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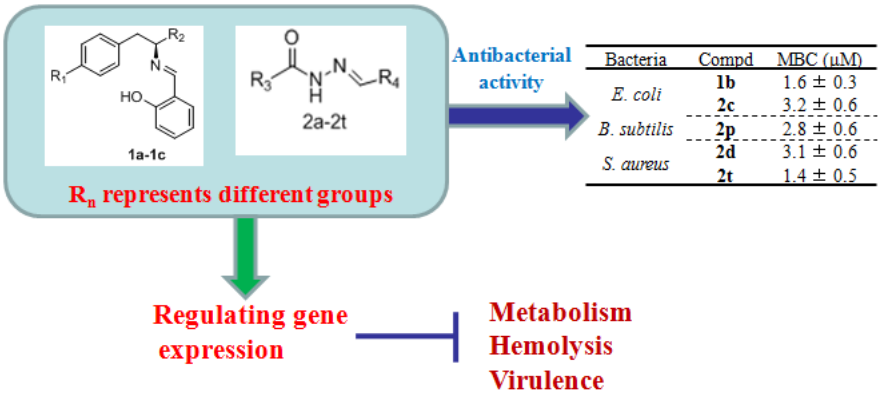
Graphical Abstract



Bacteria	Compd	MBC (μM)
E. coli	**1b**	1.6 ± 0.3
	2c	3.2 ± 0.6
B. subtilis	**2p**	2.8 ± 0.6
S. aureus	**2d**	3.1 ± 0.6
	2t	1.4 ± 0.5

Benzaldehyde Schiff bases regulation to the metabolism, hemolysis, and virulence genes expression *in vitro* and their structure–microbicidal activity relationship

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Abstract: There is an urgent need to develop new antibacterial agents because of multidrug resistance by bacteria and fungi. Schiff bases (aldehyde or ketone-like compounds) exhibit intense antibacterial characteristics, and are therefore, promising candidates as antibacterial agents. To investigate the mechanism of action of newly designed benzaldehyde Schiff bases, a series of high-yielding benzaldehyde Schiff bases were synthesized, and their structures determined by NMR and MS spectra data. The structure–microbicidal activity relationship of the derivatives was investigated, and the antibacterial mechanisms were investigated by gene assays for the expression of functional genes *in vitro* using *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis*. The

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Abbreviations: *E. coli*, *Escherichia coli*; *S. aureus*, *Staphylococcus aureus*; *B. subtilis*, *Bacillus subtilis*; KOH, potassium hydroxide; MBC, minimum bactericidal concentration; CFU, colony forming unit; ELISA, enzyme linked immunosorbent assay; RT-PCR, reverse transcription PCR; LB, Luria-Bertani; .

active compounds were selective for certain active groups. The polar substitution of the R_2 group of the amino acids in the Schiff bases, affected the antibacterial activity against *E. coli* and *S. aureus*; specific active group at the R_3 or R_4 groups of the acylhydrazone Schiff bases could improve their inhibitory activity against these three tested organisms. The antibacterial mechanism of the active benzaldehyde Schiff bases appeared to regulate the expression of metabolism-associated genes in *E. coli*, hemolysis-associated genes in *B. subtilis*, and key virulence genes in *S. aureus*. Some benzaldehyde Schiff bases were bactericidal to all the three strains and appeared to regulate gene expression associated with metabolism, hemolysis, and virulence, *in vitro*. The newly designed benzaldehyde Schiff bases possessed unique antibacterial activity and might be potentially useful for prophylactic or therapeutic intervention of bacterial infections.

Keywords: Antibacterial activity; Benzaldehyde Schiff base; Synthesis; Regulating gene expression

1. Introduction

Currently, multidrug resistance in several bacteria is one of the major causes of increasing rate of mortality associated with infectious diseases in humans [1]. The main reason behind this problem is lack of effective methods and drugs for treatment [2-3], and hence, there is an urgent need of developing new antibacterial agents with novel and more efficient mechanisms of action [4]. Schiff bases, named after Hugo Schiff, exhibit a broad range of physicochemical and biological activities [5]. Structurally, they are aldehyde or ketone-like compounds in which the carbonyl group (C=O) is replaced by an imine or azomethine group; they are formed by the reaction of primary amines with aldehydes or ketones under specific conditions. Previous studies have established that the presence of imine or azomethine subunits in various natural, natural-derived, and non-natural compounds was critical to their biological activities [6-8]. Many Schiff bases also reversibly bind with oxygen, catalyze the hydrogenation of olefins, coordinate with and show fluorescent variability with some toxic metals [9-11]. In addition, some Schiff bases also exhibit a broad range of biological activities, such as antifungal, antibacterial, antimalarial, antiproliferative, anti-inflammatory, antiviral, and

antipyretic properties [12-13].

In recent years, hydrazone derivatives have received attention due to their various biological properties and their wide application in medicinal chemistry. The biological activity of these compounds was attributed to the presence of the (–CONHN=CH–) moiety. Some studies have successfully identified that several hydrazide-hydrazone derivatives exhibited broad spectrum of biological activities such as anticonvulsant [14], antidepressant [15], antimicrobial [16-17], antitumor [18-19], analgesic, and anti-inflammatory [20] activities. Our aim was to confirm whether Schiff bases, derived from benzaldehyde, possessed antibacterial property involved regulating genes related to metabolism, hemolysis and virulence expression. Therefore, we designed and synthesized a series of benzaldehyde Schiff bases based on the acylhydrazone structure, and assessed their antibacterial activity against *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Bacillus subtilis* (*B.subtilis*). In addition, the expression of some genes associated with metabolism, hemolysis and virulence was determined at the transcriptional level in bacteria treated with selected derivatives, using quantitative real-time reverse transcriptase PCR (RT-PCR). The results presented here validate the antibacterial efficacy and structure–activity relationships of benzaldehyde Schiff bases *in vitro* and also supported the development of these Schiff bases and related systemic antibacterial benzaldehyde Schiff bases as potential antibacterial agents, targeting genes that regulate metabolism, hemolysis, and virulence.

2. Results and discussion

2.1. Chemistry

Benzaldehyde Schiff bases were produced when the amidogen of amino acids was ground with 2-hydroxybenzaldehyde, in the presence of an alkali [21]. The synthetic pathway and chemical structures of benzaldehyde Schiff bases (compounds **1a**–**1c**) were outlined in Scheme 1. The starting compounds of benzaldehyde Schiff bases: L-phenylalanine, L-tyrosine, and L-phenylalanine methyl ester are commercially available. The target compounds could be generated by grinding the mixture

of amino acid and KOH, which was followed by grinding the mixture with 2-hydroxybenzaldehyde over a period of 30 minutes. The synthesis of acylhydrazone Schiff bases (compounds **2a**–**2t)** were described in Scheme 2. The starting materials, 4-methoxyphenylacetic acid or 4-chlorophenylacetic acid were suspended in methanol, into which thionyl chloride ($SOCl_2$) was added to generate methyl ester **I**, which was then hydrazinolysized to produce acylhydrazide **II** [22, 23]. Isoniazide and salicylhydrazide are commercially available. Twenty acylhydrazone Schiff bases were synthesized via condensation of the acylhydrazide **II** with corresponding 2-hydroxybenzaldehyde by the previous strategy [21, 24]. The structures of compounds **1a**–**1c** and **2a**–**2t** were determined on the basis of NMR and ESI-MS data.

2.2. Antibacterial activity of benzaldehyde Schiff bases

Preliminary evaluation of the antibacterial activity was performed by assessing the anti-growth activity of 100 μmol/L purified benzaldehyde Schiff bases (results are summarized in Fig. 1 and Table 1). Compounds which could inhibit ≥50% bacterial growth at a concentration of 100 μmol/L were chose for further analysis. It could be observed that compounds **1b, 2c,** and **2q** exhibited better activity against *E. coli*; especially **1b** and **2c** possessed similar antibacterial activity to that of control. Similar inhibitory activity was observed for compounds **2p** and **2r** against *B. subtilis.* In addition, it was also found that five compounds, **1c, 2d, 2f, 2h,** and **2t**, could selectively suppress the growth of *S. aureus*, and similar anti-growth activity could be observed for **1c**, **2d** and **2t** on the tested strain. However, further studies are required to confirm the antibacterial activity of these compounds by determining their MBC values.

The bacteria were incubated in LB medium containing compounds of varied concentrations for 8 hours at 37 °C, with shaking, to determine whether the screened compounds demonstrated antibacterial activity of the compounds at a concentration of <100 μmol/L. The optical density was measured at 450 nm after 8 hours of incubation to calculate the inhibitory activity and MBC value of every compound. The MBC value of each screened compound for each bacterium was defined as the

lowest concentration of the compound that reduced growth by 1% as compared to the inoculum control seeded with normal strains [25, 26]. By the concentration inhibited curves (Fig. 2) and MBC (Table 2) values, it can be concluded that there were some benzaldehyde Schiff bases, which inhibited the growth of three bacteria in a concentration-dependent manner. Nevertheless, the inhibitory activity of compounds **1b** and **2c** against *E. coli* had no evident difference at different concentrations. In addition, a moderate difference was seen in the MBC values of **1b**, **2c,** and control, which indicated that **1b** and **2c** exhibited an inhibitory effect on the growth of *E. coli*, similar to ampicillin, which is routinely used in clinical practice. Likewise, compound **2p** showed a closed inhibitory activity for *B. subtilis*, and two compounds **2d** and **2t** displayed a perfect antibacterial activity for *S. aureus*, when compared with that of streptomycin.

2.3. Analysis on structure–activity relationships

Firstly, the potent inhibitory action of compound **1b** on the growth of *E. coli* suggested that higher polar substitution at R_2 might improve the antibacterial activity for the amino acid-containing Schiff bases. Meanwhile, the substitution at R_2 by less polar groups (**1c**) might improve their inhibitory activity on *S. aureus*.

Secondly, in case of acylhydrazone Schiff base series, their inhibitory activity against the growth of *E. coli* was improved by the *meta*-substitution of a bromine-group at R_4. The substitution of chloride(s) at both the R_3 and R_4 positions might improve their inhibitory activity against the growth of *B. subtilis*. As far as the antibacterial activity against *S. aureus* is concerned, compounds **2d** and **2t** were found to be the most active ones, which indicated that the presence of electron-deficient aromatic rings, such as pyridinyl or *para*-chloro-phenyl groups at R_3 and electron-donating groups (lipophilic as well), such as 3,5-di-*tert*-butyl or 3,4-benzyl on the phenyl ring of R_4 might be potential pharmacophores for the inhibitory activity against *S. aureus* of the acylhydrazone Schiff base.

Finally, the most important point is that all these active compounds showed good selectivity in their action against the three different bacteria and the selectivity was related to the presence of specific active groups, which implied that they might possess different mechanisms of action on different bacteria, respectively.

2.4. Effect of the compounds on the expression of the genes related to metabolism in E. coli

The effect of mechanism of the screened compounds on the growth of *E. coli* was determined by measuring the expression of metabolism associated genes *cysB*, *acrA*, *tsh*, *iroD*, and the housekeeping gene *gapA*. Real-time RT-PCR, (Fig. 3A) and semi-quantitative RT-PCR (using agarose gel, Fig. 3B) were used for determining the expression level of the above-mentioned genes. Results showed that compound **1b** had significantly high ($P < 0.01$) inhibitory effect, **2c** had a significant ($P < 0.05$) promotion, and **2q** displayed a slight ($P < 0.05$) inhibitory effect for the expression of *cysB* (a structural gene of the transcriptional regulator CysB in *E. coli* that activates the expression of the genes involved in the cysteine biosynthetic pathway [27, 28]. The above findings demonstrated that three benzaldehyde Schiff bases inhibited the growth of bacteria (probably through up or down regulation of *cysB*), and further interfered with the genes involved in the cysteine biosynthetic pathway.

In order to evaluate the effects of the three screened compounds on the membranes of *E. coli*, the expression level of *acrA* at the transcriptional level was assayed by RT-PCR. The *acrA* gene maps between *proC* and *purE* at 10.5 min on the *E. coli* chromosome, contributes to the integrity of *E. coli* membranes [29], and is responsible for the susceptibility to the basic dyes, detergents, and certain antibiotics [30]. The results revealed that not every compound could affect the expression of *acrA*; however, only the compound **2c** and **2q** could significantly ($P < 0.01$) promote and slightly inhibit the expression of the gene, respectively compared to the control gene *gapA*. The up or down regulation for the expression of *E. coli* membrane gene, suggests that Schiff bases might differentially regulate a large number of membrane genes and then change the integrity and

permeability, but not every compound can regulate the gene expression, that might be linked with the structural and chemical properties of the compounds [29, 30].

