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Research paper

Pharmacomodulations of the benzoyl-thiosemicarbazide scaffold reveal antimicrobial agents targeting D-alanyl-D-alanine ligase *in bacterio*

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ABSTRACT

D-Alanyl-D-alanine ligase (Ddl) is a validated and attractive target among the bacterial enzymes involved in peptidoglycan biosynthesis. In the present work, we investigated the pharmacomodulations of the benzoylthiosemicarbazide scaffold to identify new Ddl inhibitors with antibacterial potency. Five novel series of thiosemicarbazide analogues, 1,2,4-thiotriazole-3-thiones, 1,3,4-thiadiazoles, phenylthiosemicarbazones, diacylthiosemicarbazides and thioureas were synthesized via straightforward procedures, then tested against Ddl and on susceptible or resistant bacterial strains. Among these, the thiosemicarbazone and thiotriazole were identified as the most promising scaffolds with Ddl inhibition potency in the micromolar range. Antimicrobial evaluation of salicylaldehyde-4(*N*)-(3,4-dichlorophenyl) thiosemicarbazone **33**, one of the best compounds in our study, revealed interesting antimicrobial activities with values of 3.12–6.25 μM (1.06–2.12 μg/mL) against VRE strains and 12.5–25.0 μM (4.25–8.50 μg/mL) towards MRSA and VRSA strains. A detailed mechanistic study was conducted on the Ddl inhibitors 4-(3,4-dichlorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3*H*-1,2,4-triazole-3-thione **20** and compound **33**, and revealed a bactericidal effect at 5 × MIC concentration after 7 h and 24 h, respectively, and a bacteriostatic effect at 1 × MIC or 2 × MIC without any sign of bacterial membrane disruption at these lower concentrations. Finally, **20** and **33** were proved to target Ddl *in bacterio via* intracellular LC-MS dosage of D-Ala, L-Ala and D-Ala-D-Ala. Although, at this stage, our results indicate that other mechanisms might be involved to explain the antimicrobial potency of our compounds, their ability to inhibit the growth of strains resistant to usual antibiotics, as well as strains that express alternative ligases, sets the stage for the development of new antimicrobial agents potentially less sensitive to resistance mechanisms.

1. Introduction

Nowadays, the threat of antibiotic resistance drives medicinal chemists to seek for new structural classes of antibiotics that could escape known resistance mechanisms [1]. Efforts are notably focused on the development of effective antibacterial drugs acting on original targets. Among them, peptidoglycan biosynthesis is a validated and attractive target as this major cell wall component does not exist in mammalian cells and the last steps of its synthesis are the site of action of clinically important β-lactams and glycopeptides [2,3]. The main function of peptidoglycan is to conserve cell integrity by resisting the turgor, thus avoiding cell lysis. It is present on the outside of the cytoplasmic membrane of both Gram-negative and Gram-positive bacteria, allowing the development of broad spectrum antimicrobials [4]. However, resistance to both β-lactams and glycopeptides has emerged, indicating the necessity to target earlier steps of peptidoglycan synthesis.

D-Alanyl-D-alanine ligase (Ddl) appears as an interesting target

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because of its crucial function in this cross-linked glycopeptide polymer biosynthesis. It is an ATP-dependent enzyme providing D-Ala-D-Ala, an essential component of peptidoglycan intracellular pentapeptidic precursor. This terminal dipeptide plays a necessary role in the reticulation of peptidoglycan via peptide bonds in the extracellular stage of this pathway [5]. Further interest in this enzyme arose from the acquisition of glycopeptide resistance. It consists in the elimination of the normally produced D-Ala-D-Ala for which vancomycin has a high affinity, and in the concomitant production of others peptidoglycan precursors by alternative Ddl ligases (D-Ala-D-Lac for VanA, -B and -D types and D-Ala-D-Ser for VanC, -E, -G and -L types) with lower affinity for the antibiotic [6]. Consequently, the discovery of inhibitors also acting on these alternative ligases would broaden their potential spectrum of activity.

Until today, four major classes of Ddl inhibitors were described [7]: (i) the substrate analogues [8–12], like D-cycloserine (DCS), used for tuberculosis second line treatment [13–16], (ii) transition state analogues, such as the tight-binding inhibitor 1-(*S*)-aminoethyl-(2(*R*)-carboxy-1-*n*-propyl)phosphinic acid (K_i = 4 μM) [17–19], (iii) D-Ala-D-Ala itself and other DD-dipeptides [8,20] and, finally, (iv) other compounds discovered by screening of chemical libraries or rational drug design [21–31].

In recent studies, we identified the 4-(3,4-dichlorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **9** (Scheme 1) as a promising Ddl inhibitor with submicromolar inhibition potency possessing good antimicrobial efficacy on susceptible and resistant strains of bacteria (MICs = 17.81–35.62 μg/mL) [32]. The activity of **9** was also demonstrated *in bacterio* by the determination of intracellular pools of L-Ala, D-Ala, and D-Ala-D-Ala using a UPLC-MS/MS method.

In the present work, we investigated the pharmacomodulations of the benzoylthiosemicarbazide scaffold to identify new Ddl inhibitors with antibacterial potency.

2. Results and discussion

2.1. Organic synthesis

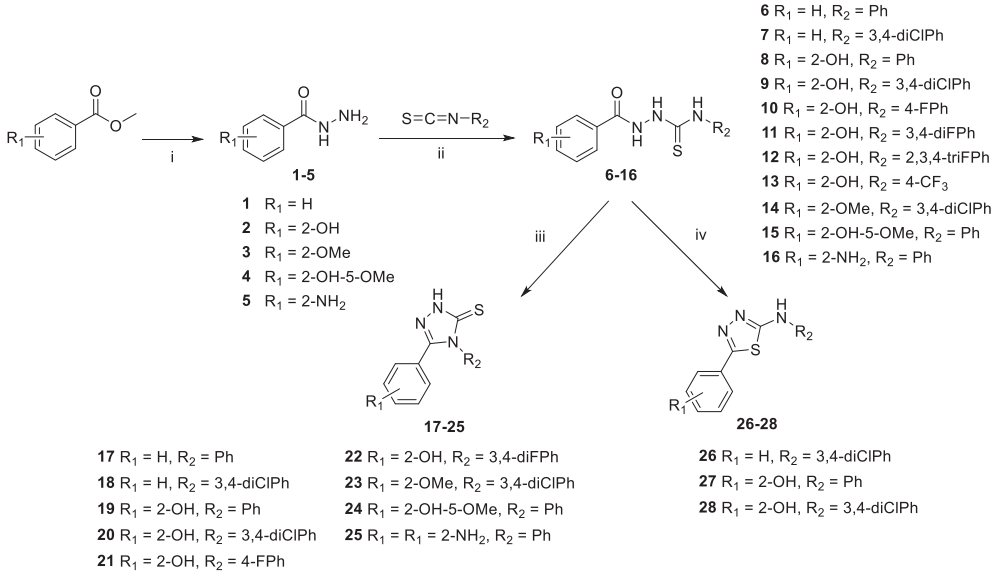
To further explore the structure-activity relationships of our compounds on Ddl, several pharmacomodulations of the benzoylthiosemicarbazide central linker were performed, knowing that the 2-hydroxyl substituent on the left phenyl group (R_1) was previously shown to be crucial for enzymatic inhibition. The cyclization of this linker was first considered to rigidify the structure and the syntheses of 1,2,4-thiotriazole-3-thiones or 1,3,4-thiadiazoles were achieved in three steps as presented in Scheme 1. Briefly, the benzohydrazides **2** and **3** were obtained as described previously by refluxing hydrazine hydrate and the appropriate methyl benzoate in ethanol while **1**, **4** and **5** were obtained commercially [33,34]. Then, benzohydrazides **1**–**5** were reacted with various isothiocyanates to yield the corresponding benzoylthiosemicarbazides **6**–**16** [32,35,36]. Finally, these compounds were refluxed in either 2% NaOH [37] or concentrated sulfuric acid/EtOH [38,39] to obtain the cyclized products, 1,2,4-thiotriazole-3-thiones **17**–**25** or 1,3,4-thiadiazoles **26**–**28** respectively.

The analysis of the ^{1}H NMR signals of the labile –NH– for the two cyclized forms (δ 14 ppm and 11 ppm for the thiotriazoles **17**–**25** and thiadiazoles **26**–**28**, respectively) revealed that, on the contrary to what we initially observed for the parent 1-(2-hydroxybenzoyl)-thiosemicarbazides **8**–**15** [32], only one tautomer, the thione form (Scheme 2), is visible on the thiotriazoles spectra (see Supporting Information). Indeed, the chemical shift of the labile –NH– was δ 14 ppm, while the range of the -SH of the thiol form is around δ 13 ppm [40].

