6.63 (m, 2H), 6.58 – 6.55 (m, 1H), 4.53 (s, 2H), 4.28 (br s, 1H); ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 163.9 (d, $J = 240.4$ Hz), 162.1, 150.9 (d, $J = 11.1$ Hz), 140.1, 130.8 (d, $J = 11.1$ Hz), 127.5, 124.9, 124.3, 115.6, 108.9 (d, $J = 2.0$ Hz), 102.6 (d, $J = 21.2$ Hz), 98.8 (d, $J = 25.3$ Hz), 41.1; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{10}\text{FN}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 261.0681, found 261.0679; Melting Point: 122-123°C.

2-(((4-fluorophenyl)amino)methyl)-4-nitrophenol (39)

The title compound was prepared according to general procedure (B) using **6** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography

(Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 10.20 (br s, 1H), 8.15 (dd, $J = 8.8, 2.8$ Hz, 1H), 8.12 (d, $J = 2.8$ Hz, 1H), 7.02 – 6.97 (m, 2H), 6.95 (d, $J = 8.8$ Hz, 1H), 6.85 (dd, $J = 9.0, 4.4$ Hz, 2H), 4.53 (s, 2H), 4.07 (s, 1H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.3, 158.4 (d, $J = 242.4$ Hz), 142.1 (d, $J = 2.0$ Hz), 140.8, 125.5, 124.5, 122.7, 118.0 (d, $J = 8.1$ Hz), 117.2, 116.2 (d, $J = 23.2$ Hz), 49.7; HRMS (ESI) calcd for $\text{C}_{13}\text{H}_{10}\text{FN}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 261.0681, found 261.0682; Melting Point: 131-132°C.

4-nitro-2-((o-tolylamino)methyl)phenol (40)

The title compound was prepared according to general procedure (B) using **7** (100 mg, 0.39 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 10.10 (br s, 1H), 8.17 – 8.15 (m, 2H), 7.22 – 7.18 (m, 2H), 6.98 – 6.94 (m, 2H), 6.88 (d, $J = 8.1$ Hz, 1H), 4.59 (s, 2H), 3.95 (br s, 1H), 2.29 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.3, 143.9, 140.8, 130.7, 127.4, 125.9, 125.4, 124.7, 123.0, 121.8, 117.1, 114.1, 48.5, 17.6; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 257.0932, found 257.0928; Melting Point: 105-106°C.

4-nitro-2-((m-tolylamino)methyl)phenol (41)

The title compound was prepared according to general procedure (B) using **8** (100 mg, 0.39 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 8.14 (d, $J = 10.1$ Hz, 2H), 7.18

(t, $J = 7.6$ Hz, 1H), 6.95 (d, $J = 8.8$ Hz, 1H), 6.84 (d, $J = 7.6$ Hz, 1H), 6.71 (d, $J = 5.5$ Hz, 2H), 4.54 (s, 2H), 2.33 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.4, 146.0, 140.8, 139.6, 129.4, 125.4, 124.5, 123.0, 123.0, 117.5, 117.1, 113.6, 49.2, 21.5; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_3$ (M - H^+) 257.0932, found 257.0928; Melting Point: 140-141°C.

4-nitro-2-((p-tolylamino)methyl)phenol (42)

The title compound was prepared according to general procedure (B) using **9** (100 mg, 0.39 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography (hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 10.49 (br s, 1H), 8.17 – 8.12 (m, 2H), 7.10 (d, $J = 8.3$ Hz, 2H), 6.94 (d, $J = 8.8$ Hz, 1H), 6.81 (d, $J = 8.4$ Hz, 2H), 4.54 (s, 2H), 4.02 (br s, 1H), 2.31 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.5, 143.4, 140.7, 131.8, 130.1, 125.4, 124.5, 122.9, 117.1, 116.9, 49.7, 20.6; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_3$ (M - H^+) 257.0932, found 257.0927; Melting Point: 118-119°C.

2-(((2-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (43)

The title compound was prepared according to general procedure (B) using **10** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H 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.

2-(((3-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (44)

The title compound was prepared according to general procedure (B) using **11** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography (hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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°C.

2-(((4-(tert-butyl)phenyl)amino)methyl)-4-nitrophenol (45)

The title compound was prepared according to general procedure (B) using **12** (100 mg, 0.34 mmol), yielding the pure product as a yellow solid (94 mg, 93%) after flash chromatography (Hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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°C.

2-(((2-hydroxyphenyl)amino)methyl)-4-nitrophenol (46)

The title compound was prepared according to general procedure **(B)** using **13** (100 mg, 0.39 mmol), yielding the pure product as a red solid (90 mg, 89%) after flash chromatography (DCM : MeOH = 100 : 1); ^1H 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 $\text{C}_{13}\text{H}_{11}\text{N}_2\text{O}_4$ ($\text{M} - \text{H}^+$) 259.0724, found 259.0723; Melting Point: 136-137°C.

2-(((3-hydroxyphenyl)amino)methyl)-4-nitrophenol (47)

The title compound was prepared according to general procedure **(B)** using **14** (100 mg, 0.39 mmol), yielding the pure product as a red solid (87 mg, 86%) after flash chromatography (DCM : MeOH = 100 : 1); ^1H 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 $\text{C}_{13}\text{H}_{11}\text{N}_2\text{O}_4$ ($\text{M} - \text{H}^+$) 259.0724, found 259.0723; Melting Point: 142-143°C.