We analyzed the *tsh* gene expression of *E. coli* following treatment with three Schiff bases derivatives, because the limitation of the compounds to the virulence reflected directly the pharmaceutical effect. The *tsh* gene encodes a temperature-sensitive hemagglutinin [31] and resides on transmissible R plasmids encoding aerobactin and colicin V that is associated with avian *E. coli* virulence [32–34]. The expression of the *tsh* gene was significantly ($P < 0.01$) decreased upon treatment with three compounds, and this reduction was not due to a significant decrease in bacterial burden; this indicated that the three Schiff bases were involved in the down regulation of expression of *tsh* gene, and in inhibiting the biosynthesis of temperature-sensitive hemagglutinin in *E. coli*. Similar results were found in the expression of *IroD* gene as the *tsh* gene in *E. coli*. The *IroD* gene, belongs to the salmochelin gene cluster that encodes a cytoplasmic esterase with hydrolytic activity that preferentially cleaves iron-free enterobactin [35]. IroD protein can hydrolyze the ester bonds of both enterobactin and drug molecules, such as salmochelin, which is required for subsequent iron release from the contained iron drugs [36, 37]. The data suggested that the three Schiff bases had antimicrobial activity against *E. coli in vitro* and may be related with the reduced expression of *IroD* to limit the hydrolytic activity of cytoplasmic esterase.

2.5. Effect of the compounds on the expression of hemolysis-associated genes in B. subtilis

The pathogenicity of the bacteria was closely related with hemolysis vitality because of the hemolysin as an important virulence factor of pathopoiesis [38, 39]. There exists an increasing threat to the human health with the wide application of *B. subtilis* and the direct relationship of its production with human life. The whole genome sequencing of *B. subtilis* has been completed, and the hemolysis-associated genes from the genome annotation were *yqhC*, *yhdP*, *yhdT*, *yqhB*, *yugS*, and *ytjA* [40]. Except for the *ytjA* gene that was identified as hemolysis gene of *B. subtilis* [41], the functions of the other several genes have not been identified yet. The change in expression level of

yqhC, *yhdP*, *yhdT*, *yqhB*, and *yugS* genes was assayed, compared to the control housekeeping gene *16sRNA*, of the test strain after treatment with two compounds by RT-PCR (Fig. 4A) and semi-quantitative RT-PCR methods (Fig. 4B). Results showed that the expressions of five hemolysis genes were significantly ($P < 0.01$) decreased when regulated by the treatment of **2p** and **2r**, suggesting that the hemolysis genes were strongly involved in the inhibition of *B. subtilis*.

2.6. Effect of the compounds on the expression of virulence genes in S. aureus

In the results, it was found that the five Schiff base compounds were obtained had a better inhibitory action towards the growth of *S. aureus*. To determine whether the screened compounds had any influence on the expression of virulence factors, *S. aureus* were incubated with a sublethal dose of the compounds (50 μmol/L) and 8 hours post-exposure transcript abundance of key *S. aureus* virulence factors *saeR*, *hla*, *sbi*, and *mecA* was measured (gyrB housekeeping gene as control) by RT-PCR (Fig. 5A) and semi-quantitative RT-PCR methods (Fig. 5B). The results showed that there were significant differences ($P < 0.01$) in the inhibitory action for the expression of *saeR* gene by compounds **1c**, **2d**, **2f** and **2h**. Transcript abundance of *saeR* gene, the regulatory gene component of the global virulence regulatory system SaeR/S, which is essential for the development of staphylococcal skin lesions in mice [42], was upregulated about two-fold higher with other compounds as compared to the control in *S. aureus*. The transcript expression of *hla* gene, which encodes for α-toxin, a virulence factor responsible for dermonecrosis in mouse skin infections [43], was up regulated by compounds **1c**, **2d**, **2f** and **2t**, and was markedly down regulated by **2h**. The transcript expression of *sbi*, a gene encoding an immunomodulatory protein that is important in antibody and complement evasion [44] was markedly up regulated by **1c**, **2h** and **2t**. In addition, the expression of *mecA* gene that encodes an altered penicillin-binding protein 2a to confer resistance to β-lactam antibiotics was analyzed using the same methods. The transcript expression level of *mecA* [45] was upregulated by **1c** with varying degrees**; it** was down regulated two-fold higher degree by **2d**, **2h,** and **2t** than that in controls. The reduction in transcript expression of virulence genes of *S.*

aureus, including the SaeR/S virulence regulatory system, suggests that the Schiff bases may differentially regulate a considerably large number of *S. aureus* virulence genes, which may support the hypothesis that the inhibitory effect of Schiff bases to *S. aureus* may correlate with the up- or down regulation of the virulence gene expression.

3. Experimental sections

3.1. General

Varian Inova-400 spectrometer (USA) was employed to record the NMR spectra with tetramethylsilane as an internal standard, and MS studies were carried out on a HP1100-MSD spectrometer (ESI-MS mode) (USA). Column chromatography was performed using silica gel (200–300 & 300–400 mesh, Qingdao Marine Chemical Co. Ltd., Qingdao, P R China).

Chemicals used in all the experiments performed during the study were of the analytical reagent grade (AR). Organic solvents (spectroscopically pure from Acros, USA) used in biological screening included ethyl alcohol, diethylether, and dimethylformamide (DMF). Deionization of water was done entirely in glass equipments and this water was normally used in all preparations. Luria-Bertani (LB) broth growth media was purchased from Oxoid (Basingstoke, UK); ampicillin and streptomycin were obtained from Sigma; *Escherichia coli* (*E. coli*) ATCC 25922, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, and *Bacillus subtilis* (*B. subtilis*) ATCC 6051 were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China).

3.2. General procedure for synthesis of amino acid-containing Schiff bases

Compound **1a** was synthesized according to the method described by Zhong *et al*. [46]. In brief, L-phenylalanine (1.0 g, 6.1 mmol) and potassium hydroxide (KOH) (0.406 g, 7.3 mmol) were ground together in an agate mortar to obtain a viscous mixture; 2-hydroxybenzaldehyde (0.8 mL, 7.3 mmol) was added drop-by-drop to the mixture, which was ground for 30 minutes prior to addition of 50 mL methanol to dissolve it homogenously; the mixture was then transferred to another reaction vessel. Next, diethylether (30 mL) was added slowly into this mixture until a yellow precipitate was

formed, which was filtered and washed with diethylether to obtain the new compound **1a**.

*3.2.1. Potassium-(S)-2-(2-hydroxybenzylideneamino)-3-phenylpropanoic (**1a**)*

Yellow solid; yield: 95.4%; IR (KBr) ν_{max}:3418, 3025, 1632, 1487, 1375, 1144, 1082 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 3.03 (m, 1H, 3-H_a), 3.36 (m, 1H, 3-H_b), 4.05 (m, 1H, 2-CH), 6.66 (m, 1H, 5′-H), 6.77 (m, 1H, 3′-H), 6.96 (m, 1H, 5-H), 7.05 (m, 1H, 7-H), 7.13 (m, 1H, 9-H), 7.17-7.25 (m, 4H, 6, 8, 4′, 6′-H), 7.86 (m, 1H, -N=CH); ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 36.1, 77.8, 116.9, 120.6, 123.6, 124.9, 127.7, 128.6, 132.3, 136.9, 160.8, 161.1, 175.4; ESI-MS: *m/z* 292.0 $[M+Na]^+$.

*3.2.2. Potassium-(S)-2-(2-hydroxybenzylideneamino)-3-(4-hydroxy)-phenylpropanoic (**1b**)*

The title compound was obtained by condensing L-tyrosine with salicylaldehyde, as a brown solid with yield of 44.6%. IR (KBr) ν_{max}:3421, 2925, 1636, 1484, 1376, 1150, 1105 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 2.86 (m, 1H, 3-H_a), 3.48 (m, 1H, 3-H_b), 3.99 (m, 1H, 2-CH), 6.57 (m, 2H, 8-H), 6.64 (d, 1H, *J* = 8.0 Hz, 3′-H), 6.73 (d, 1H, *J* = 8.0 Hz, 6′-H), 6.89 (d, 2H, *J* = 8.4 Hz, 5, 9-H), 7.01 (m, 1H, 4′-H), 7.23 (m, 1H, 5′-H), 7.69 (s, 1H, -N=CH); ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 37.1, 75.8, 114.3, 116.9, 120.6, 123.6, 128.4, 128.7, 132.1, 155.1, 159.2, 159.7, 176.4; ESI-MS: *m/z* 308.0 $[M+Na]^+$.

*3.2.3. (S)-2-(2-hydroxybenzylideneamino)-3-phenylpropanoic acid methyl ester (**1c**)*

The title compound was obtained by condensing L-phenylalanine methyl ester with salicylaldehyde, as yellow oil with yield of 84.4%. IR (KBr) ν_{max}:2926, 1743, 1630, 1493, 1458, 1230, 1202 cm^{-1}; ^{1}H-NMR (CD_3OD, 400 MHz) δ (ppm): 3.12 (m, 1H, 3-H_a), 3.36 (m, 1H, 3-H_b), 3.75 (s, 3H, 1-OCH_3), 4.16 (m, 1H, 2-CH), 6.83 (m, 1H, 5′-H), 6.96 (d, 1H, *J* =8.2, 3′-H), 7.09 (m, 1H, 7-H), 7.14 (d, 2H, *J* =8.6, 5, 9-H), 7.19 (m, 1H, 4′-H), 7.24 (d, 2H, *J* =8.6, 6, 8-H), 7.32 (d, 1H, *J* =8.2, 6′-H), 7.96 (s, 1H, -N=CH); ^{13}C-NMR (CD_3OD, 125 MHz) δ (ppm): 37.5, 50.3, 72.9, 116.4, 120.7, 123.6, 124.9, 126.4, 127.5, 130.1, 136.9, 157.8, 158.4, 172.7; ESI-MS: *m/z* 306.1 $[M+Na]^+$.

3.3. General synthetic procedure for acylhydrazone Schiff bases

An esterification procedure devised by Park *et al*. was used to synthesize methyl eater I [22], as follows: 4-methoxy benzene acetic acid (10.0 g, 60.0 mmol) was suspended in methanol (100 mL) and thionyl chloride (26 mL, 361.1 mmol) was added into the solution placed over an ice bath (-10 °C). The reaction mixture was refluxed at 90 °C for 3 hours under argon, and then excess methanol and thionyl chloride were evaporated. The crude product was purified by crystallization (methanol/ethylacetate) and 4-methoxy benzene methyl acetate (9.84 g), with a yield of 90%, was obtained.

Further, 4-methoxy benzene methyl acetate (9.84 g, 54 mmol) and hydrazine hydrate (27 mL, 300 mmol) were dissolved in methanol (100 mL). The mixture was refluxed at 80°C for 5 hours. After cooling down to room temperature, the white crystal was filtered and washed with cold methanol. 4-methoxy phenylacetyl hydrazine (4.66 g), with yield of 43.0% was obtained as a white crystal by crystallization using methanol. Another compound named 4-chlorobenzene oxygen acetyl hydrazine (white needle like crystals) was synthesized by the same procedure with a final yield of 73.1%.

Twenty acylhydrazone Schiff bases were synthesized as described previously [21, 24]. Under an argon atmosphere, a solution of 2-hydroxybenzaldehyde (5 mmol) in 20 mL anhydrous ethanol was added in acylhydrazine II (5 mmol; suspended in 30 mL anhydrous ethanol). The mixture was then refluxed for 4–8 h at 80°C and monitored by TLC. The resulting solid was filtered and washed with cold ethanol after cooling down to room temperature. Recrystallization in ethanol provided the final derivatives **2a**–**2t**.

*3.3.1. N'-(2-hydroxybenzylidene)-isoniazide (**2a**)*

The title compound **2a** was synthesized by condensing isoniazide with 2-hydroxybenzaldehyde. Light green crystals; yield: 53.2%; IR (KBr) ν_{max}:3454, 3183, 3005, 1683, 1617, 1490, 1408, 1291, 1160 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.81 (d, 1H, J = 9.2 Hz, 3′-H), 6.93 (m, 1H, 5′-H), 7.30 (m, 1H, 4′-H), 7.59 (d, 1H, J = 6.0 Hz, 6′-H), 7.82 (d, 1H, J = 6.0 Hz, 3-H), 7.83 (d, 1H, J

= 6.0 Hz, 5-H), 8.66 (s, 1H, CH=N), 8.77 (d, 1H, *J* = 6.0 Hz, 2-H), 8.78 (d, 1H, *J* = 6.0 Hz, 6-H); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):116.6, 118.8, 119.6, 121.7, 129.4, 131.9, 140.1, 149.1, 150.5, 157.6, 161.5; ESI-MS: *m/z* 264.0 $[M+Na]^+$.

*3.3.2. N'-(2-hydroxy-5-nitrobenzylidene)-isoniazide (**2b**)*

The title compound **2b** was synthesized by condensing isoniazide with 2-hydroxy-5-nitrobenzaldehyde. Orange powder; yield: 84.1%; IR (KBr) ν_{max}:3454, 3230, 3071, 1658, 1549, 1483, 1439, 1298, 1081 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 7.09 (d, 1H, *J* = 9.2 Hz, 3′-H), 7.81 (d, 1H, *J* = 6.0 Hz, 3-H), 7.83 (d, 1H, *J* = 6.0 Hz, 5-H), 8.15 (m, 1H, 4′-H), 8.57 (s, 1H, 6′-H), 8.72 (s, 1H, CH=N), 8.77 (d, 1H, *J* = 5.2 Hz, 2-H), 8.78 (d, 1H, *J* = 5.2 Hz, 6-H); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):117.3, 120.2, 121.7, 123.6, 127.1, 140.1, 140.1, 145.2, 150.5, 161.9, 162.7; ESI-MS: *m/z* 309.0 $[M+Na]^+$.