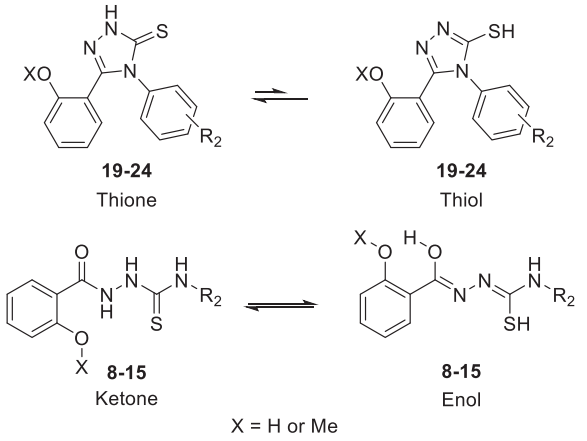
This was further confirmed by analysis of the X-ray structures of **19** as previously reported [41], **18** and **26**, as depicted on Fig. 1. We can also observe that the presence of a hydroxyl group for compound **19** constrains the thiotriazole cycle to be in the same plane as the 2-hydroxy-phenyl group.

To further study the role of this hydroxyl group in Ddl inhibition and thiotriazole conformation, the [1,2,4]triazolo[4,3-d][1,4]oxazepine-3(2H)-thione **29** was also synthetized via the procedure described above for the cyclization of benzoylthiosemicarbazides in basic conditions (Scheme 3). The nucleophilic aromatic substitution occurred through attack of the phenolate on the *ortho*-fluorine substituted carbon of the right aromatic ring.

Then, the oxygen from the carbonyl group of the central linker was removed to assess its role in Ddl inhibition. To this end, the benzoylthiosemicarbazide function was replaced by a



Scheme 1. Synthetic route for the cyclization of the benzoylthiosemicarbazide central linker.[a] Reagents and conditions: (i) 65% hydrazine hydrate (5 equiv), EtOH, reflux (ii) MeOH or EtOH, reflux or r.t. (iii) 2% NaOH, reflux (iv) H_2SO_4 cc., EtOH.



Scheme 2. Only the thione form of 5-(2-hydroxyphenyl) or 5-(2-methoxyphenyl)-1,2,4-thiotriazoles **19**–**24** is visible on ^{1}H NMR spectrum in DMSO, contrary to the 1-(2-hydroxy) or 1-(2-methoxybenzoyl)-thiosemicarbazides **8**–**15**.

phenylthiosemicarbazone motif (Scheme 4). Compounds **32** and **33** were synthesized via a straightforward procedure [42] consisting in the condensation of salicylaldehyde with appropriate thiosemicarbazides in ethanol at reflux in presence of acetic acid as catalyst. The thiosemicarbazides **30** and **31** were previously obtained via the addition of hydrazine hydrate on commercially available phenylisothiocyanates in ethanol at room temperature.

Finally, the central linker was either extended or shortened thanks to the replacement of the acylthiosemicarbazide function by a diacylthiosemicarbazide or a thiourea respectively (Scheme 5). 4-Benzoyl-1-benzoylthiosemicarbazides **34** and **35** were obtained according to the literature [43] by addition of benzoyl chloride to a solution of potassium cyanate in acetonitrile at 70 °C, followed by reaction of the crude product with 2-hydroxybenzohydrazide **5**. Phenylthioureas **36** and **37** were in turn synthesized using a known procedure from 2-aminophenol and the corresponding phenylisothiocyanates in methanol at room temperature [44].

All compounds were analyzed by ^{1}H NMR, ^{13}C NMR, HRMS and HPLC. The detailed procedures and spectral data of the target compounds are provided in Experimental and Supplementary information sections. These molecules were then assessed on *His*-tagged Ddl and their antimicrobial activity was subsequently evaluated on susceptible and resistant strains of bacteria.

2.2. Study of in vitro Ddl inhibition

To evaluate the activity of these compounds on recombinant *His*-tagged Ddl, a previously optimized colorimetric malachite green assay was used for the determination of inorganic phosphate produced during the enzymatic reaction [32,45]. A first screening at 100 μM ([D-Ala] = 1 mM, [ATP] = 500 μM) allowed to select inhibitors that significantly decrease Ddl activity compared to the

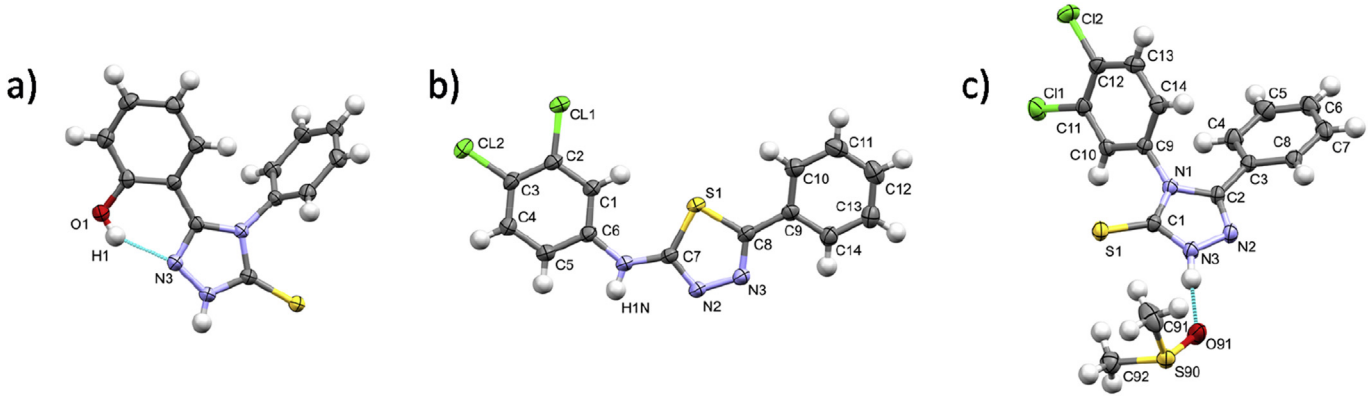
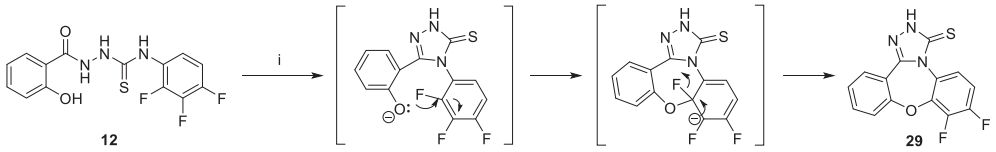
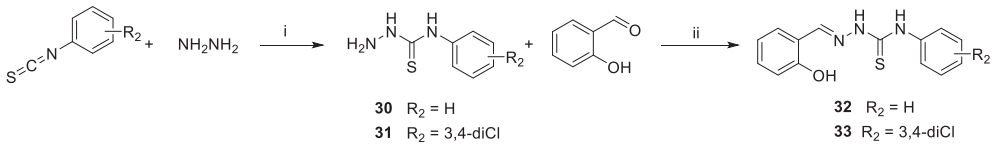


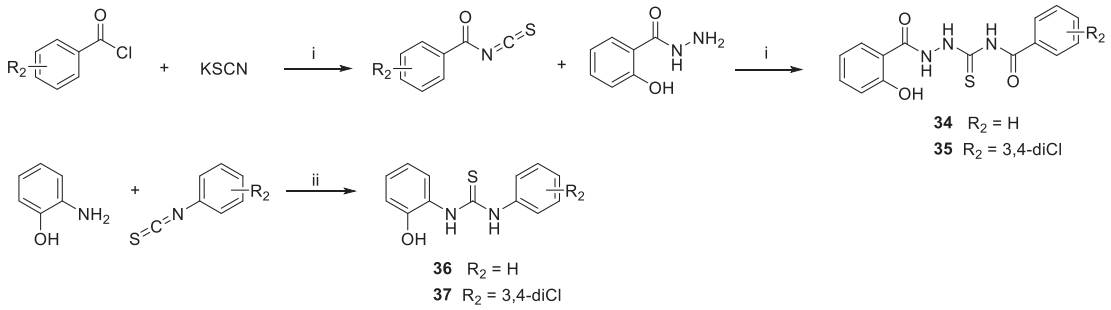
Fig. 1. Molecular structures of **a) 19** from Ref. [41] **b)** 2-(3,4-dichlorophenylamino)-5-phenyl-1,3,4-thiadiazole **26** and **c)** 4-(3,4-dichlorophenyl)-5-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione **18** co-crystallized with one DMSO molecule from the crystallization solvent. Displacement ellipsoids are drawn at the 50% probability level and H atoms are shown as spheres of arbitrary radii.