2-(((2-chlorophenyl)amino)methyl)-4-nitrophenol (48)

The title compound was prepared according to general procedure (B) using **16** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₃H₁₀ClN₂O₃ (M - H⁺) 277.0385, found 277.0384; Melting Point: 104-105°C.

2-(((3-chlorophenyl)amino)methyl)-4-nitrophenol (49)

The title compound was prepared according to general procedure (B) using **17** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₃H₁₀ClN₂O₃ (M - H⁺) 277.0385, found 277.0387; Melting Point: 116-117°C.

2-(((4-chlorophenyl)amino)methyl)-4-nitrophenol (50)

The title compound was prepared according to general procedure (B) using **18** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₃H₁₀ClN₂O₃ (M - H⁺) 277.0385, found 277.0386; Melting Point: 123-124°C.

2-(((2-methoxyphenyl)amino)methyl)-4-nitrophenol (51)

The title compound was prepared according to general procedure (B) using **19** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (88 mg, 87%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.39 (br s, 1H), 8.20 – 8.11 (m, 2H), 7.02 – 6.80 (m, 5H), 4.70 (br s, 1H), 4.54 (s, 2H), 3.90 (s, 3H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 163.5, 148.9, 140.7, 135.8, 125.3, 124.4, 123.3, 121.7, 121.2, 117.1, 114.8, 110.1, 55.6, 49.1; HRMS (ESI) calcd for C₁₄H₁₃N₂O₄ (M - H⁺) 273.0881, found 273.0878; Melting Point: 124-125°C.

2-(((3-methoxyphenyl)amino)methyl)-4-nitrophenol (52)

The title compound was prepared according to general procedure (B) using **20** (100 mg, 0.37 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography

(hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 9.93 (br s, 1H), 8.17 – 8.14 (m, 2H), 7.20 (t, J = 8.1 Hz, 1H), 6.95 (d, J = 8.6 Hz, 1H), 6.56 (dd, J = 8.2, 2.4 Hz, 1H), 6.48 (dd, J = 7.9, 2.3 Hz, 1H), 6.43 (d, J = 2.4 Hz, 1H), 4.54 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 163.1, 160.7, 147.6, 140.8, 130.4, 125.4, 124.5, 123.1, 117.0, 109.0, 106.8, 102.8, 55.2, 48.7; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_4$ ($\text{M} - \text{H}^+$) 273.0881, found 273.0876; Melting Point: 98-99°C.

2-(((4-methoxyphenyl)amino)methyl)-4-nitrophenol (53)

The title compound was prepared according to general procedure (B) using **21** (100 mg, 0.37 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 10 : 1); ^1H NMR (400 MHz, DMSO-*d*₆) δ 8.09 (d, J = 2.9 Hz, 1H), 8.02 (dd, J = 9.0, 3.0 Hz, 1H), 6.99 (d, J = 8.9 Hz, 1H), 6.70 (d, J = 8.4 Hz, 2H), 6.51 (d, J = 8.5 Hz, 2H), 4.20 (s, 2H), 3.62 (s, 3H); ^{13}C NMR (101 MHz, DMSO-*d*₆) δ 162.1, 151.4, 142.9, 140.1, 128.4, 124.6, 124.2, 115.5, 115.1, 113.7, 55.7, 42.1; HRMS (ESI) calcd for $\text{C}_{14}\text{H}_{13}\text{N}_2\text{O}_4$ ($\text{M} - \text{H}^+$) 273.0881, found 273.0881; Melting Point: 140-141°C.

methyl 2-((2-hydroxy-5-nitrobenzyl)amino)benzoate (54)

The title compound was prepared according to general procedure (B) using **22** (100 mg, 0.33 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, Chloroform-*d*) δ 8.65 (br s, 1H), 8.18 (d, J = 2.8

Hz, 1H), 8.14 (dd, $J = 8.8, 2.8$ Hz, 1H), 8.04 (br s, 1H), 8.01 (dd, $J = 7.9, 1.7$ Hz, 1H), 7.42 (ddd, $J = 8.5, 7.3, 1.7$ Hz, 1H), 6.94 (d, $J = 8.8$ Hz, 1H), 6.89 – 6.82 (m, 2H), 4.60 (d, $J = 4.9$ Hz, 2H), 3.92 (s, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 168.5, 162.6, 150.7, 139.8, 135.3, 131.7, 127.0, 125.2, 124.4, 115.8, 115.4, 112.1, 110.1, 52.1, 41.2; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{13}\text{N}_2\text{O}_5$ ($\text{M} - \text{H}^+$) 301.0830, found 301.0833; Melting Point: 175-176°C.

methyl 3-((2-hydroxy-5-nitrobenzyl)amino)benzoate (55)

The title compound was prepared according to general procedure (B) using **23** (100 mg, 0.33 mmol), yielding the pure product as a yellow solid (88 mg, 87%) after flash chromatography (hexane : EtOAc = 10 : 1); ^1H NMR (400 MHz, DMSO- d_6) δ 11.36 (br s, 1H), 8.08 – 8.02 (m, 2H), 7.22 – 7.14 (m, 3H), 7.02 (d, $J = 8.9$ Hz, 1H), 6.82 (d, $J = 8.1$ Hz, 1H), 6.63 (br s, 1H), 4.29 (s, 2H), 3.79 (s, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 167.1, 162.1, 149.0, 140.1, 130.8, 129.8, 127.5, 124.9, 124.1, 117.3, 117.0, 115.6, 112.9, 52.4, 41.1; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{13}\text{N}_2\text{O}_5$ ($\text{M} - \text{H}^+$) 301.0830, found 301.0831; Melting Point: 130-131°C.