*3.3.3. N'-(5-bromo-2-hydroxybenzylidene)-isoniazide (**2c**)*

The title compound **2c** was synthesized by condensing isoniazide with 5-bromo-2-hydroxybenzaldehyde. Light green crystals, yield: 76.5%; IR (KBr) ν_{max}:3454, 3175, 3001, 1676, 1618, 1475, 1410, 1285, 1066 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.92 (d, 1H, *J* = 8.4 Hz, 3′-H), 7.46 (m, 1H, 4′-H), 7.84 (d, 1H, *J* = 6.0 Hz, 3-H), 7.85 (d, 1H, *J* = 6.0 Hz, 5-H), 7.86 (s, 1H, 6′-H), 8.66 (s, 1H, CH=N), 8.80 (d, 1H, *J* = 6.0 Hz, 6-H), 8.81 (d, 1H, *J* = 6.0 Hz, 2-H); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):110.7, 118.8, 121.4, 121.6, 130.2, 134.0, 140.0, 140.1, 146.4, 150.5, 156.5, 161.6; ESI-MS: *m/z* 342.0 $[M+Na]^+$.

*3.3.4. N'-(3,5-di-tert-butyl-2-hydroxybenzylidene)-isoniazide (**2d**)*

The title compound was obtained as solid white crystals by condensing isoniazide with 3,5-di-tert-butyl-2-hydroxybenzaldehyde. Yield: 57.2%; IR (KBr) $\nu_{max:}$3441, 3191, 2960, 2866, 1656, 1611, 1436, 1361, 1250, 1067 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.27 (s, 9H, 3′-t-Bu), 1.39 (s, 9H, 5′-t-Bu), 7.21 (s, 1H, 4′-H), 7.31 (s, 1H, 6′-H), 7.82 (d, 1H, *J* = 6.0 Hz, 3-H), 7.83 (d, 1H, *J* = 6.0 Hz, 5-H), 8.56 (s, 1H, CH=N), 8.78 (d, 1H, *J* = 6.0 Hz, 2-H), 8.79 (d, 1H, *J* = 6.0 Hz, 6-H);

^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 31.8, 34.7, 115.6, 120.6, 121.9, 126.3, 135.4, 138.2, 147.0, 150.2, 153.5, 161.6; ESI-MS: *m/z* 376.1 $[M+Na]^+$.

*3.3.5. N'-(2-hydroxybenzylidene)-salicylhydrazide (**2e**)*

The title compound **2e** was obtained by condensing salicylhydrazide with 2-hydroxybenzaldehyde. White powder; yield: 82.8%; IR (KBr) $\nu_{max.}$ 3454, 3100, 1620, 1559, 1454, 1376, 1231, 1158 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.89 (m, 1H, 3-H), 6.93 (m, 1H, 3′-H), 6.96 (m, 1H, 5′-H), 6.99 (m, 1H, 5-H), 7.30 (m, 1H, 4′-H), 7.44 (m, 1H, 4-H), 7.55 (d, 1H, J = 9.6 Hz, 6′-H), 7.88 (d, 1H, J = 9.2 Hz, 6-H), 8.67 (s, 1H, CH=N); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):115.7, 116.5, 117.3, 118.7, 119.1, 119.5, 128.7, 129.5, 131.7, 134.0, 149.0, 157.6, 159.0, 164.5; ESI-MS: *m/z* 279.0 $[M+Na]^+$.

*3.3.6. N'-(5-bromine-2-hydroxybenzylidene)-salicylhydrazide (**2f**)*

The title compound **2f** was synthesized by condensing salicylhydrazide with 5-bromo-2-hydroxybenzaldehyde in yield of 82.6%. White powder; IR (KBr) $\nu_{max.}$3442, 3087, 1629, 1527, 1459, 1338, 1237, 1103 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.92 (d, 1H, J = 8.4 Hz, 3-H), 6.96 (d, 1H, J = 8.8 Hz, 3′-H), 6.99 (m, 1H, 5-H), 7.44 (m, 1H, 4′-H), 7.48 (m, 1H, 4-H), 7.80 (s, 1H, 6′-H), 7.88 (d, 1H, J = 9.2 Hz, 6-H), 8.64 (s, 1H, CH=N); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):115.8, 117.2, 117.4, 119.9, 123.9, 126.8, 128.8, 134.2, 140.0, 145.2, 159.0, 162.7, 164.9; ESI-MS: *m/z* 357.0 $[M+Na]^+$.

*3.3.7. N'-(2-hydroxy-5-nitrobenzylidene)-salicylhydrazide (**2g**)*

The title compound **2g** was synthesized by condensing salicylhydrazide with 2-hydroxy-5-nitrobenzaldehyde in yield of 82.1%. Light yellow powder; IR (KBr) ν_{max}:3418, 3195, 1716, 1614, 1483, 1456, 1232, 1091 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 6.94 (d, 1H, J = 8.4 Hz, 3-H), 6.97 (m, 1H, 5-H), 7.10 (d, 1H, J = 8.4 Hz, 3′-H), 7.44 (m, 1H, 4-H), 7.86 (s, 1H, 6′-H), 8.15 (d, 1H, J = 8.4 Hz, 4′-H), 8.55 (s, 1H, 6-H), 8.72 (s, 1H, CH=N); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):110.6, 115.8, 117.4, 118.8, 119.2, 121.3, 128.8, 130.5, 133.9, 134.1, 146.4, 156.6,

160.0, 164.8; ESI-MS: *m/z* 324.0 $[M+Na]^+$.

*3.3.8. N'-(3,5-Di-tert-butyl-2-hydroxybenzylidene)-salicylhydrazide (**2h**)*

The title compound **2h** was synthesized by condensing salicylhydrazide with 3,5-di-tert-butyl-2-hydroxybenzaldehyde in yield of 57.6%. White lumpy crystals; IR (KBr) ν_{max}:3236, 3074, 2956, 2907, 1796, 1641, 1233, 1098 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.27 (s, 9H, 3′-t-Bu), 1.40 (s, 9H, 5′-t-Bu), 6.96 (d, 1H, *J* = 7.6 Hz, 3-H), 6.99 (m, 1H, 5-H), 7.23 (s, 1H, 6′-H), 7.31(s, 1H, 4′-H), 7.45 (m, 1H, 4-H), 7.86 (d, 1H, *J* = 9.2 Hz, 6-H), 8.61 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm):31.6, 34.9, 116.2, 117.0, 119.7, 121.7, 126.3, 126.9, 131.5, 135.4, 138.2, 144.0, 152.5, 157.4, 161.2; ESI-MS: *m/z* 391.1 $[M+Na]^+$.

*3.3.9. N'-(2-hydroxybenzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2i**)*

The title compound **2i** was synthesized by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 2-hydroxybenzaldehyde with yield of 92%. White soild; IR (KBr) ν_{max}:3443, 2924, 1680, 1641 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.68 (s, 2H, 2-CH_2), 6.83 (d, 1H, *J* = 8.6 Hz, 5-H), 6.85 (d, 1H, *J* = 8.6 Hz, 9-H), 6.93 (m, 1H, 5′-H), 7.02 (d, 1H, *J* = 6.4 Hz, 3′-H), 7.29 (d, 1H, *J* = 8.8 Hz, 6-H), 7.35 (d, 1H, *J* = 8.8 Hz, 8-H), 7.51 (m, 1H, 4′-H), 7.69 (d, 1H, *J* = 7.2 Hz, 6′H), 8.53 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 69.0, 116.2, 116.8, 117.5, 120.4, 125.5, 126.5, 128.9, 130.4, 144.0, 154.2, 155.2, 169.0; ESI-MS: *m/z* 328.1 $[M+Na]^+$.

*3.3.10. N'-(2-hydroxy-5-nitrobenzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2j**)*

The title compound **2j** was synthesized by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 2-hydroxybenzaldehyde with yield of 90%. Yellow solid; IR (KBr) ν_{max}:3442, 3302, 2931, 1653, 1607 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.69 (s, 3H, 6-OCH_3), 3.88 (s, 2H, 2-CH_2), 6.86 (d, 1H, *J* = 9.2 Hz, 5-H), 6.88 (d, 1H, *J* = 8.4 Hz, 7-H), 7.05 (d, 1H, *J* = 9.2 Hz, 4-H), 7.06 (d, 1H, *J* = 8.4 Hz, 8-H), 7.22 (d, 1H, *J* = 9.2 Hz, 3′-H), 8.14 (d, 1H, *J* = 8.4 Hz, 4′-H), 8.24 (s, 1H, 6′-H), 8.47 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 42.2, 55.4, 114.2, 116.7, 119.3, 125.3, 127.7, 128.4, 130.3, 140.2, 146.0, 159.2, 167.2, 171.0; ESI-MS: *m/z* 352.0 $[M+Na]^+$.

*3.3.11. N'-(5-bromo-2-hydroxybenzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2k**)*

The title compound **2k** was obtained by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 5-bromo-2-hydroxybenzaldehyde with yield of 43%. White solid; IR (KBr) ν_{max}:3668, 3200, 3059, 1667, 1610 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.71 (s, 3H, 6-OCH_3), 3.86 (s, 2H, 2-CH_2), 6.85 (d, 1H, J = 8.8 Hz, 5-H), 6.88 (d, 1H, J = 8.8 Hz, 7-H), 6.94 (d, 1H, J = 8.8 Hz, 3′-H), 7.17 (d, 1H, J = 8.8 Hz, 4-H), 7.19 (d, 1H, J = 8.8 Hz, 8-H), 7.51 (d, 1H, J = 8.8 Hz, 4′-H), 7.88 (s, 1H, 6′-H), 8.34 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 42.4, 55.7, 110.4, 114.6, 119.2, 120.6, 127.7, 130.6, 132.0, 135.3, 146.0, 159.5, 160.1, 169.7; ESI-MS: *m/z* 385.0 $[M+Na]^+$.

*3.3.12. N'-(5-chloro-2-hydroxybenzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2l**)*

The title compound **2l** was obtained by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 5-chloro-2-hydroxybenzaldehyde with yield of 84%. White crystals; IR (KBr) ν_{max}:3454, 3199, 3059, 1668, 1612 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.69 (s, 2H, 2-CH_2), 3.70 (s, 3H, 6-OCH_3), 3.86 (s, 1H, 2′-CH_2), 6.85 (d, 1H, J = 8.8 Hz, 5-H), 6.87 (d, 1H, J = 8.8 Hz, 7-H), 6.91 (d, 1H, J = 8.8 Hz, 3′-H), 7.17 (d, 1H, J = 8.8 Hz, 4-H), 7.19 (d, 1H, J = 8.8 Hz, 8-H), 7.20 (d, 1H, J = 8.8 Hz, 4′-H), 7.27 (s, 1H, 6′-H), 8.35 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 40.2, 55.2, 113.9, 118.2, 120.7, 123.1, 127.3, 130.2, 130.4, 130.8, 144.8, 157.9, 158.2, 167.1; ESI-MS: *m/z* 341.0 $[M+Na]^+$.

*3.3.13. N'-(3,5-di-tert-butyl-2-hydroxybenzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2m**)*

The title compound **2m** was obtained by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 3,5-di-tert-2-hydroxybenzaldehyde with yield of 50%. White crystals; IR (KBr) ν_{max}:3454, 3199, 2957, 1650, 1613 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.25 (s, 9H, 3′-t-Bu), 1.36 (s, 9H, 5′-t-Bu), 3.48 (s, 2H, 2-CH_2), 3.71 (s, 3H, 6-OCH_3), 6.85 (d, 1H, J = 8.8 Hz, 5-H), 6.88 (d, 1H, J = 8.8 Hz, 7-H), 7.16 (s, 1H, 4′-H), 7.21 (d, 1H, J = 8.0 Hz, 4-H), 7.23 (d, 1H, J = 8.0 Hz, 8-H), 7.26 (s, 1H, 6′-H), 8.32 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 31.3, 34.4, 40.5, 55.8, 114.6, 117.5, 123.6, 127.5, 128.2, 130.2, 137.7, 146.0, 153.7, 159.5, 169.1; ESI-MS: *m/z* 419.2

$[M+Na]^+$.

*3.3.14. N'-(2-hydroxybenzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2n**)*

The title compound **2n** was obtained by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 2-hydroxybenzaldehyde with yield of 74%. White crystals; IR (KBr) ν_{max}:3500, 3166, 3036, 1662, 1614 cm^{-1};^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.70 (s, 3H, 6-OCH_3), 3.85 (s, 2H, 2-CH_2), 6.84 (d, 1H, *J* = 8.8 Hz, 5-H), 6.89 (d, 1H, *J* = 8.8 Hz, 7-H), 7.18 (d, 1H, *J* = 8.8 Hz, 3′-H), 7.20 (m, 1H, 5′-H), 7.22 (d, 1H, *J* = 8.4 Hz, 4-H), 7.26 (d, 1H, *J* = 8.4 Hz, 8-H), 7.49 (m, 1H, 4′-H), 7.66 (d, 1H, *J* = 9.2 Hz, 6′-H), 8.37 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 42.3, 55.6, 113.5, 116.8, 118.5, 120.4, 126.6, 127.2, 128.6, 131.4, 146.0, 156.2, 158.5, 170.1; ESI-MS: *m/z* 307.1 $[M+Na]^+$.