Scheme 3. Synthesis of [1,2,4]triazolo[4,3-d][1,4]oxazepine-3(2H)-thione **29** occurs through a nucleophilic aromatic substitution.[a]
[a] Reagents and conditions: (i) 2% NaOH, reflux.



Scheme 4. Synthesis of phenylthiosemicarbazones **32** and **33**.[a]
[a] Reagents and conditions: (i) 65% hydrazine hydrate (5 equiv), EtOH, r.t. (ii) EtOH, $AcOH_{cat.}$, reflux.



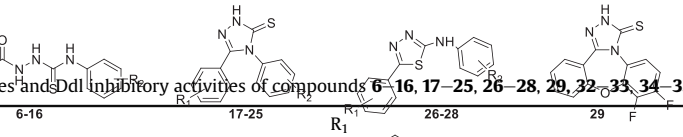
Scheme 5. Synthetic route for 4-benzoyl-1-benzoylthiosemicarbazides **34** and **35** and phenylthioureas **36** and **37**.[a] [a] Reagents and conditions: (i) Acetonitrile, 70 °C (ii) MeOH, r.t.

control. Afterwards, IC_{50} determination was performed for active compounds after an incubation of 30 min with Ddl. The results are shown in Table 1.

First, it should be noted that for compounds indicated with a "[b]" in Table 1, such as compounds **11**, **24**, **32** and **34**–**35**, the maximum Ddl inhibition observed was less than 50% (residual Ddl activity > 50% at the highest inhibitor concentration). A similar observation has already been made with some of the benzoylthiosemicarbazides from our previous work [32]. A dose-response curve illustrating this incomplete inhibition profile can be found in Supplementary Information. The addition of 0.01% Triton X-100 was used to verify if that phenomenon was not due to drug aggregation but no change in the IC_{50} curve was noticed [46,47].

From our past studies, it was known that a 2-hydroxy substituent on the left phenyl group (R_1 = 2-OH) of the benzoylthiosemicarbazides was crucial for Ddl inhibition. Therefore, we first evaluated whether this substitution was also needed for the cyclized analogues by assessing Ddl inhibition of the thiotriazoles **17**–**18** and thiadiazole **26**. As no inhibition was detected without this 2-hydroxy group, we further evaluated 2-hydroxy substituted analogues with different linkers, without any substituent on the

Table 1
Structures and Ddl inhibitory activities of compounds **6**–**16**, **17**–**25**, **26**–**28**, **29**, **32**–**33**, **34**–**35** and **36**–**37**.



Cpd	R_1	R_2	IC_{50} (μM)[a]
		Benzoylthiosemicarbazides	
6	H	H	n.a.
7	H	3,4-dichloro	n.a.
8	2-OH	H	0.740 ± 0.038
9	2-OH	3,4-dichloro	1.17 ± 0.048
10	2-OH	4-fluoro	0.890 ± 0.052
11	2-OH	3,4-difluoro	0.920 ± 0.032[b]
12	2-OH	2,3,4-trifluoro	0.640 ± 0.047
13	2-OH	4-trifluoromethyl	0.630 ± 0.032
14	2-OMe	3,4-dichloro	n.a.
15	2-OH-5-OMe	H	0.630 ± 0.047
16	2-NH_2	H	n.a.
		1,2,4-Thiotriazole-3-thiones	
17	H	H	n.a.
18	H	3,4-dichloro	n.a.
19	2-OH	H	n.a.
20	2-OH	3,4-dichloro	299 ± 38.4
21	2-OH	4-fluoro	60.0 ± 6.04
22	2-OH	3,4-difluoro	n.a.
23	2-OMe	3,4-dichloro	n.a.
24	2-OH-5-OMe	H	133 ± 22.5[b]
25	2-NH_2	H	n.a.
		1,3,4-Thiadiazoles	
26	H	3,4-dichloro	n.a.
27	2-OH	H	n.a.
28	2-OH	3,4-dichloro	n.a.
29	/	/	n.a.
		Phenylthiosemicarbazones	
32	2-OH	H	2.60 ± 0.21[b]
33	2-OH	3,4-dichloro	1.48 ± 0.064
		4-Benzoyl-1-benzoylthiosemicarbazides	
34	2-OH	H	0.810 ± 0.056[b]
35	2-OH	3,4-dichloro	1.15 ± 0.11[b]
		Phenylthioureas	
36	2-OH	H	n.a.
37	2-OH	3,4-dichloro	n.a.
DCS	/	/	262 ± 43.4

n.a. = not active; 100% residual activity at 100 μM.

[a] IC_{50} values are presented as the (mean ± SD) of measures performed in triplicate (n ≥ 2).

[b] These values are EC_{50} (concentration at which 50% of the maximal effect is observed) as compounds did not lower the residual activity more than 50% at their maximal tested concentration (50 μM for benzoylthiosemicarbazides, phenylthiosemicarbazones and 4-benzoyl-1-benzoylthiosemicarbazides, and 1 mM for cyclic compounds).

right phenyl group (R_2 = H). Whereas compounds **19**, **27** and **36** were devoid of any Ddl inhibition, some activity could be observed for the thiosemicarbazone **32** and the dibenzoylthiosemicarbazide **34**.

Next, we set out to investigate Ddl inhibition of analogues bearing at the R_2-position either a 3,4-dichloro substitution or fluorine substituent(s), as these groups were previously shown to positively contribute to the antibacterial potency of our compounds. In the benzoylthiosemicarbazide series, the introduction of either a 3,4-dichloro (**9**) or fluorine substituents (**10**–**13**) resulted in promising Ddl inhibitors with activity in the low μM range. In the 1,2,4-thiotriazole-3-thione series, the introduction of a 3,4-dichloro (**20**) or a 4-fluoro (**21**) substituent enhanced Ddl inhibition compared to the unsubstituted analogue (**19**), although these compounds remain relatively modest Ddl inhibitors. In both series, the replacement of the 2-hydroxy substituent in the R_1-position with a 2-NH_2 or a 2-OCH_3 resulted in the complete loss of Ddl inhibitory potency (see compounds **16**, **25**, and **14**, **24** respectively), hence reinforcing the crucial role of the 2-hydroxy-substituent at the R_1-position.

A positive impact of the introduction of the 3,4-dichloro substitution was also noted in the thiosemicarbazone series with **33** being more potent than the unsubstituted compound **32.** On the other hand, no improvement of Ddl inhibition was observed for the thiadiazole **28**, the thiourea **37** and the diacylthiosemicarbazide **35**.

Comparing the benzoylthiosemicarbazides **10** and **11** and their cyclized thiotriazole analogues **21** and **22**, respectively, revealed that, with the cyclized form, only the 4-fluoro substituent is tolerated for Ddl inhibition (**21**: IC_{50} = 60 μM).

Finally, the [1,2,4]triazolo[4,3-d][1,4]oxazepine-3(2H)-thione **29** was synthesized to evaluate the impact of a conformational restriction. Because **29** is devoid of any Ddl activity, it seems very clear that an appropriate geometrical conformation is needed for Ddl inhibition.

As a result of our pharmacomodulations, the thiosemicarbazones and thiotriazoles were identified as potent Ddl inhibitors, albeit a 2-hydroxy substituent as R_1 and lipophilic substituents (4-F or 3,4-diCl) as R_2 seem absolutely required.

2.3. Biological activities

2.3.1. MIC determination

All compounds were evaluated for their *in vitro* antibacterial activity against two Gram-positive bacterial strains, *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212. MICs values were determined using maximal concentrations of 100 μM–800 μM, depending on the solubility of the compounds, with 2.5% DMSO (v:v) and are presented in Table 2. Compounds with antibacterial potency >400 μM are considered inactive.

As already observed from our previous work, compounds from the benzoylthiosemicarbazide series bearing no hydroxyl group in the 2-position or no lipophilic substituent are not antimicrobial agents. Compound **13** was the most active derivative with MIC values of 50.0 μM on both strains. Compound **9** is also a moderate antibacterial agent with MIC values of 100 μM and 50.0 μM against *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 respectively.

As for the two cyclized series, the only active compound was the thiotriazole **20**, bearing 3,4-dichloro substituents in R_2, with MIC values of 400 μM for both strains.

The thiosemicarbazone **33** was characterized with MICs of 12.5 μM (4.25 μg/mL) on both strains, which is 4–8 times better than the parent thiosemicarbazide analogue.