methyl 4-((2-hydroxy-5-nitrobenzyl)amino)benzoate (56)

The title compound was prepared according to general procedure (B) using **24** (100 mg, 0.33 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H NMR (400 MHz, DMSO- d_6) δ 11.38 (br s, 1H), 8.05 (d, $J = 7.8$ Hz, 2H), 7.69 (d, $J = 8.5$ Hz, 2H), 7.11 (t, $J = 6.0$ Hz, 1H), 7.02 (d, $J = 8.1$ Hz, 1H), 6.62 (d, $J =$

8.6 Hz, 2H), 4.33 (d, $J = 5.9$ Hz, 2H), 3.74 (s, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 166.7, 162.1, 152.9, 140.0, 131.5, 127.1, 125.0, 124.2, 116.9, 115.7, 111.7, 51.7, 40.8; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{13}\text{N}_2\text{O}_5$ ($\text{M} - \text{H}^+$) 301.0830, found 301.0826; Melting Point: 203-204°C.

4-nitro-2-(((2-(trifluoromethyl)phenyl)amino)methyl)phenol (57)

The title compound was prepared according to general procedure (B) using **25** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H 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 $\text{C}_{14}\text{H}_{10}\text{F}_3\text{N}_2\text{O}_3$ ($\text{M} - \text{H}^+$) 311.0649, found 311.0651; Melting Point: 114-115°C.

4-nitro-2-(((3-(trifluoromethyl)phenyl)amino)methyl)phenol (58)

The title compound was prepared according to general procedure (B) using **26** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1); ^1H 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°C.

4-nitro-2-(((4-(trifluoromethyl)phenyl)amino)methyl)phenol (59)

The title compound was prepared according to general procedure (B) using **27** (100 mg, 0.32 mmol), yielding the pure product as a yellow solid (89 mg, 88%) after flash chromatography (Hexane : EtOAc = 15 : 1); 1H 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°C.

2-(((3-ethynylphenyl)amino)methyl)-4-nitrophenol (60)

The title compound was prepared according to general procedure (B) using **29** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (hexane : EtOAc = 15: 1); 1H 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.

2-(((3-(hydroxymethyl)phenyl)amino)methyl)-4-nitrophenol (61)

The title compound was prepared according to general procedure (B) using **31** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (85 mg, 84%) after flash chromatography (hexane : EtOAc = 15: 1); 1H NMR (400 MHz, DMSO- d_6) δ 11.29 (br s, 1H), 8.09 (d, $J = 2.9$ Hz, 1H), 8.03 (dd, $J = 9.0, 2.9$ Hz, 1H), 7.02 – 6.98 (m, 2H), 6.59 – 6.49 (m, 2H), 6.41 (dd, $J = 7.9, 2.3$ Hz, 1H), 5.01 (br s, 1H), 4.36 (s, 2H), 4.25 (s, 2H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.0, 148.7, 143.8, 140.1, 129.1, 128.3, 124.7, 124.0, 115.5, 114.9, 110.9, 110.8, 63.7, 41.3; HRMS (ESI) calcd for $C_{14}H_{13}N_2O_4$ ($M - H^+$) 273.0881, found 273.0879; Melting Point: 136-137°C.

3-((2-hydroxy-5-nitrobenzyl)amino)benzoic acid (62)

The title compound was prepared according to general procedure (B) using **33** (100 mg, 0.35 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (DCM : MeOH = 50 : 1); 1H 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 C₁₄H₁₁N₂O₅ (M - H⁺) 287.0673, found 287.0673; Melting Point: 190-191°C.

methyl (*E*)-3-(((3-ethynylphenyl)imino)methyl)-4-hydroxybenzoate (63)

step 1: methyl 3-formyl-4-hydroxybenzoate (**63a**). 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); ¹H 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 **63a** (200 mg, 1.11 mmol) and 3-aminophenylacetylene **29a** (125 μL, 1.11 mmol, 1 equiv), yielding the pure product as a white solid (257 mg, 83%); ¹H 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); ¹³C 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 C₁₇H₁₂NO₃ (M - H⁺) 278.0823, found 278.0822; Melting Point: 153-154°C.

(*E*)-2-(((3-ethynylphenyl)imino)methyl)-4-fluorophenol (64)

The title compound was prepared according to general procedure (A) using 5-fluorosalicylaldehyde **64a** (300 mg, 2.14 mmol) and 3-aminophenylacetylene **29a** (241 μ L, 2.14 mmol, 1 equiv), yielding the pure product as a red solid (346 mg, 68%); ^1H 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 $\text{C}_{15}\text{H}_9\text{FNO}$ ($\text{M} - \text{H}^+$) 238.0674, found 238.0672; Melting Point: 113-114°C.