*3.3.15. N'-(3,4-di-benzyloxy-benzylidene)-2-(4-methoxyphenyl)-acetyl hydrazide (**2o**)*

The title compound **2o** was obtained by condensing 2-(4-methoxyphenyl)-acetyl hydrazide with 3,4-di-benzyloxy-benzaldehyde with yield of 82%. White crystals; IR (KBr) ν_{max}:3669, 3216, 3007, 1725, 1663 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 3.70 (s, 3H, 6-OCH_3), 3.84 (s, 2H, 2-CH_2), 5.16 (s, 2H, 3′-OC<u>*H*</u>$_2$Ph), 5.18 (s, 2H, 4′-OC<u>*H*</u>$_2$Ph), 6.82 (d, 1H, *J* = 8.4 Hz, 5-H), 6.86 (d, 1H, *J* = 8.4 Hz, 7-H), 7.11 (d, 1H, *J* = 9.6 Hz, 5′-H), 7.15 (d, 1H, *J* = 8.4 Hz, 4-H), 7.18 (d, 1H, *J* = 8.4 Hz, 8-H), 7.21(d, 1H, *J* = 9.6 Hz, 6′-H), 7.28-7.40 (m, 10H, 3′, 4′-OCH_2<u>*Ph*</u>), 7.43 (s, 1H, 2′-H), 8.08 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 41.6, 54.8, 71.1, 111.8, 114.5, 122.5, 127.1, 127.9, 128.9, 130.4, 130.6, 136.6, 146.2, 149.7, 151.9, 159.2, 170.8; ESI-MS: *m/z* 503.1 $[M+Na]^+$.

*3.3.16. N'-(2-hydroxy-5-nitrobenzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2p**)*

The title compound **2p** was obtained by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 2-hydroxy-5-nitrobenzaldehyde with yield of 81%. Yellow powder; IR (KBr) ν_{max}:3284, 3071, 1721, 1660 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.68 (s, 2H, 2-CH_2), 7.09 (d, 1H, *J* = 7.6 Hz, 5-H), 7.11 (d, 1H, *J* = 7.6 Hz, 9-H), 7.35 (d, 1H, *J* = 8.4 Hz, 3′-H), 7.60 (d, 1H, *J* = 7.6 Hz, 6-H),

7.61 (d, 1H, *J* = 7.6 Hz, 8-H), 7.94 (d, 1H, *J* = 8.4 Hz, 4′-H), 8.40 (s, 1H, 6′-H), 8.79 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 68.6, 116.7, 117.3, 119.2, 125.3, 126.3, 128.6, 130.9, 140.6, 146.2, 156.2, 167.2, 171.3; ESI-MS: *m/z* 372.0 $[M+Na]^+$.

*3.3.17. N'-(5-bromo-2-hydroxybenzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2q**)*

The title compound was obtained by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 5-bromo-2-hydroxybenzaldehyde with yield of 78%. White solid; IR (KBr) ν_{max}:3645, 2978, 1683, 1630 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.68 (s, 2H, 2-CH_2), 6.85 (d, 1H, *J* = 8.4 Hz, 3′-H), 6.93 (d, 1H, *J* = 9.6 Hz, 5-H), 7.02 (d, 1H, *J* = 9.6 Hz, 9-H), 7.30 (d, 1H, *J* = 8.4 Hz, 4′-H), 7.37 (d, 1H, *J* = 9.6 Hz, 6-H), 7.41 (d, 1H, *J* = 9.6 Hz, 8-H), 7.73 (s, 1H, 6′-H), 8.49 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 69.0, 110.5, 117.1, 118.0, 119.8, 125.8, 128.9), 130.6, 134.4, 146.2, 154.7, 165.1, 171.0; ESI-MS: *m/z* 405.0 $[M+Na]^+$.

*3.3.18. N'-(5-chloro-2-hydroxy-benzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2r**)*

The title compound was obtained by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 5-chloro-2-hydroxybenzaldehyde in yield of 78%. White solid; IR (KBr) ν_{max}:3732, 2982, 1683, 1610 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.67 (s, 2H, 2-CH_2), 6.89 (d, 1H, *J* = 6.8 Hz, 3′-H), 6.93 (d, 1H, *J* = 8.0 Hz, 5-H), 7.00 (d, 1H, *J* = 8.0 Hz, 9-H), 7.25 (d, 1H, *J* = 6.8 Hz, 4′-H), 7.30 (d, 1H, *J* = 10.0 Hz, 6-H), 7.34 (d, 1H, *J* = 10.0 Hz, 8-H), 7.60 (s, 1H, 6′-H), 8.48 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 68.7, 117.4, 118.2, 119.6, 123.4, 126.8, 130.6, 131.7, 133.5, 146.0, 156.3, 158.9, 171.0; ESI-MS: *m/z* 361.0 $[M+Na]^+$.

*3.3.19. N'-(3,5-di-tert-butyl-2-hydroxy-benzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2s**)*

The title compound was obtained by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 3,5-di-tert-butyl-2-hydroxybenzaldehyde in yield of 30%. White solid; IR (KBr) ν_{max}:3680, 3004, 1726, 1661 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 1.25 (s, 9H, 3′-t-Bu), 1.37 (s, 3H, 5′-t-Bu), 4.72 (s, 2H, 2-CH_2), 7.02 (d, 1H, *J* = 9.2 Hz, 5-H), 7.03 (d, 1H, *J* = 9.2 Hz, 9-H), 7.19 (s, 1H, 6′-H), 7.29 (s, 1H, 4′-H), 7.35 (d, 1H, *J* = 8.8 Hz, 6-H), 7.37 (d, 1H, *J* = 8.8 Hz, 8-H), 8.47 (s, 1H,

N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 32.7, 34.4, 69.0, 117.4, 117.7, 122.8, 126.2, 128.3, 129.7, 137.9, 146.0, 153.5, 156.2, 171.2; ESI-MS: *m/z* 439.3 $[M+Na]^+$.

*3.3.20. N'-(3,4-di-benzyloxy-benzylidene)-2-(4-chlorophenyl-oxyl)-acetyl hydrazide (**2t**)*

The title compound was obtained by condensing 2-(4-chlorophenyl-oxyl)-acetyl hydrazide with 3,4-di-benzyloxy-benzaldehyde in yield of 79%. White solid; IR (KBr) ν_{max}:3668, 3216, 3007, 1708, 1512 cm^{-1}; ^{1}H-NMR (DMSO-d_6, 400 MHz) δ (ppm): 4.63 (s, 2H, 2-CH_2), 5.15 (s, 2H, 3′-OC$\underline{H_2}$Ph), 5.16 (s, 2H, 4′-OC$\underline{H_2}$Ph), 6.92 (d, 1H, *J* = 9.2 Hz, 5′-H), 6.99 (d, 1H, *J* = 8.4 Hz, 9-H), 7.09 (d, 1H, *J* = 8.4 Hz, 5-H), 7.13 (d, 1H, *J* = 8.0 Hz, 6-H), 7.19 (d, 1H, *J* = 8.0 Hz, 8-H), 7.27-7.43 (m, 10H, 3′, 4′-OCH$_2$$\underline{Ph}$), 7.44 (d, 1H, *J* = 9.2 Hz, 6′-H), 7.86 (s, 1H, 2′-H), 8.19 (s, 1H, N=CH); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ (ppm): 68.1, 70.6, 111.8, 116.2, 121.4, 125.4, 127.1, 128.9, 130.6, 130.9, 136.7, 146.8, 149.5, 152.1, 156.2, 171.0; ESI-MS: *m/z* 523.0 $[M+Na]^+$.

3.4. Turbidimetry test

The antibacterial activity of the test compounds were assessed *in vitro* by turbidimetric assays [47-48]. Test compounds were prepared in 0.1 mol/L DMSO and diluted with LB, the final concentration of DMSO was maintained at ≤1% (v/v). The bacteria were seeded at a concentration of 1×10^5 CFU/mL in 96-well microculture plates (90 μL/well) containing LB; thereafter, the solutions of prepared compounds were added to each well to obtain a final concentration of 100 μM. Thereafter, the microplates were vigorously shaken on a vibrating platform for 20 hours at 37°C. Further, the plates were then examined in an ELISA plate reader at 450 nm for absorbance, to determine the growth inhibitory activity. Results were expressed as the mean values of the three independent variables.

$$\text{Inhibitory activity} = [(OD_{control} - OD_{sample}) / OD_{control}] \times 100\%$$

$OD_{control}$ was the optical density of bacteria suspension added the vehicle. OD_{sample} was the optical density of bacteria suspension treated with tested compounds.

The MBC values were measured by broth microdilution method in the 96-well microculture

plates [49]. The bacteria were seeded in 96-well microculture plates at the concentration of 1×10^5 CFU/mL in LB, in a series of different concentrations of tested compounds and incubated for 20 hours at 37°C. The minimum bactericidal concentration was the lowest concentration of compound whose absorbance by ELISA was parallel to the control in three independent experiments.

3.5. In vitro gene expression

Bacteria (2×10^7 CFU/mL) were resuspended in LB containing 50 μmol/L compounds or in LB alone. Samples were incubated for 8 hours at 37 °C and, the total RNA was harvested and confirmed by TaqMan real-time reverse transcriptase polymerase chain reaction (RT-PCR), as described by the report [42]. The relative quantification of genes was determined by changes in expression of transcripts relative to their expression in untreated bacteria. Samples were normalized to the housekeeping gene *gapA* of *E. coli*, *16sRNA* gene of *B. subtilis*, and *gyrB* gene of *S. aureus*. Data were analyzed as change in transcript level after treatment with the screened compounds. The sequences of the primers used for analysis are listed in Table 3.

3.6. Statistical analysis

The data were expressed as mean ± SD of the number of experiments indicated, and analyzed using the SPSS 19.0 Windows Students version software (SPSS Inc., Chicago, USA). The comparisons between multiple treatments were made with analysis of variance (ANOVA). One-way ANOVA followed by a student's t-test was performed to assess the statistical significance of difference between control and chemotherapeutic agents treated for all the measurements. A difference of $P < 0.05$ was considered statistically significant.

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Appendix A. Supplementary data.

These data include NMR spectra of all new derivatives.

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Figure captions

Fig. 1. The inhibition of series of benzaldehyde Schiff bases on the growth of three bacteria *in vitro*. The compounds were added into 96-well microculture plates containing the bacteria at a concentration of 1×10^5 CFU/mL in LB. The plates were analyzed in an ELISA plate reader at 450 nm after cultures were shaken on a vibrating platform for 8 h at 37°C. The concentration of the compounds was 100 μmol/L. Data were pooled from three independent experiments, same as the below results.

Fig. 2. The concentration inhibited curves of the screened compounds against three bacterial strains *in vitro*. Different concentration of tested compounds were added to 96-well microculture plates containing (A) the *E. coli*, (B) *B. subtilis* and (C) *S. aureus* strains at concentration of 1×10^5 CFU/mL in LB, respectively. The plates were measured in an ELISA plate reader at 450 nm after cultures were shaken on a vibrating platform for 8 h at 37°C. The inhibition rate (%) was calculated with the results of the turbidimetry test (as described in Methods section).

Fig.3. The screened compounds altered gene expression of *E. coli in vitro*. (A) Change in the folds of four metabolism genes were analyzed by real-time RT-PCR. (B) The expression of four genes was assayed used semi-quantitative RT-PCR. The 2×10^7 CFU/mL *E. coli* was incubated with the 50 μmol/L compounds for 8 h. Data

were normalized to the transcript abundance of *gapA* gene, and fold change was relative to *E. coli* incubated in medium alone (time matched).

Fig. 4. The screened compounds altered the expression of hemolysis-associated gene in *B. subtilis in vitro*. (A) Changes in the folds of five hemolysis genes were analyzed by real-time RT-PCR. (B) The transcript expression level of five genes was assayed used semi-quantitative RT-PCR. The 2×10^7 CFU/mL *B. subtilis* was incubated with 50 μmol/L compounds for 8 h. Data were normalized to the transcript abundance of *16sRNA* gene, and fold change was relative to *B. subtilis* incubated in medium alone (time matched). Data were pooled from three experiments.

Fig. 5. The screened compounds altered the expression of *S. aureus* virulence genes *in vitro*. (A) Changes in the folds of four virulence genes were analyzed by real-time RT-PCR. (B) The transcript expression level of four genes was assayed used semi-quantitative RT-PCR. The 2×10^7 CFU/mL *S. aureus* was incubated with 50 μmol/L compounds for 8 h. Data were normalized to the transcript abundance of *gyrB* gene, and fold change was relative to *S. aureus* incubated in medium alone (time matched). Data were pooled from three experiments.