The diacylthiosemicarbazides series exhibited moderate to good antibacterial activities despite their poor Ddl inhibitory potency, with MIC values of 100 μM for compound **34** bearing no substituent, and 25.0–50.0 μM for the dichlorinated analog **35**.

Table 2
MIC values of compounds against fully susceptible Gram-positive reference strains.

	MIC			
	S. aureus ATCC 25923		*E. faecalis* ATCC 29212	
Cpd	μM	μg/mL	μM	μg/mL
6	>100	>27.1	>400	>108
7	>100	>34.0	>100	>34.0
8	800	230	>800	>230
9	100	35.6	50.0	17.8
10	>100	>30.5	200	61.1
11	400	129	400	129
12	200	68.3	200	68.3
13	50.0	17.8	50.0	17.8
14	>100	>37.0	>100	>37.0
15	>100	>31.7	>100	>31.7
16	>400	>114	>400	>114
17	>400	>101	>400	>101
18	>100	>35.6	>100	>35.6
19	>800	>215	>800	>215
20	400	135	400	135
21	>800	>230	>800	>230
22	>400	>122	>400	>122
23	>400	>141	>400	>141
24	>800	>239	>800	>239
25	>800	>215	>800	>215
26	>100	>32.2	>100	>32.2
27	>100	>26.9	>100	>26.9
28	>100	>37.3	>100	>37.3
29	>400	>121	>400	>121
32	>400	>108	>400	>108
33	12.5	4.20	12.5	4.20
34	100	31.5	100	31.5
35	25.0	9.60	50.0	19.2
36	>800	>195	>800	>195
37	25.0	7.80	50.0	15.7
DCS	313	32.0	1250	128

*N.D.: non determined.

Finally, among the thioureas, only **37** had an effect on bacterial growth with MICs of 25.0 μM and 50.0 μM against *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 respectively. However, for diacylthiosemicarbazides and thioureas, as no link between their antimicrobial activity and Ddl inhibition could be established, the mechanism of action of these two last series is yet to be elucidated.

Compounds demonstrating some antimicrobial potency on susceptible strains (**8**–**13**, **20**, **33**, **34**–**35** and **37**) were then assayed against various Gram-negative (*Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* PAO1) and Gram-positive bacteria, including clinical isolates resistant to current antibiotics and strains expressing alternative ligases. None of the tested compounds was active against Gram-negative bacteria.

Against Gram-positive resistant bacteria (Table 3), whereas the thiosemicarbazide analogues **8, 10** and **12** have no or weak antimicrobial potency, the CF_3 substituted compound **13** possesses MIC values of 100 μM against all the tested strains apart from the vancomycin resistant BM 4390.

Interestingly, in each series the di-chlorinated compounds (**9**, **20**, **33**, **35** and **37**) are the most potent antimicrobial agents, with the thiosemicarbazone **33** being the best compound with MIC values of 3.12–6.25 μM (1.06–2.10 μg/mL) against VRE strains and 12.5–25.0 μM (4.25–8.50 μg/mL) towards MRSA and VRSA strains.

Hence, our study led to the identification of compounds that not only prevent the growth of strains resistant to a variety of antibiotics (glycopeptides, oxazolidinones, or fluoroquinolones) but also the growth of *E. faecalis* strains that express alternative ligases. We believe that these results constitute an important step towards the

Table 3
MIC values against Gram-positive resistant strains of active compounds on susceptible strains. MIC values on susceptible strains are given as a reference.

Cpd	MIC (μM (μg/mL))									
	E. faecalis[a]				*S. aureus*[b]					
	ATCC 29212	BM 4390	JH2-2::C1	BM 4575	ATCC 25923	MU 50	NRS 119	SA 325	SA 481	VRS-1
8	>800	>800	>800	>800	800	800	>800	800	800	800
9	50.0	50.0	100	50.0	100	100	50.0	50.0	100	50.0
10	200	>100	>100	>100	>100	>100	>100	>100	>100	>100
12	200	>400	>400	400	200	400	400	400	400	400
13	50.0	>100	100	100	50.0	100	100	100	100	100
20	400	>800	400	400	400	400	400	400	800	400
33	12.5	6.25	3.12	6.25	12.5	25.0	12.5	25.0	25.0	12.5
34	100	>100	>100	>100	100	>100	>100	100	100	100
35	50.0	25.0	12.5	50.0	25.0	25.0	25.0	50.0	25.0	100
37	50.0	50.0	50.0	100	25.0	50.0	50.0	50.0	50.0	100
DCS	1250	5015	5015	>5015	313	627	627	1250	1250	157
Vm[c]	N.D.	700(**1024**)	350(**512**)	N.D.	N.D.	5(8)	0.7(1)	N.D.	N.D.	>175(>**256**)
Lzd[c]	N.D.	N.D.	N.D.	N.D.	N.D.	3(1)	190(**64**)	N.D.	N.D.	N.D.
Cip[c]	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	770(**256**)	N.D.

[a] VRE (Vancomycin resistant *enterococcus*): *E. faecalis* BM 4390 [48] (*vanB* genotype, *in vitro* mutant of a clinical isolate with inactive Ddl; constitutive expression of D-Ala-D-Lac); *E. faecalis* JH2-2:C1 [49](*vanB* genotype, engineered derivative of JH2-2; constitutive expression of Ddl and of the VanB ligase); *E. faecalis* BM 4575 [50] (*vanE* genotype, clinical isolate, constitutive expression of Ddl and of the VanE D-Ala-D-Ser ligase).

[b] *S. aureus* MU 50: MRSA (methicillin resistant *S. aureus*) and VISA (vancomycin intermediate resistant *S. aureus*); *S. aureus* NRS 119: MRSA resistant to linezolid (clinical isolate) [51]; *S. aureus* SA 325: CA-MRSA (community-acquired MRSA) resistant to erythromycin; *S. aureus* SA 481: HA-MRSA (hospital-acquired MRSA) resistant to ciprofloxacin and moxifloxacin; VRS-1: VRSA (vancomycin resistant *S. aureus*, *vanA* genotype) and HA-MRSA.

[c] VAN, vancomycin; LZD, linezolid; CIP, ciprofloxacin.

development of antimicrobial agents overcoming the problem of vancomycin resistance.

Finally, in order to establish that Ddl is the bacterial target of these derivatives, additional studies were conducted on the most promising compounds **20** and **33**, exhibiting antibacterial activity but also active in the enzymatic assay.

2.3.2. Killing curves

Killing curves were first performed to assess their effect on bacterial growth. Briefly, a 10^6 CFU/mL starting inoculum of *S. aureus* ATCC 25923 was exposed to increasing concentrations of **20** and **33** over 24 h of incubation. As observed in Fig. 2a and b, both compounds are bacteriostatic at 1 × and 2 × their MIC and a bactericidal effect is reached at 5 × MIC (2 mM for **20** and 62.5 μM for **33**) after 7 h and 24 h, respectively.

2.3.3. Membrane permeabilization assay

Next, we assessed the compounds potential to permeabilize the membrane in order to evaluate their selectivity for the bacterial cell wall. For this purpose, the fluorescence intensity of propidium iodide (PI) bounded to DNA of damaged bacterial cells was measured according to the literature [52,53]. PI was added to *S. aureus* ATCC 25923 cells incubated during 2 h with various concentrations (1 × MIC, 2 × MIC, and 5 × MIC) of compounds **9**, **20**, **33**, and the reference antibiotic, D-cycloserine. SDS detergent was used as a positive control and resulted in an increase in fluorescence intensity until 35 RFU. No increase in fluorescence was noticed in Fig. 3a, confirming that DCS specifically acts on the bacterial cell wall. As depicted in Fig. 3b, compound **9** had no disrupting effect on membrane as compared to the control. The large increase in fluorescence from 1 h of cells treatment with thiotriazole **20** (Fig. 3c) at 5 × MIC (2 mM) demonstrated that PI could enter bacteria through the damaged membrane at this concentration. At 5 × MIC (62.5 μM), compound **33** was responsible for a moderate increase in fluorescence intensity of about 15 RFU regardless of the incubation time (Fig. 3c). If we compare these results to the killing curve in Fig. 2d, a slight decrease in bacterial counts (<1 $\log_{10}$ CFU/mL) is observed after 2 h at this concentration. The reduction of 2 $\log_{10}$ CFU/mL observed in Fig. 2c for compound **20** at 5 × MIC could also be caused by its effect on membrane permeability. It would therefore appear that compounds **20** and **33** have an additional effect on the cell membrane at 5 × MIC. However, the thiosemicarbazide **9** acts specifically on bacterial cell wall as it showed no permeabilization of the membrane despite its bactericidal effect from 2 × MIC after 24 h [32].