(E)-3-(((3-ethynylphenyl)imino)methyl)-4-hydroxybenzonitrile (65)

step 1: 5-cyano-2-methoxybenzaldehyde (**65a**). 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%); ^1H 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-hydroxybenzotrile (**65b**). To a solution of compound **65a** (478 mg, 2.97 mmol) in dry DCM (10 mL) was added BBr₃ (8.9 mL, 1 M in DCM, 3 equiv) dropwisely via syringe under N₂ 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 Na₂SO₄ 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%); ¹H 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 **65b** (200 mg, 1.36 mmol) and 3-aminophenylacetylene **29a** (153 μL, 1.36 mmol, 1 equiv), yielding the pure product as a yellow solid (287 mg, 86%); ¹H 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); ¹³C 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 C₁₆H₉N₂O (M - H⁺) 245.0720, found 245.0719; Melting Point: 222-223°C.

(E)-2-(3-(((3-ethynylphenyl)imino)methyl)-4-hydroxyphenyl)acetonitrile (66)

step 1: 2-(3-formyl-4-hydroxyphenyl)acetonitrile (**66a**). 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 **66a** obtained in step 1 (486 mg) and 3-aminophenylacetylene **29a** (251 μ L, 2.23 mmol), yielding the pure product as a white solid (348 mg, 36% in two steps); ^1H 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 $\text{C}_{17}\text{H}_{11}\text{N}_2\text{O}$ ($\text{M} - \text{H}^+$) 259.0877, found 259.0876; Melting Point: 119-120°C.

(E)-2-(((3-ethynylphenyl)imino)methyl)-4-methoxyphenol (67)

step 1: 2-hydroxy-5-methoxybenzaldehyde (**67a**). 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); ^1H 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 **67a** (300 mg, 1.97 mmol) and 3-aminophenylacetylene **29a** (222 μ L, 1.97 mmol, 1 equiv), yielding the

pure product as a white solid (295 mg, 65%); ¹H 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); ¹³C 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 C₁₆H₁₂NO₂ (M - H⁺) 250.0874, found 250.0875; Melting Point: 116-117°C.

(*E*)-2-(((3-ethynylphenyl)imino)methyl)phenol (68)

The title compound was prepared according to general procedure (A) using salicylaldehyde **68a** (250 mg, 2.05 mmol) and 3-aminophenylacetylene **29a** (230 μL, 2.05 mmol, 1 equiv), yielding the pure product as a white solid (303 mg, 67%); ¹H 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); ¹³C 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 C₁₅H₁₀NO (M - H⁺) 220.0768, found 220.0772; Melting Point: 116-117°C.

(*E*)-N-(3-ethynylphenyl)-1-(3-nitrophenyl)methanimine (69)

The title compound was prepared according to general procedure (A) using 3-nitro benzaldehyde **69a** (250 mg, 1.65 mmol) and 3-aminophenylacetylene **29a** (186 μL, 1.65 mmol, 1 equiv), yielding the pure product as a yellow solid (284 mg, 69%); ¹H 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 $\text{C}_{15}\text{H}_{11}\text{N}_2\text{O}_2$ ($\text{M} + \text{H}^+$) 251.0815, found 251.0818; Melting Point: 126-127°C.

(*E*)-*N*-(3-ethynylphenyl)-1-(2-methoxy-5-nitrophenyl)methanimine (70)

step 1: 2-methoxy-5-nitrobenzaldehyde (**70a**). 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 **70a** (200 mg, 1.10 mmol) and 3-aminophenylacetylene **29a** (124 μL , 1.10 mmol, 1 equiv), yielding the pure product as a yellow solid (265 mg, 86%); ^1H 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 C₁₆H₁₃N₂O₃ (M + H⁺) 281.0921, found 281.0922; Melting Point: 156-157°C.

(E)-2-(((3-ethynylphenyl)imino)methyl)-5-nitrophenol (71)

The title compound was prepared according to general procedure (A) using 4-nitrosalicylaldehyde **71a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **29a** (135 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (278 mg, 87%); ¹H 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); ¹³C 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 C₁₅H₉N₂O₃ (M - H⁺) 265.0619, found 265.0620; Melting Point: 204-205°C.

(E)-2-(((3-ethynylphenyl)imino)methyl)-6-nitrophenol (72)

The title compound was prepared according to general procedure (A) using 3-nitrosalicylaldehyde **72a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **29a** (135 μ L, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (235 mg, 74%); ¹H 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); ¹³C 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 C₁₅H₉N₂O₃ (M - H⁺) 265.0619, found 265.0616; Melting Point: 140-141°C.

(E)-3-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (73)

The title compound was prepared according to general procedure (A) using 5-hydroxy-2-nitrobenzaldehyde **73a** (200 mg, 1.20 mmol) and 3-aminophenylacetylene **29a** (135 µL, 1.20 mmol, 1 equiv), yielding the pure product as a yellow solid (261 mg, 82%); ¹H 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); ¹³C 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 C₁₅H₉N₂O₃ (M - H⁺) 265.0619, found 265.0621; Melting Point: 168-169°C;

(E)-2-bromo-6-(((3-ethynylphenyl)imino)methyl)-4-nitrophenol (74)

The title compound was prepared according to general procedure (A) using 3-bromo-2-hydroxy-5-nitrobenzaldehyde **74a** (150mg, 0.61 mmol) and 3-aminophenylacetylene **29a** (69 µL, 0.61 mmol, 1 equiv), yielding the pure product as a yellow solid (182 mg, 86%); ¹H 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); ¹³C 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 C₁₅H₈BrN₂O₃ (M - H⁺) 342.9724, found 342.9727; Melting Point: 221-222°C.