Table 1. Anti-bacterial activity of compounds against the three strains at concentration of 100 μmol/L *in vitro.*

Compound	Inhibition rate (%)		
	E.coli	*B. subtilis*	*S. aureus*
1a	22.72 ± 4.38	ND	ND
1b	96.04 ± 8.35	4.38 ± 1.23	ND
1c	14.55 ± 1.32	4.21 ± 1.39	86.93 ± 3.10
2a	6.81 ± 1.16	ND	ND
2b	41.82 ± 1.28	ND	ND
2c	95.84 ± 7.24	ND	ND
2d	13.43 ± 1.07	ND	95.55 ± 4.36
2e	11.80 ± 3.04	ND	ND
2f	17.95 ± 4.01	ND	81.92 ± 3.79*
2g	18.30 ± 3.98	ND	ND
2h	13.86 ± 3.95	ND	70.93±5.73**

2i	0.23 ± 3.92	ND	ND
2j	5.57 ± 3.89	ND	ND
2k	ND	1.01 ± 1.98	ND
2l	ND	ND	ND
2m	ND	ND	ND
2n	ND	ND	ND
2o	ND	ND	ND
2p	ND	96.39 ± 3.96	1.39 ± 1.36
2q	73.17 ± 4.32**	15.95 ± 2.39	ND
2r	3.08 ± 3.66	83.18 ± 2.38*	ND
2s	10.48 ± 3.63	19.71 ± 3.98	ND
2t	17.56 ± 3.6	6.21 ± 2.69	99.46 ± 4.39
Ampicillin	99.34 ± 3.26	-	-
Streptomycin	-	94.12 ± 6.56	97.05 ± 4.24

Values were mean ± standard deviation of three independent experiments. The bacteria were seeded in 96-well microculture plates at a concentration of 1×10^5 CFU/mL in LB. Test compounds and positive compound solution were then added to the well to obtain the final concentration of 100 μmol/L, respectively. The growth inhibitory activity was measured in an ELISA plate reader at 450 nm after cultures were shaken on a vibrating platform at 37 ℃ for 8 h. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with the control, respectively. ND: Antibacterial activity not detected. Data were pooled from three independent experiments, same as the below results.

Table 2. MBC values of the tested compounds against three bacteria strains *in vitro*.

Bacteria	Compound	MBC (μmol/L)
E. coli	**1b**	1.6 ± 0.3
	2c	3.2 ± 0.6
	2q	18.4 ± 2.3**
	Ampicillin	1.8 ± 0.7
B. subtilis	**2p**	2.8 ± 0.6
	2r	10.8 ± 1.3**
	Streptomycin	2.1 ± 0.5
S. aureus	**1c**	13.8 ± 3.9**
	2d	3.1 ± 0.6
	2f	16.6±1.9**
	2h	19.0 ± 1.3**
	2t	1.4 ± 0.5
	Streptomycin	1.3 ± 0.6

Values were mean ± standard deviation of three independent experiments. Series of different concentration of tested compounds were added to a 96-well microculture plate containing the bacteria culture at concentration of 1×10^5

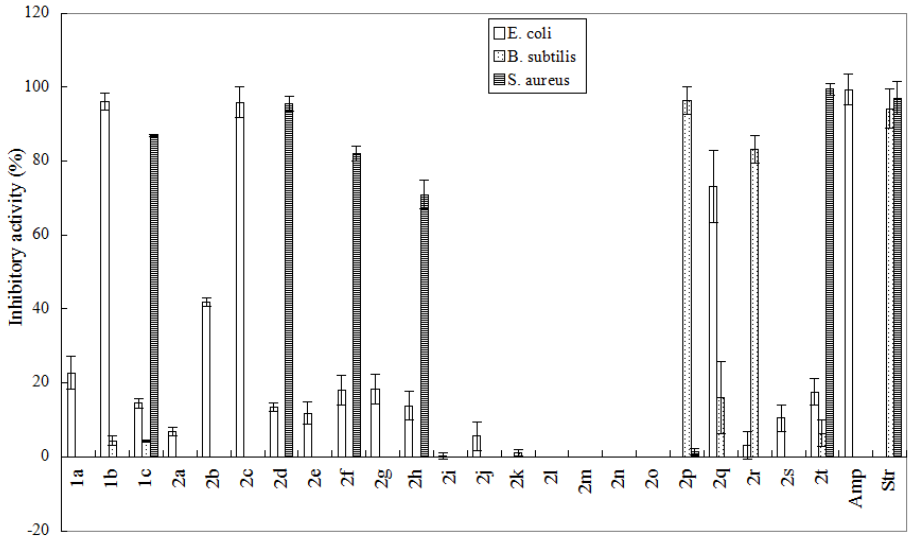
CFU/mL. The plates were analyzed by an ELISA plate reader at 450 nm after cultures were shaken on a vibrating platform for 8 h at 37°C.

** $P < 0.01$ compared with the control.

Table 3. List of the primers used in this study.

	Gene	Primer
E. coli	*cysB*	F: 5'-GTCTAGAACTGCGTTATCTGGCCGATG-3'
		R: 5'-ACCCGGGTCCTTCCGCTGTTGATGA-3'
	acrA	F: 5'-CCACCATTACCACCACCATCAC-3'
		R: 5'-ACCGCCGAACTTCAACACTC-3'
	tsh	F: 5'-TTATTCTCTTCGCTACAG-3'
		R: 5'-GATGACAGGCTACCGACAG -3'
	iroD	F: 5'-TCCTGGTTGGGTTGAATA-3'
		R: 5'-CAGCCAGAGGCCCACTA-3'
	gapA	F: 5'-CAGAACATCATCCCGTCCTCTAC-3'
		R: 5'-TACCAGTCAGTTTGCCATTCAGTT-3'
B. subtilis	*16sRNA*	F:5'-GCGTGAGTGATGAAGGTTT-3'
		R:5'-GCCGTGGCTTTCTGGTTA-3'
	yqxC	F: 5'-TATTAGTGGAAAGAGGGCT-3'
		R: 5'-GCCTTCCTGTCCCTCTCC-3'
	yhdP	F: 5'-GGCATTTTTCGTGGCATC-3'
		R: 5'-GGTCAATTAACACTTTTCC-3'
	yugS	F: 5'-GAATATTTATCAGCCTGTCA-3'
		R: 5'-GATTTCCCCGACAATTTC-3'
	yhdT	F: 5'-GCGTGCTCATTGCTTTAAC-3'
		R: 5'-CCTCAAGAATATCCTCTGTC-3'
	yqhB	F: 5'-GCTTTAGGACTGGGATGG-3'
		R: 5'-CAGGCTCAGCGTCTATGT-3'
S. aureus	*gyrB*	F: 5'-CAAATGATCACAGCTTTGGTACAG-3'
		R: 5'-CGGCATCAGTCATAATGACGAT-3'
	saeR	F: 5'CTGCCAAAACACAAGAACATGATAC-3'
		R: 5'-ATTTACGCCTTAACTTTAGGTGCAGAT-3'
	hla	F: 5'-CAACAACACTATTGCTAGGTTCCATATT-3'
		R: 5'-CCTGTTTTTACTGTAGTATTGCTTCCA-3'
	mecA	F: 5'-ACTGATTAACCCAGTACAGATCCTTTC-3'
		R: 5'-TCCAAACTTTGTTTTTCGTGTCTTT-3'

	sbi	F: 5'-ATACATCAAAACATTACGCGAACAC-3' R: 5'-CTGGGTTCTTGCTGTCTTTAAGTG-3'



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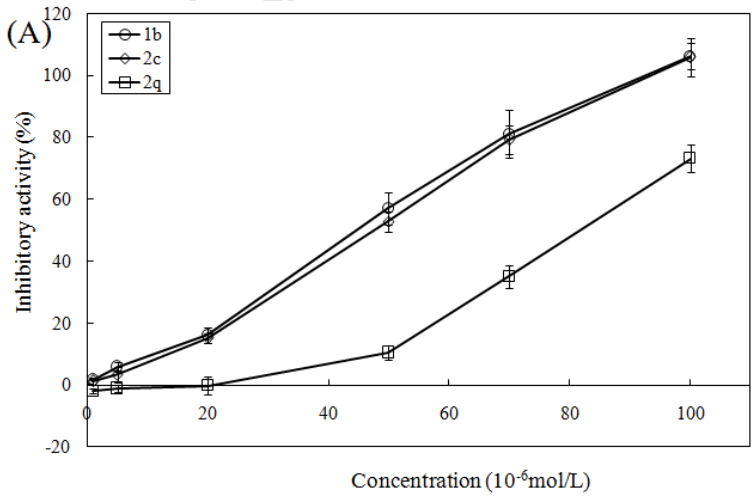

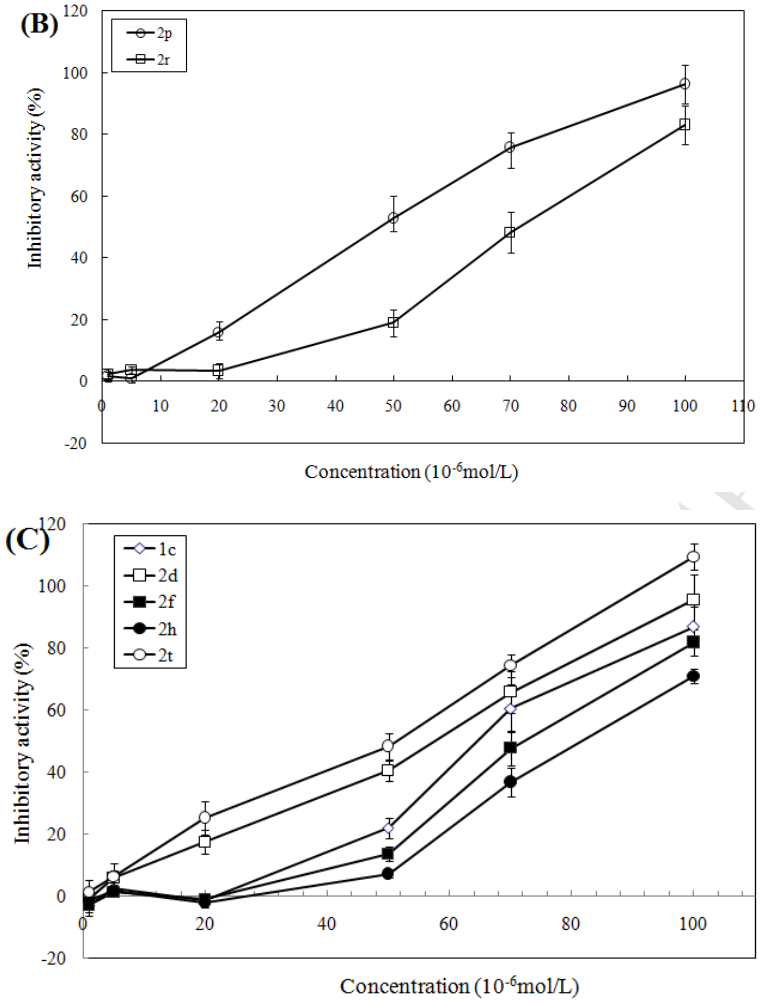

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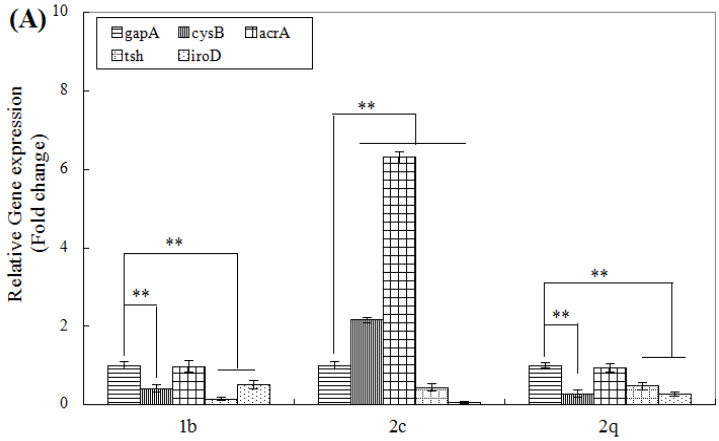


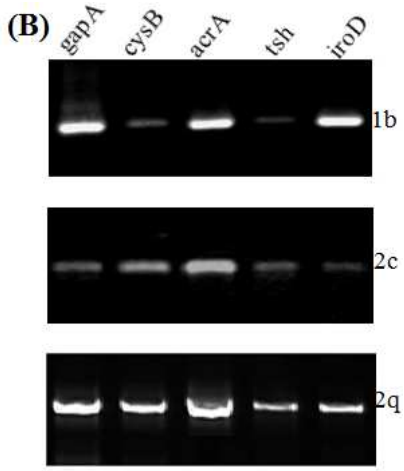


Fig. 3. The screened compounds altered *E. coli* related gene expression *in vitro*. (A) Change in the folds of four metabolism genes were analyzed by real-time RT-PCR. (B) The expression of four genes was assayed used semi-quantitative RT-PCR. The 2×10^7 CFU/mL *E. coli* was incubated with the 50 μmol/L compounds for 8 h. Data were normalized to the transcript abundance of *gapA* gene, and fold change was relative to *E. coli* incubated in medium alone (time matched).