To conclude, it seems that high concentrations of compounds **20** and **33** lead to a substantial or moderate disruption of the bacterial membrane, a phenomenon that is not observed at lower concentrations.

2.3.4. Cytotoxicity of compounds *20* and *33*

Compounds **20** and **33** were then assayed for their potential cytotoxicity using THP-1 human monocytic cell line. Fig. 4 displays the evolution of cell survival as a function of time and inhibitor concentration. After 1 h and 2 h, compounds **20** and **33** exhibited no cytotoxicity at 1 × their MIC and low cytotoxicity at 2 × their MIC (5–15% decrease in viability compared to the control). Unfortunately, from 6 h of experiment, cytotoxicity is generally observed, except for 1 × MIC of compound **33**. It thus seems that these compounds are less selective to bacteria than the parent benzoylthiosemicarbazide **9** identified previously [32].

2.3.5. Determination of in bacterio L-Ala, D-Ala and D-Ala-D-Ala levels

Finally, in order to establish that Ddl is a bacterial target of our compounds, we relied on a previously developed UHPLC-MS/MS experimental setup involving the quantification of peptidoglycan precursors, L-Ala, D-Ala and D-Ala-D-Ala [12,32,54].

In order to avoid any premature leakage of intracellular contents, bacteria were incubated with 2 × MIC of compounds **20** and **33** for short periods. From Fig. 5 it can be observed that the [D-Ala]/[D-Ala-D-Ala] ratio is increasing over 30 min for both compound **20** and **33**. As compared to DCS, it seems clear that these two compounds have an effect on Ddl *in vivo*. The slight increase in L-Ala levels might indicate either an inhibition of Alr or the interconversion of the excess of D-Ala into L-Ala by this reversible enzyme

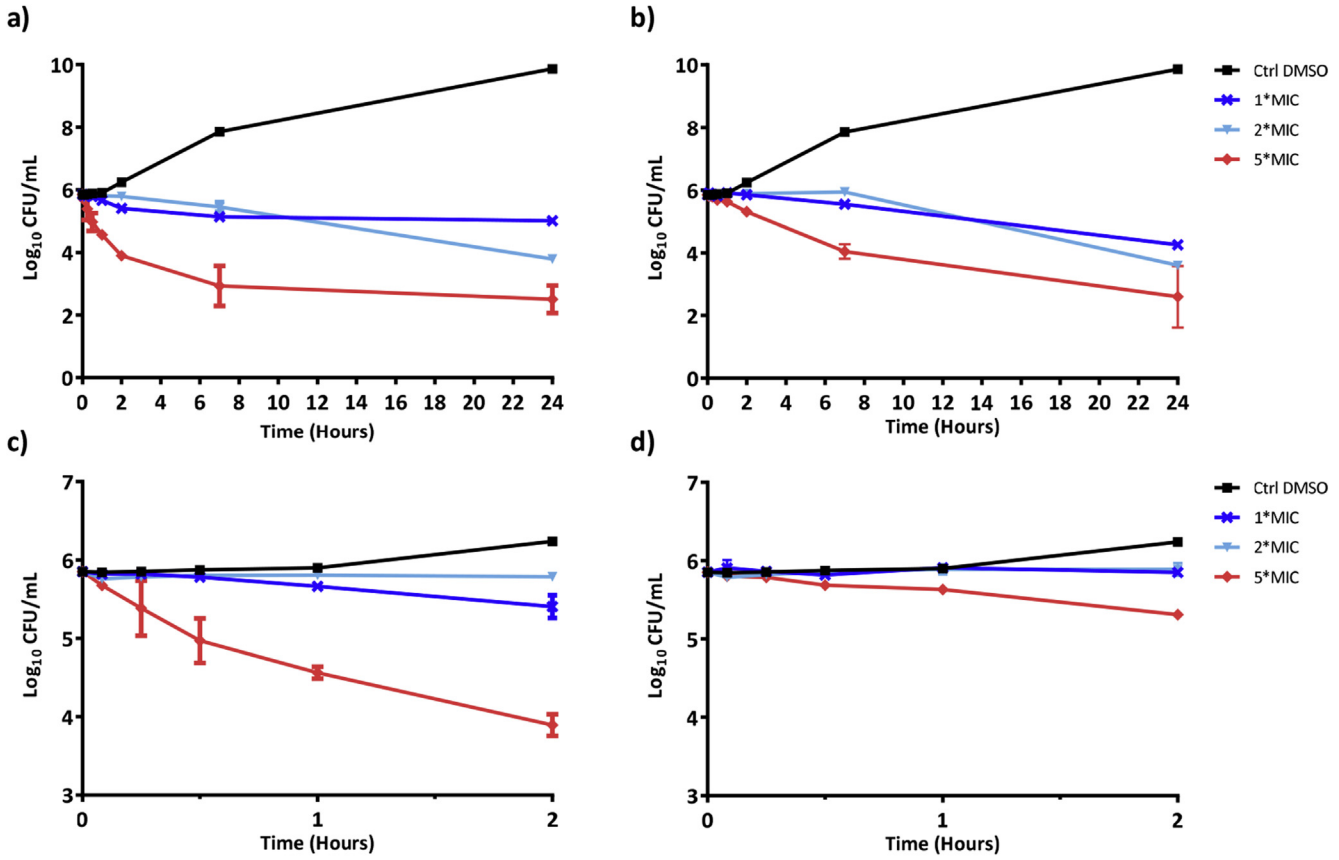


Fig. 2. Time-kill curves of *S. aureus* ATCC 25923 incubated for 24 h with **a)** the thiotriazole **20** and **b)** the thiosemicarbazone **33**. Zoom over 2 h were shown in **c)** and **d)**. The strain was incubated with growth media 5% DMSO (■) as positive control; with molecule at 1 × MIC (×), 2 × MIC (▼), and 5 × MIC (◆). All values are presented as the (mean ± SD) of measures performed in triplicate.

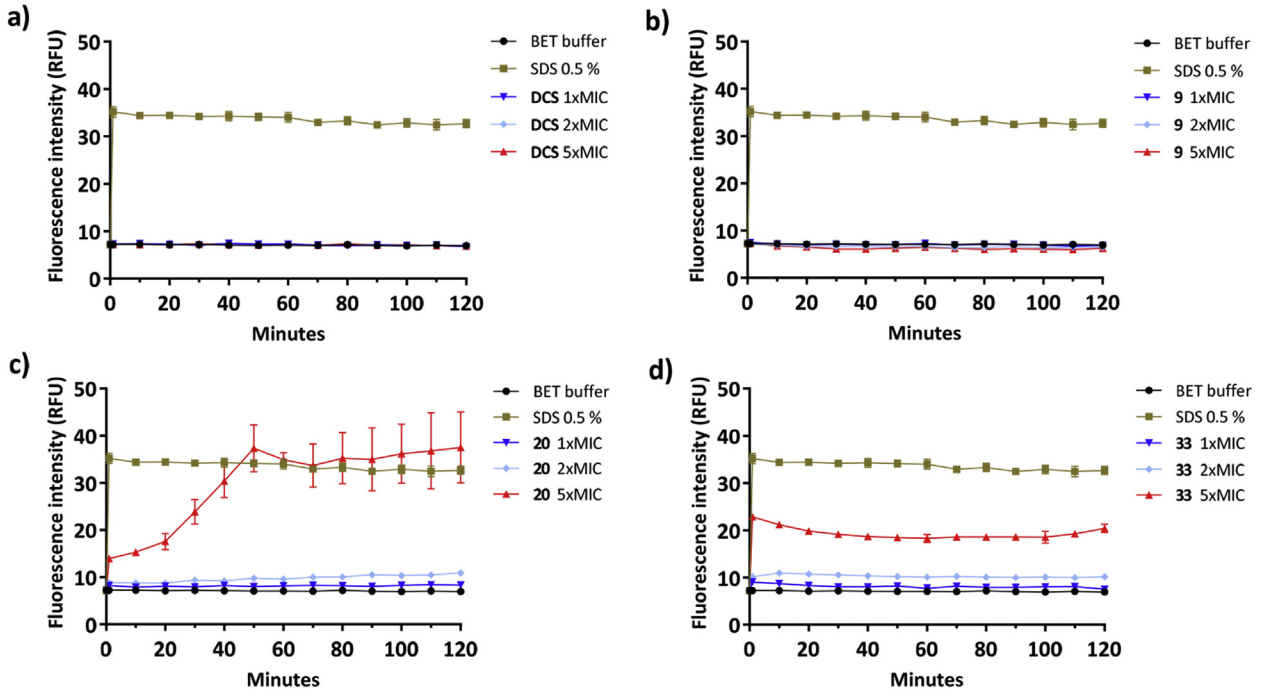


Fig. 3. Fluorescence intensity of PI as a function of time in *S. aureus* ATCC 25923 cells exposed to **a)** reference antibiotic D-cycloserine; **b)** compound **9**; **c)** compound **20**; and **d)** compound **33** at 1 × the MIC (▼), 2 × the MIC (◆), and 5 × the MIC (▲), compared to BET buffer (●) as negative control and SDS 0.5% (■) as positive control. All values are presented as the (mean ± SD) of measures performed in triplicate.