(E)-2,4-dichloro-6-(((3-ethynylphenyl)imino)methyl)phenol (75)

The title compound was prepared according to general procedure (A) using 3,5-dichlorosalicylaldehyde **75a** (200 mg, 1.05 mmol) and 3-aminophenylacetylene **29a** (118 µL, 1.05 mmol, 1 equiv), yielding the pure product as a yellow solid (224 mg, 74%); ¹H 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); ¹³C 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 C₁₅H₈Cl₂N₂O (M - H⁺) 287.9988, found 287.9987; Melting Point: 122-123°C.

methyl 3-(((3-ethynylphenyl)amino)methyl)-4-hydroxybenzoate (76)

The title compound was prepared according to general procedure (B) using **63** (100 mg, 0.36 mmol), yielding the pure product as a white solid (90 mg, 89%) after flash chromatography (hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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°C.

2-(((3-ethynylphenyl)amino)methyl)-4-fluorophenol (77)

The title compound was prepared according to general procedure (B) using **64** (100 mg, 0.41 mmol), yielding the pure product as a red solid (90 mg, 89%) after flash chromatography (hexane : EtOAc = 15 : 1); 1H 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°C.

3-(((3-ethynylphenyl)amino)methyl)-4-hydroxybenzotrile (78)

The title compound was prepared according to general procedure (B) using **65** (100 mg, 0.41 mmol), yielding the pure product as a white solid (94 mg, 93%) after flash chromatography (Hexane : EtOAc = 15 : 1); 1H 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 C₁₆H₁₁N₂O (M - H⁺) 247.0877, found 247.0876; Melting Point: 110-111°C.

2-(3-(((3-ethynylphenyl)amino)methyl)-4-hydroxyphenyl)acetonitrile (79)

The title compound was prepared according to general procedure (B) using **66** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (93 mg, 92%) after flash chromatography (hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₇H₁₃N₂O (M - H⁺) 261.1033, found 261.1032; Melting Point: 113-114°C.

2-(((3-ethynylphenyl)amino)methyl)-4-methoxyphenol (80)

The title compound was prepared according to general procedure (B) using **67** (100 mg, 0.39 mmol), yielding the pure product as a white solid (92 mg, 91%) after flash chromatography (hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₆H₁₄NO₂ (M - H⁺) 252.1030, found 252.1023; Melting Point: 92-93°C.

2-(((3-ethynylphenyl)amino)methyl)phenol (81)

The title compound was prepared according to general procedure (B) using **68** (100 mg, 0.44 mmol), yielding the pure product as a white solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 15 : 1); ¹H 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); ¹³C 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 C₁₅H₁₂NO (M - H⁺) 222.0924, found 222.0923; Melting Point: 125-126°C.

3-ethynyl-N-(3-nitrobenzyl)aniline (82)

The title compound was prepared according to general procedure (B) using **69** (100 mg, 0.40 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 20 : 1); ¹H 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); ¹³C 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 C₁₅H₁₃N₂O₂ (M + H⁺) 253.0972, found 253.0974; Melting Point: 62-63°C.

3-ethynyl-N-(2-methoxy-5-nitrobenzyl)aniline (83)

The title compound was prepared according to general procedure (B) using **70** (100 mg, 0.36 mmol), yielding the pure product as a yellow solid (95 mg, 94%) after flash chromatography (Hexane : EtOAc = 20 : 1); ¹H 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); ¹³C 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 C₁₆H₁₅N₂O₃ (M + H⁺) 283.1077, found 283.1080; Melting Point: 83-84°C.

2-(((3-ethynylphenyl)amino)methyl)-5-nitrophenol (84)

The title compound was prepared according to general procedure (B) using **71** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (87 mg, 86%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₅H₁₁N₂O₃ (M - H⁺) 267.0775, found 267.0773; Melting Point: 117-118°C.

2-(((3-ethynylphenyl)amino)methyl)-6-nitrophenol (85)

The title compound was prepared according to general procedure (B) using **72** (100 mg, 0.38 mmol), yielding the pure product as a yellow solid (90 mg, 89%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₅H₁₂N₂O₃ (M - H⁺) 267.0775, found 267.0770; Melting Point: 82-83°C.

2-bromo-6-(((3-ethynylphenyl)amino)methyl)-4-nitrophenol (86)

The title compound was prepared according to general procedure (B) using **74** (100 mg, 0.29 mmol), yielding the pure product as a yellow solid (92 mg, 91%) after flash chromatography (Hexane : EtOAc = 10 : 1); ¹H 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); ¹³C 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 C₁₅H₁₀BrN₂O₃ (M - H⁺) 344.9880, found 344.9878; Melting Point: 94-95°C.

2,4-dichloro-6-(((3-ethynylphenyl)amino)methyl)phenol (87)

The title compound was prepared according to general procedure (B) using **75** (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); ¹H 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); ¹³C 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 C₁₅H₁₀Cl₂NO (M - H⁺) 290.0145, found 290.0141. Melting point: 105-106°C.

5-nitro-2-((2-(trimethylsilyl)ethoxy)methoxy)benzaldehyde (88a).