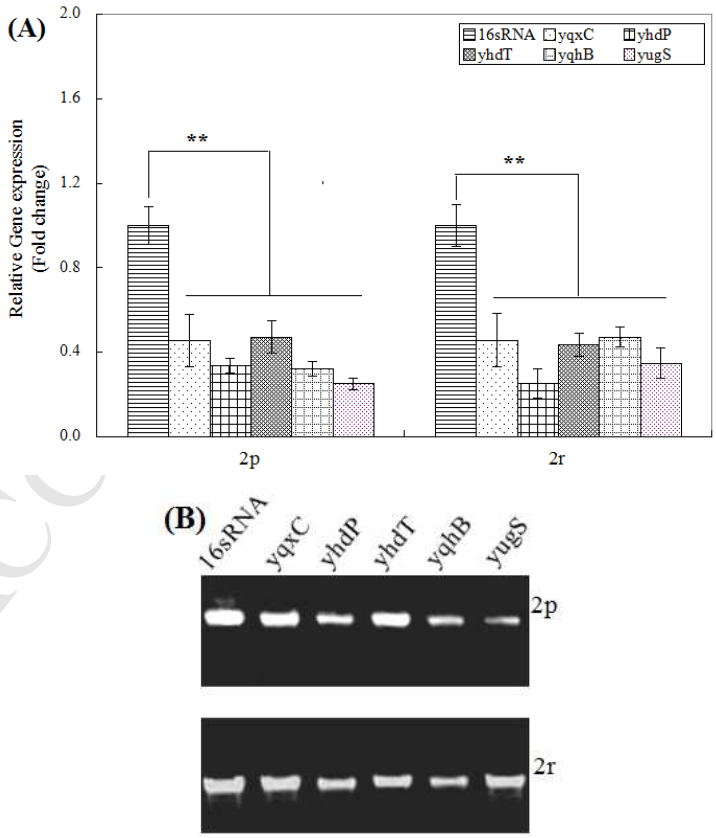


Fig. 4. The screened compounds altered *B. subtilis* hemolysis-associated gene expression *in vitro*. (A) Changes in the folds of five hemolysis genes were analyzed by real-time RT-PCR. (B) The transcript expression level of five

genes was assayed used semi-quantitative RT-PCR. The 2 × 10^7 CFU/mL *B. subtilis* was incubated with 50 μmol/L compounds for 8 h. Data were normalized to the transcript abundance of *16sRNA* gene, and fold change was relative to *B. subtilis* incubated in medium alone (time matched). Data were pooled from three experiments.

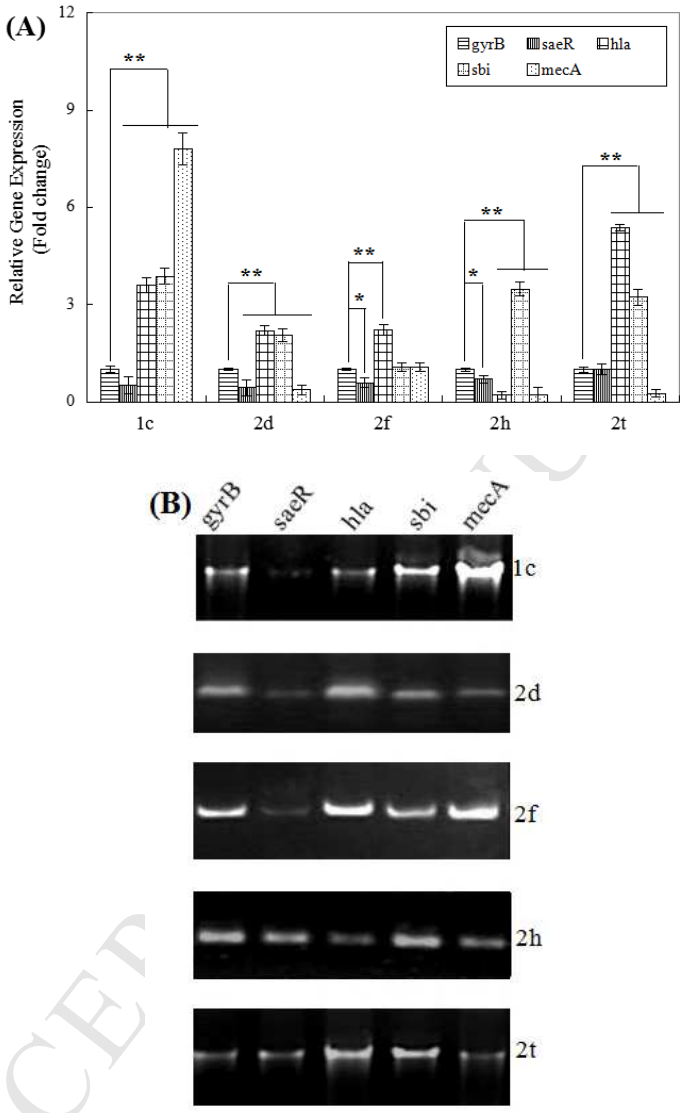
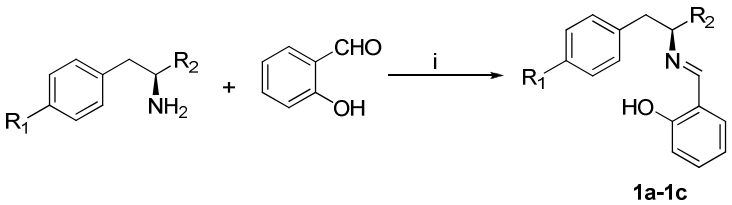


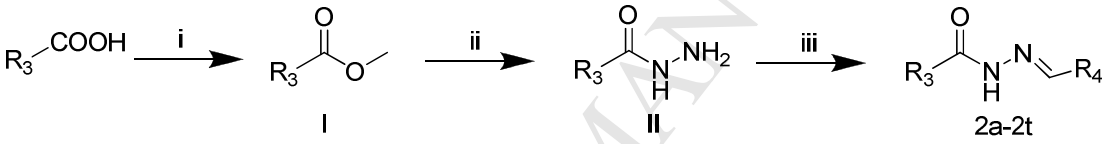
Fig. 5. The screened compounds altered the expression of *S. aureus* virulence genes *in vitro*. (A) Changes in the folds of four virulence genes were analyzed by real-time RT-PCR. (B) The transcript expression level of four genes was assayed used semi-quantitative RT-PCR. The 2 × 10^7 CFU/mL *S. aureus* was incubated with 50 μmol/L compounds for 8 h. Data were normalized to the transcript abundance of *gyrB* gene, and fold change was relative to *S. aureus* incubated in medium alone (time matched). Data were pooled from three experiments.



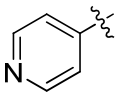
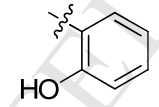
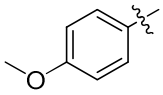
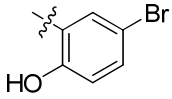
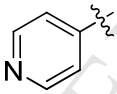
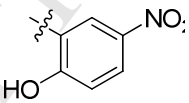
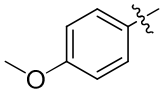
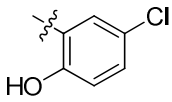
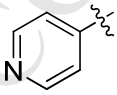
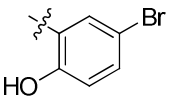
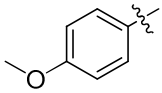
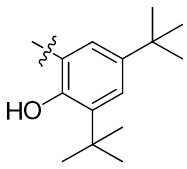
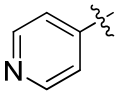
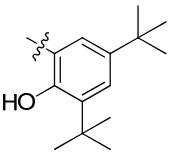
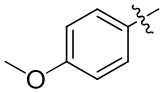
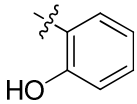
1a-1c

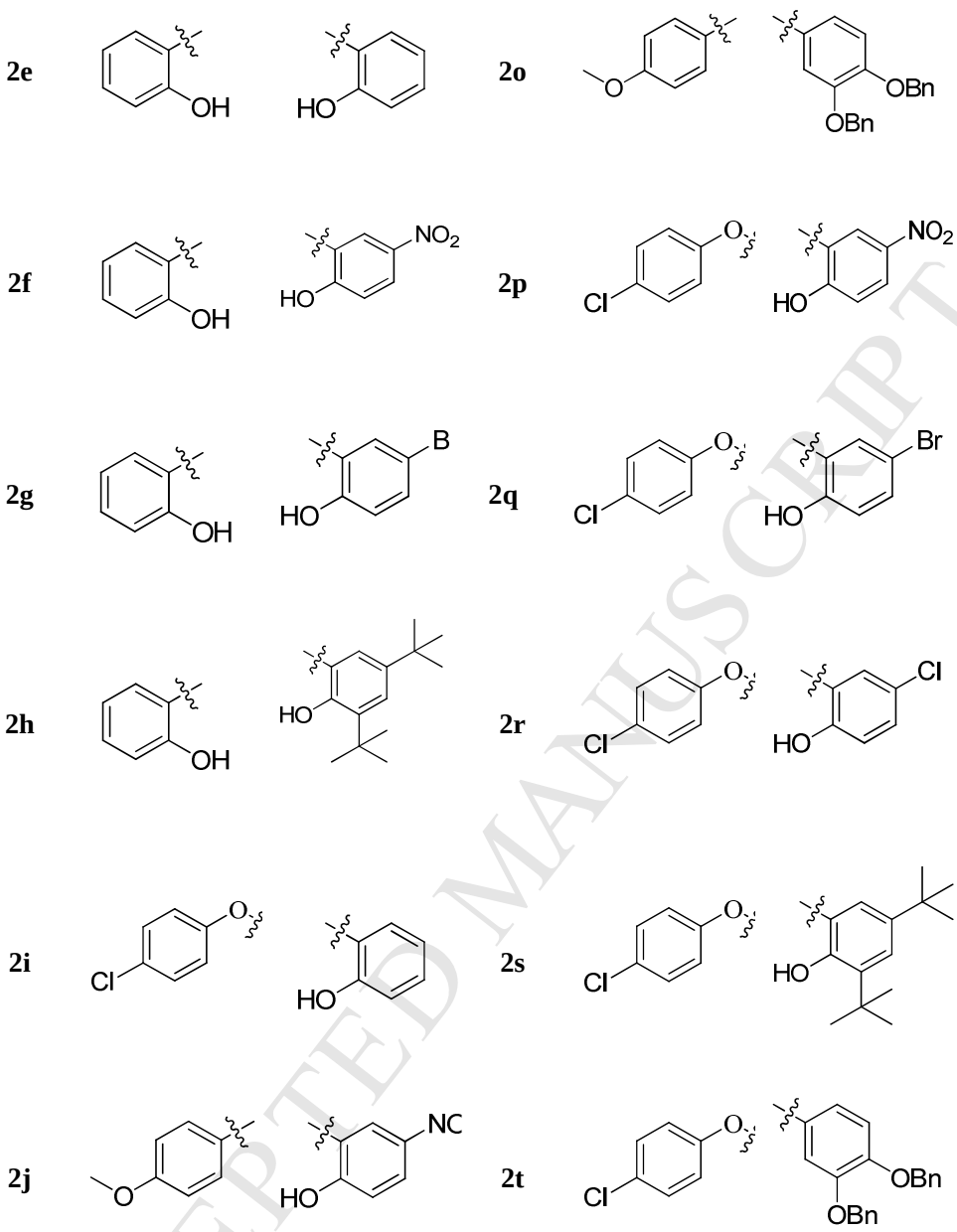
compound	R_1	R_2
1a	H	COOK
1b	OH	COOK
1c	H	$COOCH_3$

Scheme 1. The synthetic strategy and chemical structures of amino acid-containing Schiff bases. Reagents and conditions: (i) KOH, grinding for 30 min, rt.



I II 2a-2t

Comp.	R_3	R_4	Comp.	R_3	R_4
2a			**2k**		
2b			**2l**		
2c			**2m**		
2d			**2n**		



Scheme 2. The synthetic strategy and chemical structures of acylhydrazone Schiff bases. Reagents and conditions: (i) $SOCl_2$, CH_3OH, -10–90°C, reflux for 3 h; (ii) NH_2-$NH_2.H_2O$ (85%), CH_3OH, 80°C, reflux for 5 h; (iii) R_4CHO, EtOH, 80°C, reflux for 4–8 h.