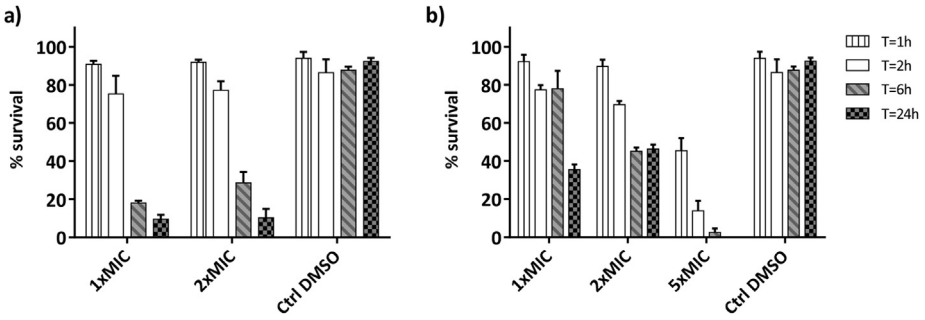


Fig. 4. Cytotoxicity on THP-1 human monocytic cell line. Percentage of cell survival as a function of time (1 h, 2 h, 6 h and 24 h) and **a)** thiotriazole **20** concentration (400 μM = 1 × MIC and 800 μM = 2 × MIC) compared to the control (DMSO 1.5%); **b)** thiosemicarbazone **33** concentration (12.5 μM = 1 × MIC, 25 μM = 2 × MIC and 62.5 μM = 5 × MIC) compared to the control (DMSO 1.5%). All values are presented as the (mean ± SD) of measures performed in triplicate.

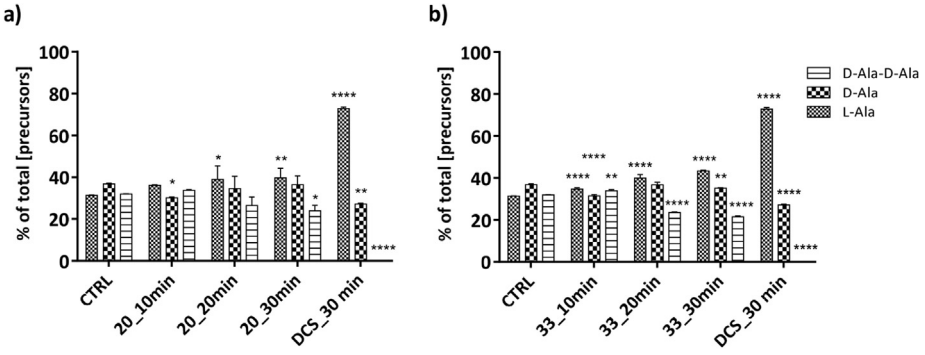


Fig. 5. Evolution over time of *S. aureus* L-Ala, D-Ala and D-Ala-D-Ala levels in presence of 2 × MIC of **a)** the thiotriazole **20** and **b)** the thiosemicarbazone **33**, compared to the control (DMSO 5%) and the reference antibiotic, DCS (2 × MIC). All values are presented as the (mean ± SD) of measures performed in triplicate. A multiple comparison (two-way ANOVA) led to the following statistical results: ****P value < 0.0001, ***P value from 0.0001 to 0.001, **P value from 0.001 to 0.01, *P value from 0.01 to 0.05, no asterisk means P value ≥ 0.05.

[55,56]. From this experiment we can conclude that the 1,2,4-thiotriazole-3-thione **20** and the thiosemicarbazone **33** exert, at least in part, their antibacterial activity through Ddl inhibition *in bacterio*.

3. Conclusions and perspectives

In the present work, five novel series of thiosemicarbazide analogues namely 1,2,4-thiotriazole-3-thiones, 1,3,4-thiadiazoles, phenylthiosemicarbazones, diacylthiosemicarbazides and thioureas were synthesized via straightforward procedures, tested against Ddl, and susceptible or resistant bacterial strains. As a result of these assays, the thiosemicarbazone and thiotriazole scaffolds were identified to potently inhibit Ddl, particularly when substituted by a 2-hydroxy group as R_1 and lipophilic substituents (4-F or 3,4-diCl) as R_2.

Results from the MIC determination revealed that the thiosemicarbazone **33** is the more potent antimicrobial of this study with values of 3.12–6.25 μM (1.06–2.10 μg/mL) against VRE strains and 12.5–25.0 μM (4.25–8.50 μg/mL) towards MRSA and VRSA strains. These promising data on strains resistant to a large variety of usual antibiotics, but also on strains that express alternative ligases, sets the stage for the development of new antimicrobial agents potentially less sensitive to resistance mechanisms.

The two most promising compounds **20** and **33** were selected for additional mechanistic studies. Both of them demonstrated a bactericidal affect at 5 × MIC after 7 h and 24 h, respectively, without any sign of bacterial membrane disruption at 1 × MIC or 2 × MIC, while the parent thiosemicarbazide **9** showed no permeabilization of the membrane at 5 × MIC either. These compounds were then proven to target Ddl *in bacterio*.

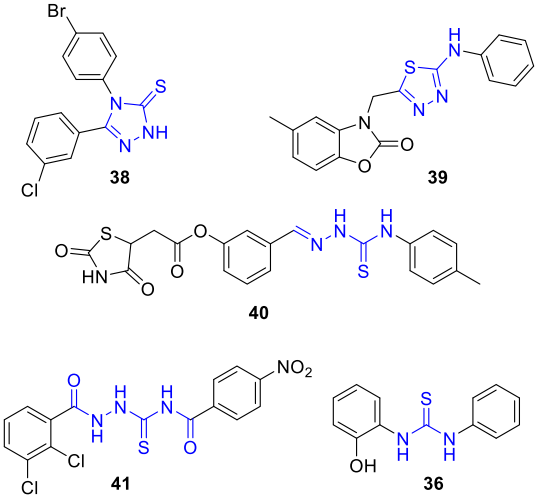


Fig. 6. Compounds **38**, **39**, **40**, **41** and metal complexes of **36**, bearing studied scaffolds, are described in the literature for their antimicrobial potencies.

Besides providing novel Ddl inhibitors with promising antibacterial potency, our works also shed light on one of the potential mechanisms-of-action of antibacterial compounds (Fig. 6) that have been previously reported but whose target are actually unknown [57–65].

This work suggests that compounds **38**, **39**, **40**, **41** from Fig. 6 may partly exert their antimicrobial potency by inhibition of Ddl, whereas the mechanism of action of **36** is yet to be elucidated, as it did not exhibit any activity on Ddl.

4. Experimental section

4.1. Chemistry

All reagents were purchased from chemical suppliers if commercially available and used without purification. Syntheses were performed under atmospheric pressure unless specified otherwise. Thin-layer chromatography (TLC) was performed using silica gel 60 F254 plates, with observation under UV. The ^{1}H and ^{13}C Nuclear Magnetic Resonance spectra were recorded respectively on an AVANCE II 400 MHz or 100 MHz Bruker spectrometer with $CDCl_3$ (residual internal $CHCl_3$ $\delta_H = 7.26$) or DMSO-d_6 (residual internal DMSO $\delta_H = 2.50$ ppm) as solvent. All coupling constants are measured in hertz (Hz), and the chemical shifts (δ_H and δ_C) are quoted in parts per million (ppm) relative to TMS (δ_0), which was used as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, br = broad, m = multiplet), coupling constant (Hz) and integration. Labile protons are not always visible in ^{1}H NMR spectrum. Melting points were measured on an Electrothermal IA9000 apparatus. High-resolution mass spectroscopy was carried out on an LTQ-Orbitrap XL hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Data were acquired in positive ion mode using full-scan MS with a mass range of 100–1000 *m/z*. The orbitrap operated at 30.000 resolution (FWHM definition). All experimental data were acquired using daily external calibration prior to data acquisition. Appropriate tuning of the electrospray ion source was done. The following electrospray inlet conditions were applied: flow rate, 100 μL min^{-1}; spray voltage, 5 kV; sheath gas (N_2) flow rate, 20 a.u.; auxiliary gas (N_2) flow rate, 10 a.u.; capillary temperature, 275 °C; capillary voltage, 45 V; tube lens, 80 V. High performance liquid chromatography analyses were performed on a Agilent (1100 series) HPLC-MS single quadrupole (InfinityLab ESI+) system equipped with a Zorbax SB-C18 5 μm (150 × 4.6 mm) and UV–vis detector. The purity of the products was determined along two methods, one using an elution gradient of 5–95% in acetonitrile in 6 min (reading at 254 nm) and the other in 9 min (reading at 210 nm). The HPLC purities of the final compounds that underwent biological assessment were ≥95%.