To a mixture of 5-nitrosalicylaldehyde (500 mg, 2.99 mmol) and DIPEA (1.24 mL, 7.48 mmol, 2.5 equiv) in dry DCM (30 mL) was added 2-(trimethylsilyl)ethoxymethyl chloride (530 μL, 2.99 mmol, 1 equiv), and the reaction mixture was stirred at room temperature until complete consumption of the starting material as determined by TLC. The product was extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 30: 1) as a light yellow liquid (716 mg, 80%); ¹H NMR (400 MHz, Chloroform-*d*) δ 10.49 (s, 1H), 8.73 (d, *J* = 2.9 Hz, 1H), 8.42 (dd, *J* = 9.3, 2.9 Hz, 1H), 7.42 (d, *J* = 9.3 Hz, 1H), 5.48 (s, 2H), 3.85 (t, *J* = 8.2 Hz, 2H), 1.00 (t, *J* = 8.2 Hz, 2H), 0.03 (s, 9H).

(E)-4-nitro-2-styrylphenol (88)

step 1: (E)-trimethyl(2-((4-nitro-2-styrylphenoxy)methoxy)ethyl)silane (**88b**). To a solution of diethyl benzylphosphonate (171 μ L, 0.84 mmol, 1 equiv) in dry THF (10 mL) at 0°C was added NaH (40 mg, 60% in mineral oil, 1.00 mmol, 1.2 equiv). The resulting mixture was stirred at the same temperature for 30 min and **88a** (250 mg, 0.84 mmol) in dry THF (5 mL) was added dropwisely. The mixture was stirred at room temperature overnight before extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were evaporated *in vacuo* and the crude product was used directly in the next step without further purification.

step 2: Crude **88b** obtained in last step was dissolved in 2% H₂SO₄-methanol solution (10 mL). The mixture was stirred at room temperature for 3h, then quenched with water and the product was extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 10: 1) as a yellow solid (71 mg, 35% in two steps); ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.48 (br s, 1H), 8.48 (d, *J* = 2.8 Hz, 1H), 8.05 (d, *J* = 9.0 Hz, 1H), 7.64 – 7.62 (m, 2H), 7.47 (d, *J* = 16.5 Hz, 1H), 7.42 – 7.38 (m, 3H), 7.30 (t, *J* = 7.3 Hz, 1H), 7.06 (d, *J* = 8.9 Hz, 1H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 158.38, 141.90, 136.62, 132.76, 128.83, 128.46, 126.87, 125.66, 124.24, 122.96, 120.60, 116.09; HRMS (ESI) calcd for C₁₄H₁₀NO₃ (M - H⁺) 240.0666, found 240.0662; Melting point: 136-137°C.

trimethyl(2-((4-nitro-2-vinylphenoxy)methoxy)ethyl)silane (89a)

To a solution of methyltriphenylphosphonium bromide (3.6 g, 10.08 mmol, 1.5 equiv) in dry THF (30 mL) at 0°C was added NaH (444 mg, 60% in mineral oil, 11.1 mmol, 1.65 equiv). The resulting mixture was stirred at the same temperature for 30 min and **88a** (2 g, 6.73 mmol) in dry THF (10 mL) was added dropwisely via syringe under N₂ atmosphere. The mixture was stirred at room temperature overnight before extracted with EtOAc (20 mL x 3). The organic layers were combined, washed with saturated brine, dried over Na₂SO₄ and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 100: 1) as a white solid (1238 mg, 62%); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.40 (d, *J* = 2.8 Hz, 1H), 8.13 (dd, *J* = 9.2, 2.9 Hz, 1H), 7.23 (d, *J* = 9.1 Hz, 1H), 7.04 (dd, *J* = 17.7, 11.1 Hz, 1H), 6.15 – 5.72 (m, 1H), 5.60 – 5.42 (m, 1H), 5.38 (s, 2H), 3.83 – 3.76 (m, 2H), 1.03 – 0.93 (m, 2H), 0.02 (s, 9H).

(*E*)-2-(2-methoxystyryl)-4-nitrophenol (89)

The title compound was prepared according to general procedure (**D**) using 2-iodoanisole **89b** (73 μL, 0.56 mmol), yielding the pure product as a yellow solid (97 mg, 64%) after flash chromatography (Hexane : EtOAc = 20 : 1); ¹H NMR (400 MHz, Chloroform-*d*) δ 8.49 (d, *J* = 2.7 Hz, 1H), 8.06 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.63 (d, *J* = 7.8 Hz, 1H), 7.59 (d, *J* = 16.6 Hz, 1H), 7.34 – 7.28 (m, 2H), 7.01 (t, *J* = 7.5 Hz, 1H), 6.94 (dd, *J* = 14.9, 8.6 Hz, 2H), 6.03 (s, 1H), 3.94 (s, 3H);

^{13}C NMR (101 MHz, DMSO- d_6) δ 161.5, 157.2, 140.4, 129.8, 127.0, 126.0, 125.9, 125.6, 124.7, 122.7, 122.7, 121.2, 116.5, 111.9, 56.0; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{12}\text{NO}_4$ ($\text{M} - \text{H}^+$) 270.0772, found 270.0769; Melting point: 183-184°C.