Highlights:

- 23 benzaldehyde Schiff bases were synthesized to evaluate antibacterial activity
- Substitution at R_2 of amino acid-containing Schiff bases affected their activity
- Aromatic ring at R_3 and R_4 acted as potential pharmacophore in acylhydrazone Schiff base
- The active compounds showed good selectivity against the different bacteria
- Benzaldehyde Schiff bases regulated expression of related genes in bacteria

Supplementary data

Benzaldehyde Schiff bases regulation to the metabolism, hemolysis, and virulence genes expression *in vitro* and their structure–microbicidal activity relationship

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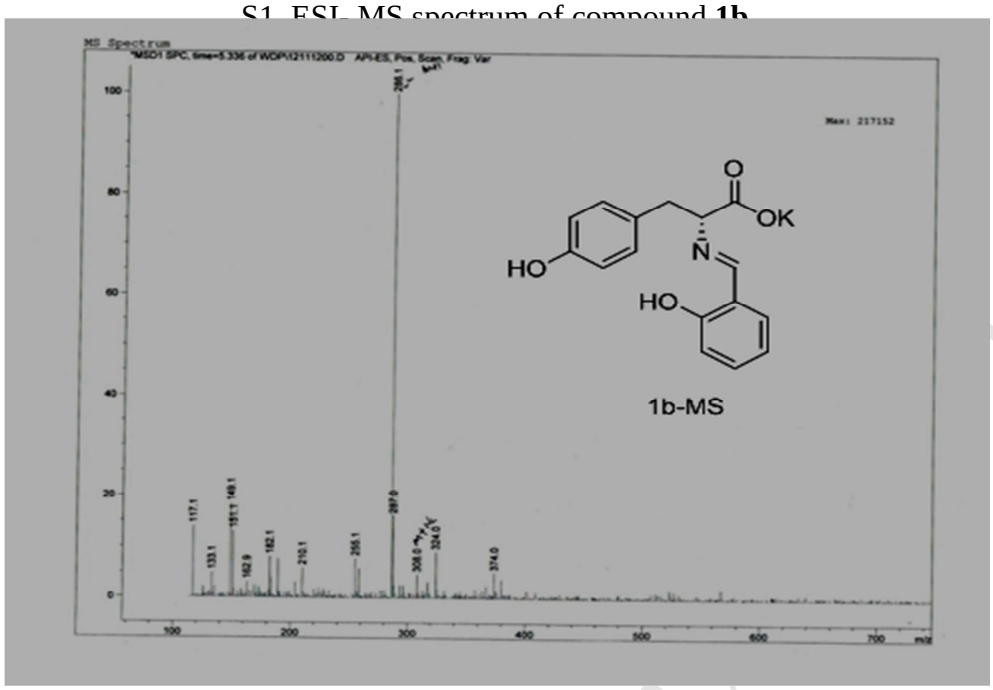
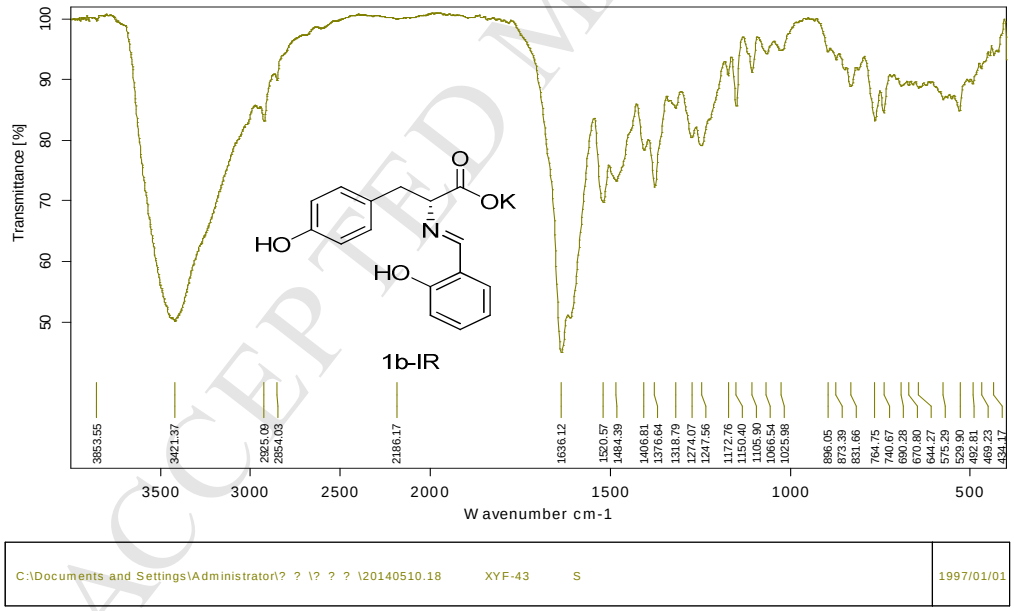
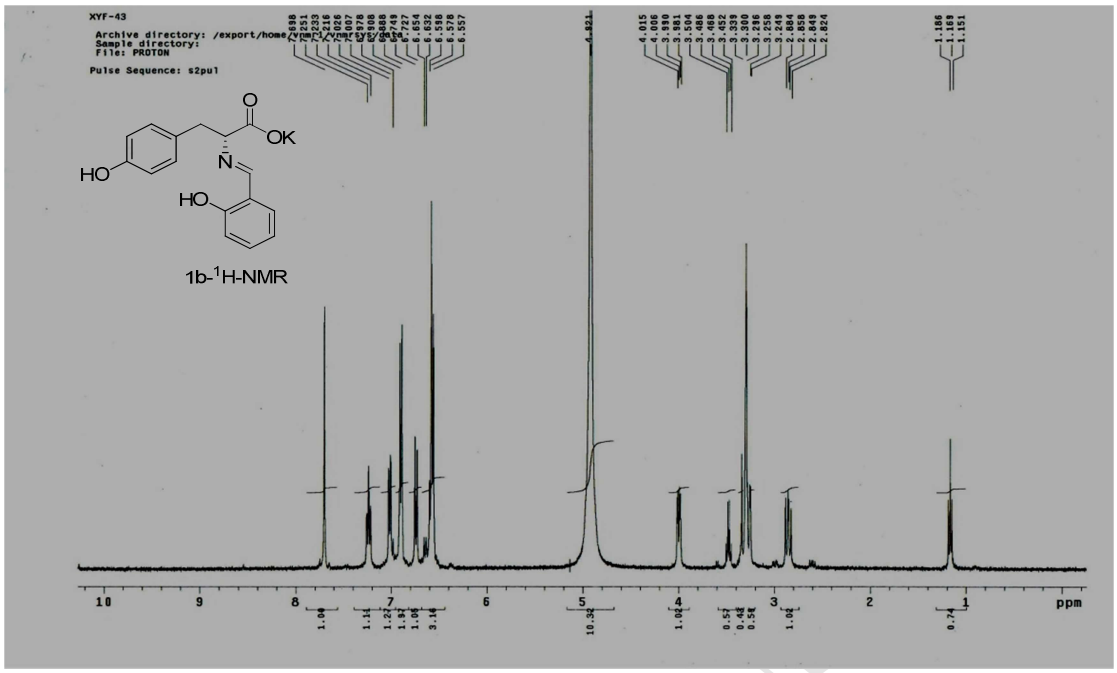


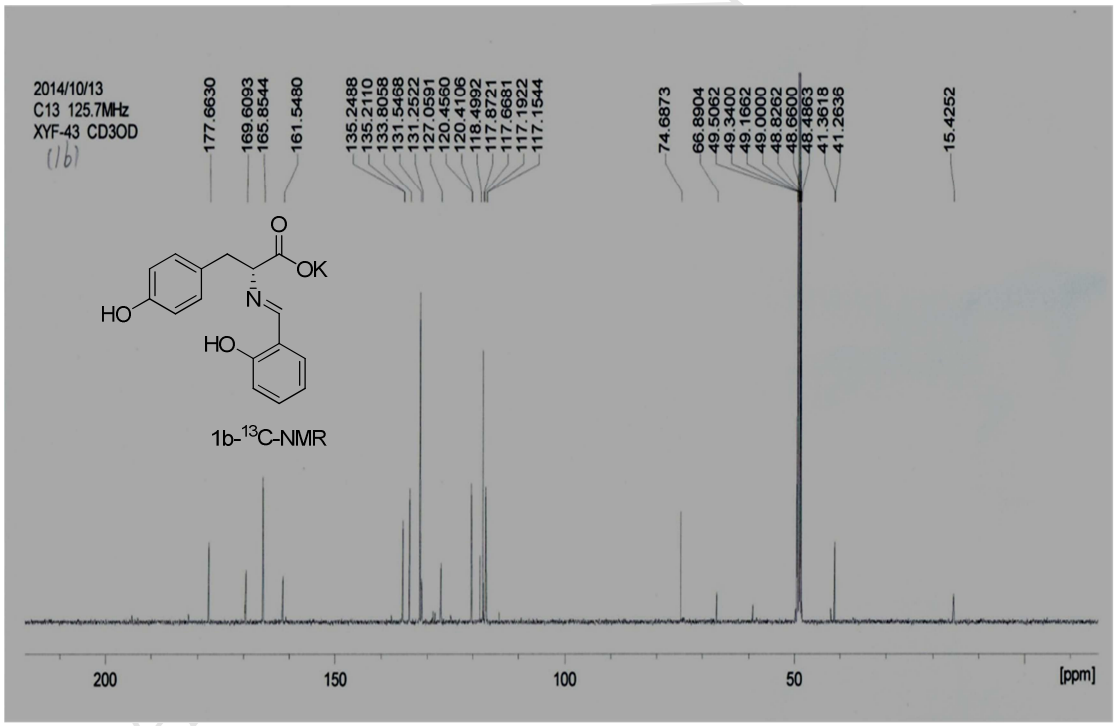
S2. IR spectrum of compound **1b**



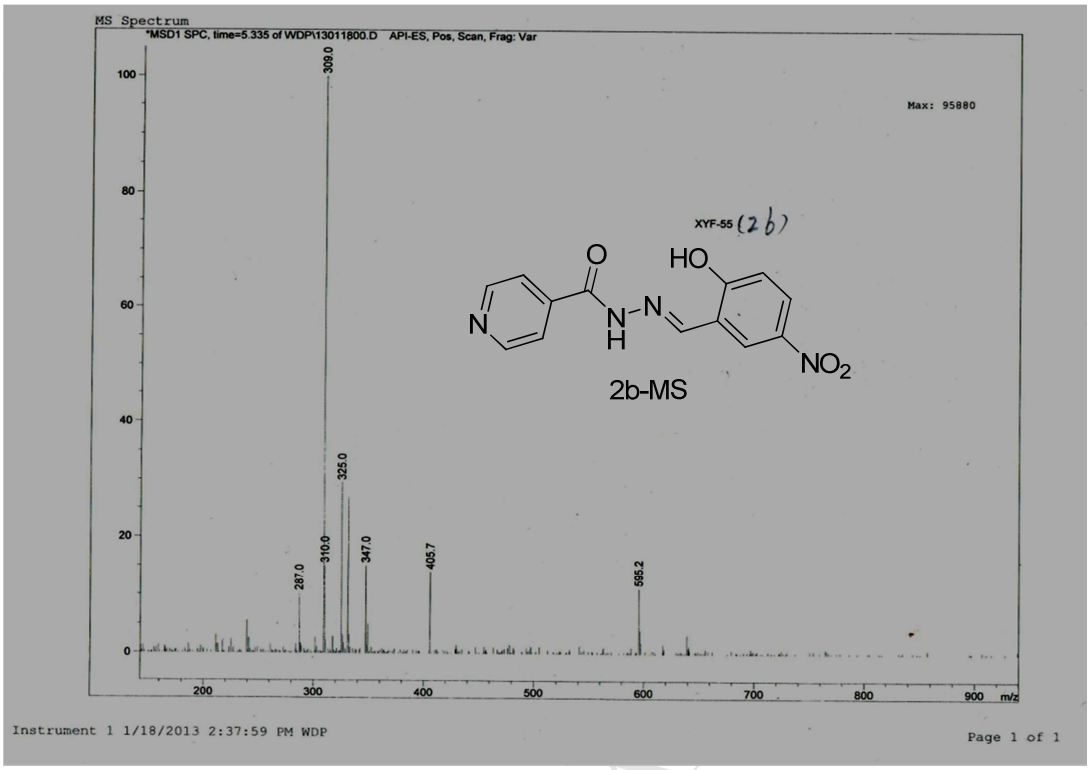
S3. ^{1}H-NMR (CD_3OD, 400 MHz) spectrum of compound **1b**



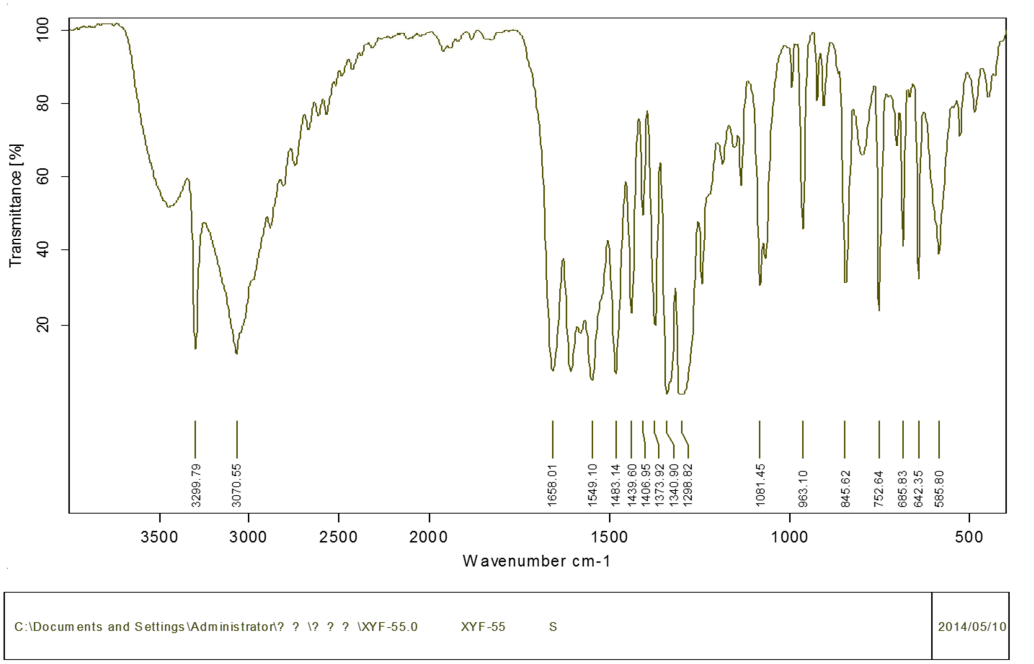
S4. ^{13}C-NMR (CD_3OD, 125 MHz) spectrum of compound **1b**