4.1.1. General procedure for the preparation of benzohydrazides precursors (*1*–*5*)

The benzohydrazides **2** and **3** were obtained according to known procedures [34,66] and benzohydrazides **1**, **4** and **5** were commercially available. A solution of the methyl benzoate (1 equiv) in ethanol was added dropwise to 65% hydrazine monohydrate (5 equiv). The reaction mixture was then heated under reflux and stirred overnight. The reaction progress was followed up by TLC. Crude product was collected by filtration after cooling of the reaction medium and finally washed with cold ethanol unless specified otherwise. The desired benzohydrazides were used without any further purification.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.2. General procedure for the preparation of benzoylthiosemicarbazides (*6*–*16*)

A series of benzoylthiosemicarbazides **6**–**16** were prepared according to a procedure adapted from the literature [32,34,35] by adding an isothiocyanate (1 equiv) dropwise to a solution of benzohydrazide (1 equiv) in methanol. The reaction mixture was stirred at room temperature or reflux if specified and its progress was followed by TLC. The precipitate was then collected by filtration, washed with cold ethanol and then recrystallized from ethanol as many times as necessary to obtain a pure product unless specified otherwise.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.3. General procedure for the preparation of 1,2,4-thiotriazoles-3-thiones (**17**–**25**).

A series of 1,2,4-thiotriazoles-3-thiones **17**–**25** was prepared according to the literature [37] by dissolving appropriate thiosemicarbazides **6**–**16** (1 equiv) in a solution of sodium hydroxide 2% (4 equiv) and heating that mixture to reflux. The reaction progress was followed by TLC. The solution was then neutralized with hydrochloric acid (3 M–0.1 M) after cooling in ice bath and the obtained precipitate was filtered and washed with distilled water. The resulting products were crystallized from ethanol.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.4. General procedure for the preparation of 1,3,4-thiadiazoles (**26**–**28**).

1,3,4-Thiadiazoles **26**–**28** were prepared according to a procedure adapted from the literature [38,39] by dissolving corresponding thiosemicarbazides **7**–**9** in ethanol and concentrated sulfuric acid. This solution was then heated to reflux and the reaction progress followed by TLC. After completion, this mixture was poured into ice-cold distilled water. The obtained precipitate was filtered and washed with potassium carbonate and distilled water. Pure product was obtained after recrystallization from ethanol.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.3. General procedure for the preparation of thiosemicarbazides precursors (*30*–*31*)

Thiosemicarbazide precursors **30**–**31** were prepared according to the literature [67] by adding an isothiocyanate (1 equiv) dropwise to a solution of hydrazine hydrate (5 equiv) in ethanol. The reaction mixture was stirred at room temperature and its progress was followed by TLC. The precipitate was then collected by filtration, washed with cold ethanol and then recrystallized from ethanol if necessary.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields and Mp these compounds are in accordance with the literature and presented in Supporting Information.

4.1.4. General procedure for the preparation of phenylthiosemicarbazones (*32*–*33*)

Phenylthiosemicarbazones **32** and **33** were prepared according to a procedure adapted from the literature [42] by adding salicylaldehyde (1 equiv) dropwise to a solution of thiosemicarbazide (1 equiv) in ethanol. The reaction mixture was stirred at reflux and a catalytic amount of acetic acid was added. Reaction progress was followed by TLC. After cooling of the reaction mixture, the precipitate was collected by filtration and recrystallized from ethanol followed by hot filtration.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.5. General procedure for the preparation of diacylthiosemicarbazides (**34**–**35**)

Diacylthiosemicarbazides **34** and **35** were prepared according to the literature [43] by adding benzoylchlorides (1 equiv) dropwise to a solution of potassium thiocyanate (1.5 equiv) in acetonitrile under nitrogen. The reaction mixture was stirred at 70 °C and its progress was followed by TLC. The obtained precipitate was filtered to afford a yellow filtrate. Salicylhydrazide (1 equiv) was then added to the filtrate and the reaction mixture was further stirred at 70 °C until completion. After cooling of the reaction mixture, the precipitate was collected by filtration and washed with cold diethyl ether.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.6. General procedure for the preparation of phenylthioureas (**36**–**37**)

Phenylthioureas **36** and **37** were prepared according to a procedure adapted from the literature [68] by adding isothiocyanates (1 equiv) dropwise to a solution of 2-aminophenol (1 equiv) in methanol. The reaction mixture was stirred at room temperature and its progress was followed by TLC. After completion of the reaction, the solution was evaporated under reduced pressure to afford an oil that solidifies as it cools. Pure thioureas were obtained after recrystallization from ethanol.

The analysis of spectral data (^{1}H and ^{13}C NMR), the yields, HRMS, Mp and R_f of these compounds are presented in Supporting Information.

4.1.7. Single-crystals X-ray diffraction of compounds **18** and **26**

Single crystals of compounds **18** and **26** were obtained by slow evaporation from a solution of EtOH/DMSO and MeOH/DMSO respectively. Data were collected using an Oxford Diffraction Gemini Ultra R diffractometer (Cu Kα radiation, fine-focus sealed tube, multilayer mirror). The data were integrated using the CrysAlisPro software [69]. The structures were solved by SIR92 and SHELXT [70], and refined by full-matrix least squares on $|F|^2$ using SHELXL-2018/3 [71], shelXLe [72], and Olex2 software [73]. Non-hydrogen atoms were refined anisotropically; in most of the cases, hydrogen atoms were located from the difference Fourier map but placed on calculated positions (except involved in hydrogen bonding) in riding mode with equivalent isotropic temperature factors fixed at 1.2 times U_{eq} of the parent atoms (1.5 times U_{eq} for methyl groups). These structures were deposited at the Cambridge Structural Database, CCDC deposition numbers 1980940–1980941.

Crystal Data for **18**: $C_{16}H_{15}N_3S_2Cl_2O$ (M = 400.33 g/mol), monoclinic, space group $P2_1/c$ (no. 14), a = 13.1785(3) Å, b = 6.30305(16) Å, c = 22.2129(7) Å, β = 91.858(3)°, V = 1844.14(9) Å^3, Z = 4, T = 295(2) K, μ(Cu Kα) = 5.356 mm^{-1}, D_{calc} = 1.442 g/cm^3, 10025 reflections measured (7.964° ≤ 2θ ≤ 134.222°), 3291 unique (R_{int} = 0.0330, R_{sigma} = 0.0286) which were used in all calculations. The final R_1 was 0.0388 (I > 2σ(I)) and wR_2 was 0.1045 (all data).

Crystal Data for **26**: $C_{14}H_9Cl_2N_3S$ (M = 322.20 g/mol), monoclinic, space group $P2_1/n$ (no. 14), a = 13.2580(4) Å, b = 5.8066(2) Å, c = 18.3029(5) Å, β = 99.790(3)°, V = 1388.52(7) Å^3, Z = 4, T = 293(2) K, μ(Cu Kα) = 5.541 mm^{-1}, D_{calc} = 1.541 g/cm^3, 15078 reflections measured (7.65° ≤ 2θ ≤ 134.186°), 2459 unique (R_{int} = 0.0534, R_{sigma} = 0.0317) which were used in all calculations. The final R_1 was 0.0365 (I > 2σ(I)) and wR_2 was 0.0957 (all data).