(E)-2-(3-methoxystyryl)-4-nitrophenol (90)

The title compound was prepared according to general procedure **(D)** using 3-iodoanisole **90b** (67 μL , 0.56 mmol), yielding the pure product as a yellow solid (89 mg, 58%) after flash chromatography (Hexane : EtOAc = 20 : 1); ^1H NMR (400 MHz, Chloroform- d) δ 8.48 (d, $J = 2.8$ Hz, 1H), 8.08 (dd, $J = 8.9, 2.7$ Hz, 1H), 7.39 – 7.29 (m, 2H), 7.23 (d, $J = 16.4$ Hz, 1H), 7.18 (d, $J = 7.6$ Hz, 1H), 7.11 (t, $J = 2.2$ Hz, 1H), 6.93 – 6.89 (m, 2H), 6.13 (s, 1H), 3.89 (s, 3H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 161.6, 160.1, 140.5, 138.9, 131.3, 130.2, 125.1, 124.9, 123.0, 122.5, 119.5, 116.6, 114.3, 112.2, 55.5; HRMS (ESI) calcd for $\text{C}_{15}\text{H}_{12}\text{NO}_4$ ($\text{M} - \text{H}^+$) 270.0772, found 270.0771; Melting point: 158-159°C.

(E)-2-(2-hydroxy-5-nitrostyryl)benzotrile (91)

The title compound was prepared according to general procedure **(D)** using 2-iodobenzotrile **91b** (128 mg, 0.56 mmol), yielding the pure product as a yellow solid (95 mg, 64%) after flash chromatography (Hexane : EtOAc = 20 : 1); ^1H NMR (400 MHz, DMSO- d_6) δ 11.82 (s, 1H), 8.45 (s, 1H), 8.11 – 8.02 (m, 2H), 7.87 – 7.46 (m, 3H), 7.66 – 7.49 (m, 2H), 7.08 (d, $J = 9.1$ Hz, 1H); ^{13}C NMR (101 MHz, DMSO- d_6) δ 162.3, 140.6, 140.4, 133.9, 133.7, 128.9, 128.5,

127.1, 126.2, 125.8, 125.0, 124.0, 118.3, 116.9, 110.7; HRMS (ESI) calcd for C₁₅H₉N₂O₃ (M - H⁺) 265.0619, found 265.0617; Melting point: 216-217°C.

methyl (*E*)-3-(2-hydroxy-5-nitrostyryl)benzoate (92)

The title compound was prepared according to general procedure (D) using methyl 3-iodobenzoate **92b** (147 mg, 0.56 mmol), yielding the pure product as a yellow solid (102 mg, 61%) after flash chromatography (Hexane : EtOAc = 20 : 1); ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.53 (br s, 1H), 8.53 (d, *J* = 2.9 Hz, 1H), 8.19 (s, 1H), 8.06 (dd, *J* = 9.0, 2.9 Hz, 1H), 7.92 (d, *J* = 7.9 Hz, 1H), 7.88 (d, *J* = 7.7 Hz, 1H), 7.61 – 7.53 (m, 2H), 7.48 (d, *J* = 16.6 Hz, 1H), 7.07 (d, *J* = 9.0 Hz, 1H), 3.89 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.6, 161.6, 140.5, 138.0, 131.5, 130.6, 130.1, 129.7, 128.8, 127.5, 125.1, 124.8, 123.3, 123.0, 116.6, 52.7; HRMS (ESI) calcd for C₁₆H₁₂NO₅ (M - H⁺) 298.0721, found 298.0717; Melting point: 200-201°C.

methyl (*E*)-4-(2-hydroxy-5-nitrostyryl)benzoate (93)

The title compound was prepared according to general procedure (D) using methyl 4-iodobenzoate **93b** (147 mg, 0.56 mmol), yielding the pure product as a yellow solid (113 mg, 67%) after flash chromatography (Hexane : EtOAc = 20 : 1); ¹H NMR (400 MHz, DMSO-*d*₆) δ 11.60 (br s, 1H), 8.52 (d, *J* = 2.7 Hz, 1H), 8.07 (dd, *J* = 9.0, 2.8 Hz, 1H), 7.97 (d, *J* = 8.1 Hz, 2H), 7.77 (d, *J* = 8.1 Hz, 2H), 7.55 (s, 2H), 7.07 (d, *J* = 9.0 Hz, 1H), 3.86 (s, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 166.4, 161.8, 142.2, 140.5, 130.1, 129.0, 127.2, 125.4, 124.9, 124.6, 123.3, 116.7,

52.5; HRMS (ESI) calcd for $C_{16}H_{12}NO_5$ ($M - H^+$) 298.0721, found 298.0719; Melting point: 258-259°C.

N-(3-ethynylphenyl)-2-hydroxy-5-nitrobenzamide (94)

To a solution a of 5-nitrosalicylic acid (50 mg, 0.27 mmol) and triphenyl phosphite (86 μ L, 0.33 mmol, 1.2 equiv) in toluene (5 mL) was added 3-aminophenylacetylene (37 μ L, 0.33 mmol, 1.2 equiv) in toluene (2 mL) via syringe under N_2 atmosphere. The resulting mixture was refluxed for 6h. 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 yellow solid (57 mg, 74%); 1H NMR (400 MHz, $DMSO-d_6$) δ 10.64 (s, 1H), 8.74 (d, $J = 3.0$ Hz, 1H), 8.30 (dd, $J = 9.1, 3.0$ Hz, 1H), 7.91 (s, 1H), 7.74 – 7.72 (m, 1H), 7.41 (t, $J = 7.9$ Hz, 1H), 7.27 (d, $J = 7.6$ Hz, 1H), 7.17 (d, $J = 9.1$ Hz, 1H), 4.23 (s, 1H); ^{13}C NMR (101 MHz, $DMSO-d_6$) δ 164.7, 163.6, 139.8, 138.7, 129.7, 128.9, 128.0, 126.3, 124.0, 122.6, 121.8, 120.0, 118.5, 83.7, 81.2; HRMS (ESI) calcd for $C_{15}H_9N_2O_4$ ($M - H^+$) 281.0568, found 281.0571; 181°C (decomposed).