S5. ESI- MS spectrum of compound **2b**



S6. IR spectrum of compound **2b**



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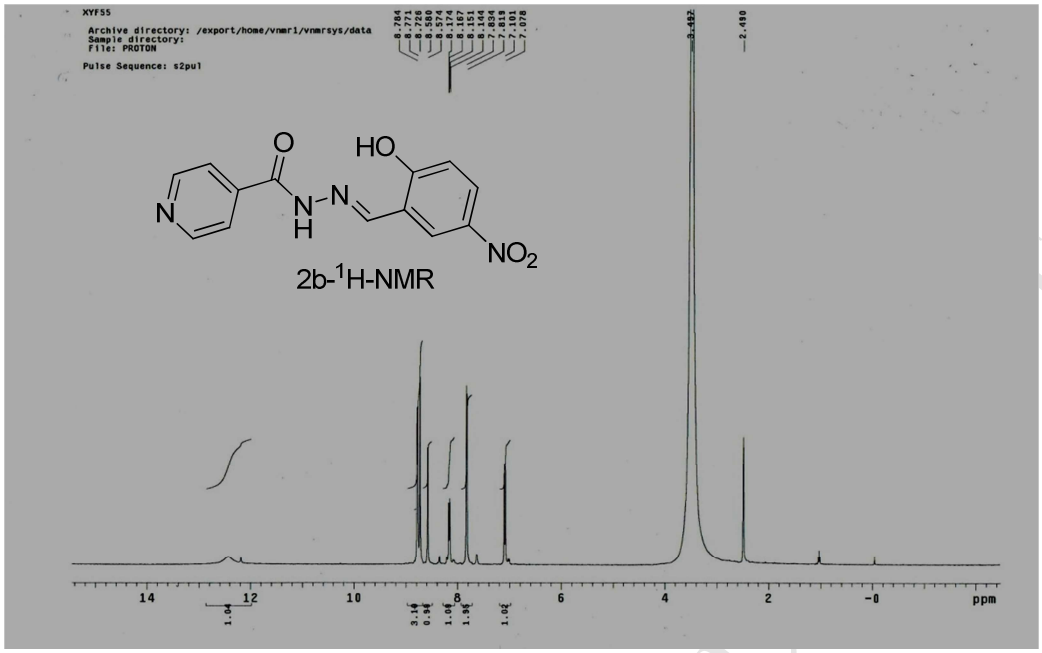
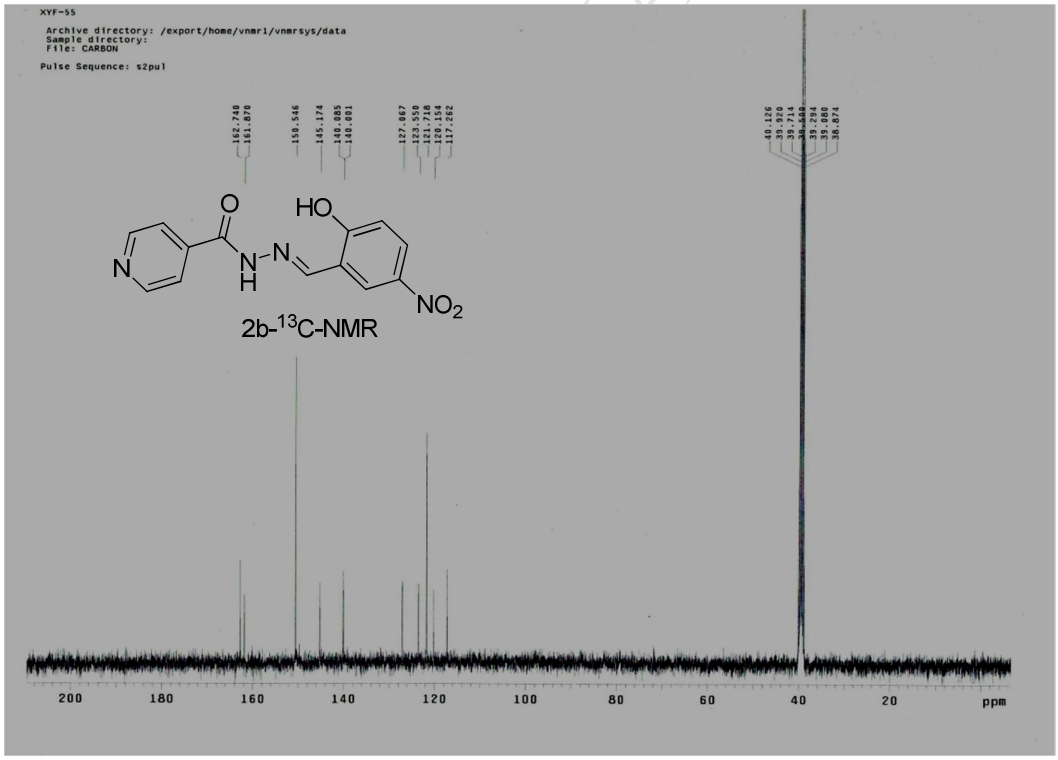
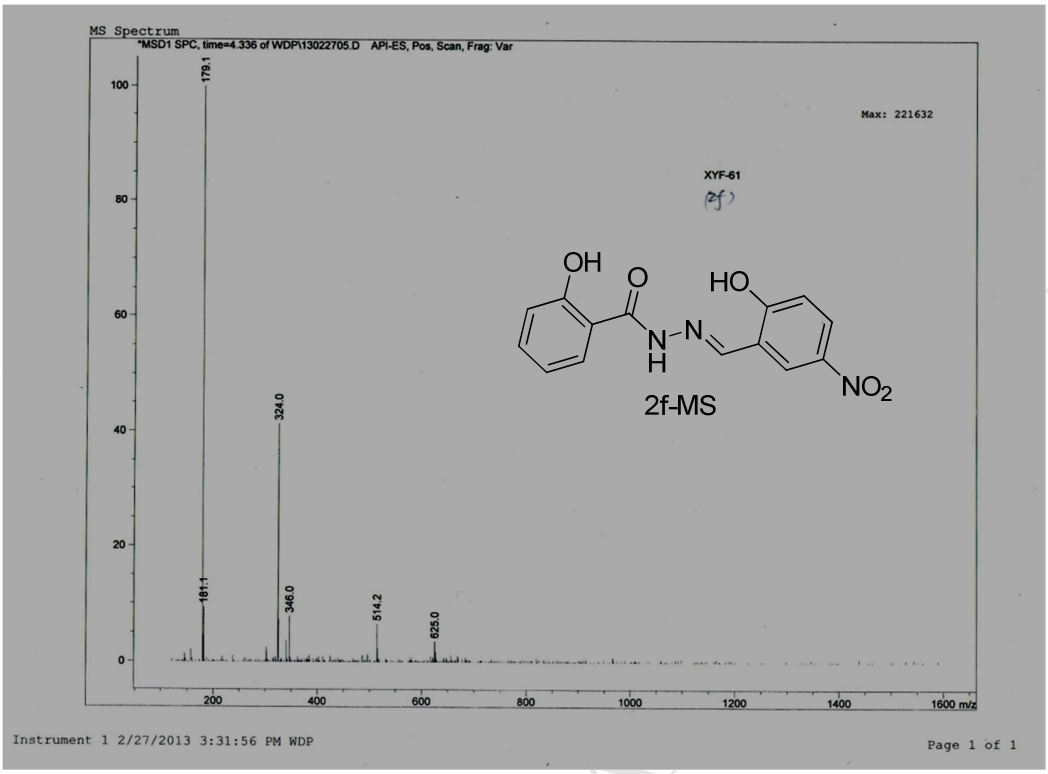
S7. ^{1}H-NMR (DMSO-d_6, 400 MHz) spectrum of compound **2b**



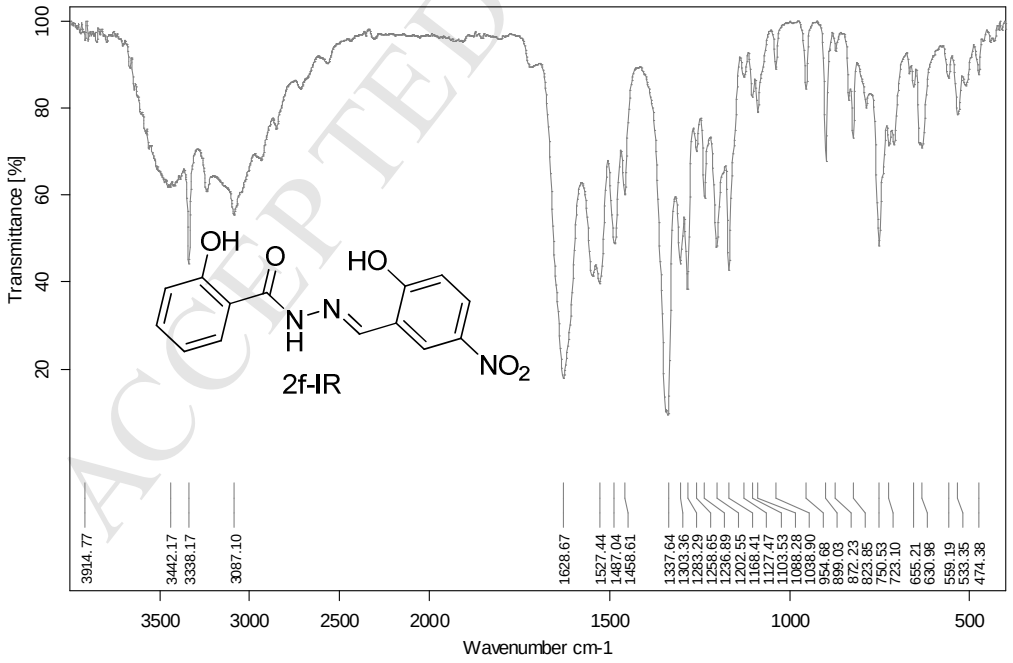
S8. ^{13}C-NMR (DMSO-d_6, 125 MHz) spectrum of compound **2b**



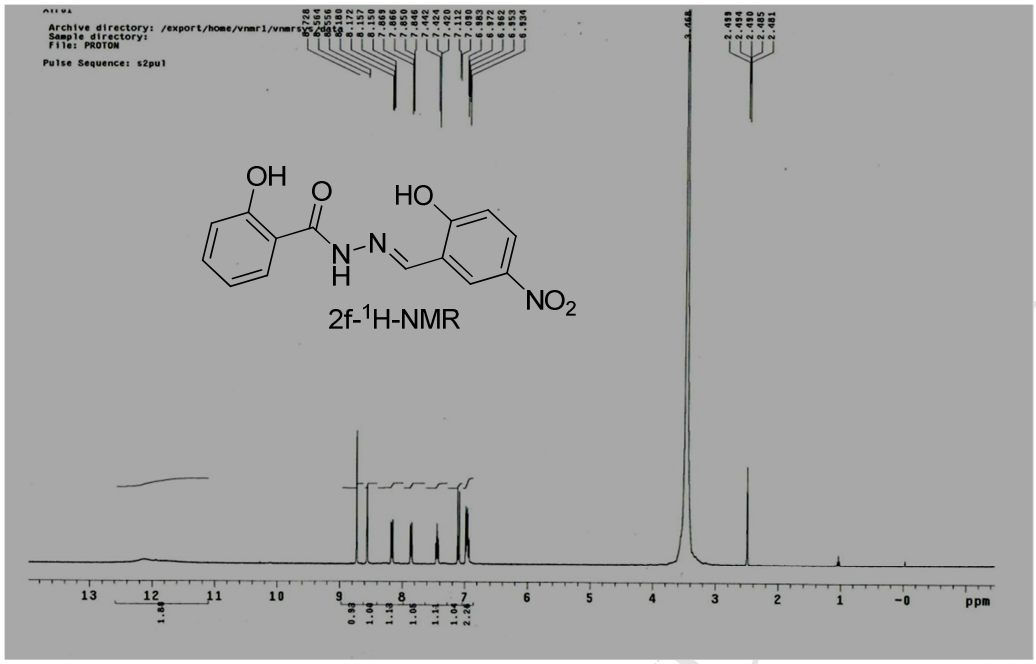
S9. ESI- MS spectrum of compound **2f**



S10. IR spectrum of compound **2f**



S11. ^{1}H-NMR (DMSO-d_6, 400 MHz) spectrum of compound **2f**



S12. ^{13}C-NMR (DMSO-d_6, 125 MHz)spectrum of compound **2f**