(2-hydroxy-5-nitrophenyl)(phenyl)methanone (95)

To a solution of 5-nitrosalicylaldehyde (100 mg, 0.60 mmol), $PdCl_2$ (5 mg, 0.03 mmol, 0.05 equiv), $LiCl$ (5 mg, 0.12 mmol, 0.2 equiv) and Na_2CO_3 (127 mg, 1.20 mmol, 2equiv) in DMF

(5 mL) was added iodobenzene (135 μ L, 1.2 mmol, 2equiv) via syringe under N₂ atmosphere. The resulting solution was stirred at 120°C for 6h. 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 Na₂SO₄ and filtered. All volatiles were evaporated *in vacuo* and the product was obtained after flash chromatography (hexane : EtOAc = 20: 1) as a yellow solid (108 mg, 74%); ¹H NMR (400 MHz, Chloroform-*d*) δ 12.67 (s, 1H), 8.63 (d, *J* = 2.8 Hz, 1H), 8.42 (dd, *J* = 9.2, 2.8 Hz, 1H), 7.76 – 7.69 (m, 3H), 7.61 (t, *J* = 7.6 Hz, 2H), 7.21 (d, *J* = 9.3 Hz, 1H); ¹³C NMR (101 MHz, Chloroform-*d*) δ 200.5, 168.0, 139.5, 136.4, 133.2, 130.9, 129.6, 129.3, 128.9, 119.6, 118.0; HRMS (ESI) calcd for C₁₃H₈NO₄ (M - H⁺) 242.0459, found 242.0454; Melting point: 121-122°C.

Determination of inhibitory activity against the NusB-NusE interaction. For inhibitor testing, chemical compounds were dissolved to 10 mg/ml in DMSO and 2-fold serial diluted in PBS. 40 μ l N-LgBiT-NusB (2.5 μ M in PBS) was added to 96-well plates, and then mixed with 20 μ l compound at desired concentrations. The mixture was incubated for 10 minutes at 37°C. 40 μ l C-SmBiT-NusE (2.5 μ M 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.

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 $\mu\text{g/ml}$ to 0.5 $\mu\text{g/ml}$, and the bacterial cell inoculum was adjusted to approximately 5×10^5 CFU per 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.

Time kill kinetics. *S. aureus* cells were suspended to $\sim 1.5 \times 10^6$ 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.

Assessment of ATP production. *S. aureus* cells were suspended to $\sim 1.5 \times 10^6$ 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 was taken at defined time points (0, 2, 4, 6, 16, 20, 24hours) for each treatment group. The ATP production was measured using the BacTiter-Glo™ Microbial Cell Viability Assay Kit (Promega™) according to the manufacturer's instructions. Experiment was performed in triplicate. Technical repeats were taken to ensure consistent results were obtained.

Protein overproduction and purification. Purification of the His-tagged proteins have been done as described previously (Yang and Lewis, 2008). Briefly, *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 NaH₂PO₄, 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 NaH₂PO₄, 500 mM NaCl, 40 mM imidazole, pH 8.0) and protein eluted with elution buffer (20 mM NaH₂PO₄, 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.

Binding inhibition assay. For inhibitor testing, chemical compounds were dissolved to 10 mg/ml in DMSO. 40 μ l N-LgBiT-NusB (2.5 μ M in PBS) was added to 96-well plates, and then mixed with 20 μ l compound. The mixture was incubated for 10 minutes at 37°C. 40 μ l C-SmBiT-NusE (2.5 μ M 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 μ M. 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.

Cytotoxicity assay. Human cell lines A549 lung carcinoma and HaCaT immortalized keratinocytes were used in this study. The cells will be seeded at 2.5×10^5 per well. After 24 hr incubation, the tested compounds will be added in a 2-fold serial dilution ranging from $1.562 \mu\text{g/ml}$ to $50 \mu\text{g/ml}$. 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.

Hemolysis assay

To separate erythrocytes from human whole blood, 35 mL of human whole blood was centrifuged at $500 \times g$, $+4^\circ\text{C}$ 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 $10 \mu\text{g/mL}$ were incubated at 37°C with 2% blood solution in PBS (pH 7.4) for 45 min. $1600 \mu\text{L}$ of the 40x-diluted erythrocyte suspension was added to $400 \mu\text{L}$ 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 ($200 \mu\text{L}$ 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:

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

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-cm² flasks. The cells (passage number: 45) were seeded at a density of 60,000 cells/cm² on polycarbonate membranes of Transwells (12 mm inner diameter, 0.4 μm pore size, 1 cm²; 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 cm² 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 μ L 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.

ASSOCIATED CONTENT

Supporting Information.

The Supporting Information are available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.XXX.

Supplemental figures, copies of NMR spectra (PDF)

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

MRSA, methicillin-resistant *S. aureus*; PPI, protein-protein interaction; MIC, minimum inhibition concentration; SAR, structure-activity relationship; CD, circular dichroism; QSAR, quantitative structure–activity relationship.

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