4.2. Biology

All the graphs were obtained with *GraphPad Prism 6* software (San Diego, CA). The reagents used for the enzymatic assay (BIOMOL® Green reagent, phosphate standard 800 μM) were purchased from Enzo Life Sciences, Inc (Farmingdale, NY). The UV spectra were recorded at room temperature on a Spectramax® M2E spectrophotometer (Molecular Devices, LLC, Sunnyvale, CA) in 96-well plates. The enzyme used was *Enterococcus faecalis* polyHis-DdlB produced and purified by our care. *E. faecalis* BM 4390, *E. faecalis* JH2-2:C1 and *E. faecalis* BM 4575 were received from Prof Patrice Courvalin, Institut Pasteur, Paris, France. *S. aureus* MU 50, VRS-1 and *S. aureus* NRS 119 were obtained from the NARSA (Network on Antimicrobial Resistance in Staphylococcus Aureus), BEI Resources, Manassas, VA. *S. aureus* SA 325 and *S. aureus* SA 481 were received from Prof. Peter Appelbaum, Hershey Medical Center, Hershey, PA. PI was purchased from Thermo Fisher Scientific (Waltham, MA). D-Ala, L-Ala and Marfey's reagent (1-fluoro-2,4-dinitripheny-L-5-alanine amide) were purchased from TCI Europe N.V. (Zwijndrecht, Belgium) and D-Ala-D-Ala from Fluorochem Ltd (Derbyshire, UK). Solvent used in LC-MS runs had the quality required for such analyses. LC–MS/MS was performed on a UHPLC system (Acquity H-Class, Waters) coupled to a tandem-quadrupole mass spectrometer (Xevo TQ-S, Waters). All chromatographic separations were achieved on an Acquity UPLC® BEH C18 column (1.7 μm, 2.1 mm × 50 mm, Waters, Milford, Massachusetts) equipped with an inline filter. All centrifuge operations were performed on an Eppendorf 5810R refrigerated centrifuge.

4.2.1. Ddl-His$_6$ enzymatic assay

The production and purification of Ddl-His$_6$ enzyme were performed as reported previously by our group [27,32]. Colorimetric malachite green method was used to monitor the activity of DdlB by measuring orthophosphate generated during the reaction [45]. Compounds were first evaluated at 100 μM in triplicate to assess their activity on DdlB. In brief, enzyme, inhibitor and ATP were pre-incubated (30 min, 30 °C) in assay buffer (20 mM Tris.HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM KCl) before addition of the substrate, final volume 50 μL. After 20 min of incubation (30 °C), 100 μL of Biomol® Green reagent were added and absorbance was read at 650 nm subsequent to 25 min in the dark. Substrates and enzyme concentrations were as follows: 500 μM ATP, 1 mM D-Ala and 20 mg/L of purified DdlB. The final concentration of DMSO in the assay mixture was set to 10%.

For compounds showing significant inhibitory activity at 100 μM with respect to a similar assay without the inhibitor, IC_{50} values were determined under similar conditions at 11 different concentrations. These compounds were also tested with the addition of 0.01% Triton X-100 to eliminate potential promiscuous inhibitors.

4.2.2. Microbiological evaluation

4.2.2.1. Antimicrobial activities. MICs were determined by microdilution method in cation-adjusted Muller-Hinton broth (CAMHB) (Becton-Dickinson, NJ, USA), following the recommendations of the US Clinical and Laboratory Standards Institute (CLSI) [74], using a 10^6 bacteria/mL inoculum and final concentration of 2.5% DMSO (proved not to impair bacterial growth). Compounds were prepared in a two-fold dilution series in CAMHB with 5% DMSO (Sigma-Aldrich), and diluted with the same volume of bacterial suspension. Maximal concentrations used were 0.1 mM for compounds **6**–**7**, **9**–**10**, **13**–**15**, **18**, **26**–**28** and **33**–**35**, 0.4 mM for compounds **11**–**12**, **16**–**17**, **22**–**23**, **29**, **32**, and **37**, and 0.8 mM for compounds **8**, **19**, **20**–**21**, **24**–**25**, **36**. Microwell plates with 96 wells were then incubated for 18–24 h at 37 °C. MIC was lowest concentration of potential antimicrobial agent that prevented the visible growth of bacteria [23,27].

4.2.2.2. Time-kill studies. Time-kill curves were performed

according to CLSI method [75]. Briefly *S. aureus* ATCC 25923 was grown overnight in CAMHB and then centrifuged for 7 min at 4000 rpm. Cell pellet was resuspended in medium to obtain 2.10^6 CFU/mL. Compounds **20** and **33** were then added to bacterial suspension to obtain a starting inoculum of 10^6 CFU/mL with 5% DMSO (5% DMSO alone was tested in parallel) at a final concentration of 1, 2 or 5 times their MIC. Aliquots (20 μL) of the cultures were removed at 0 min, 5 min, 15 min, 30 min, 1 h, 2 h, 7 h and 24 h of incubation. A series of 10-fold dilutions were prepared in phosphate buffer saline (PBS) and plated on tryptic soy agar (TSA) containing charcoal (2 g/L). The number of viable cells on TSA was determined after 24 h of incubation at 37 °C. The rate of killing was determined by calculating the reduction of viable bacteria ($\log_{10}$ CFU/mL) at different sampling times for all the inhibitor concentrations. Bactericidal activity is defined as a $\geq$3-$\log_{10}$ reduction of the initial CFU amount in 24 h.

4.2.2.3. Membrane permeabilization assay. The permeabilization of membrane was evaluated according to the literature [52,53] by measuring the fluorescence intensity after exposure to compounds **8**, **20**, **33** and reference antibiotic D-cycloserine, in presence of propidium iodide (PI), at a final concentration of 1, 2 or 5 times their MIC. Fluorescence was detected when PI entered the bacteria through damaged cell membrane and bound to DNA. The protocol from Li et al. [53] was adapted to use BET buffer (NaCl 110 mM; KCl 7 mM; NH_4Cl 40 mM; Na_2HPO_4 0.4 mM; Tris base 62 mM; Glucose 0.2%; pH 7.5 adjusted with HCl) rather than PBS containing 10% LB medium due to an increased fluorescence when bacteria were suspended in that buffer and treated only with PI. Briefly, *S. aureus* ATCC 25923 was grown overnight at 37 °C in MHB and then centrifuged for 7 min at 4000 rpm. Cell pellet was resuspended in same medium to reach an OD_{600} of 0.05 and the culture was subsequently grown to mid logarithmic phase. The bacterial suspension was harvested, washed and suspended in BET buffer to obtain 1.11×10^6 CFU/mL. Aliquots of 10 μL of compounds in DMSO 50% were deposited in 96-wells plate and 90 μL of bacterial suspension containing PI (12 nM) were added to reach concentrations of 1, 2 and 5 × MIC. The final concentration of 5% DMSO was proved not to impact membrane integrity. SDS 0.5% (v/v in buffer) was used as a positive control to disrupt cell membrane and BET buffer as a negative control. The increase in fluorescence was monitored in the dark spectrophotometrically every 10 min during 2 h with an excitation and emission wavelengths of 532 nm and 620 nm respectively.

4.2.2.4. Determination of toxicity for mammalian cells. Cell viability was determined on human THP-1 cells by use of the Trypan blue exclusion assay [76]. Human myelomonocytic THP-1 cells (ATCC TIB-202) [77] were cultivated in RPMI-1640 medium supplemented with 10% fetal calf serum (Gibco/Life Technologies Corporation (Paisley, UK)) as described previously [78]. Compounds **20** and **33** were added to cell suspension to obtain final concentrations of 1, 2 and 5 times their MIC for *S. aureus* ATCC 25923 1% DMSO. Compound **20** was not soluble in these conditions at 5 × MIC. The medium was incubated at 37 °C in a 5% CO_2 atmosphere and aliquots (50 μL) were removed at 1 h, 2 h, 6 h and 24 h. Trypan blue solution 0.4% (Gibco/Life Technologies Corporation (Paisley, UK)) was then added in a 1:1 (v:v) proportion to the cell suspension. After 5 min of incubation with the dye, the percentage of dead cells was calculated as the number of cells stained in blue *vs* the total number of cells as counted using optical microscopy.

4.2.2.5. LC-MS/MS determination of L-Ala, D-Ala and D-Ala-D-Ala levels in bacterio. Detailed procedure for the determination of L-Ala, D-Ala and D-Ala-D-Ala levels was described previously [32]. *S. aureus* ATCC 25923 was grown overnight at 37 °C in MHB 5% DMSO under shaking. The bacterial suspension was then centrifuged and cell pellet was suspended in MHB 5% DMSO until an OD_{600} of 0.05. Compounds **20** and **33** were then added to 30 mL-portions of the actively growing log-phase culture to a final concentration of 2 × MIC (0.8 mM and 0.025 mM respectively) so that the incubation time ranges from 0 to 30 min. Control cultures were grown without antibiotic (same levels of peptidoglycan precursors were detected after 30 min than for T_0) or with 2 × MIC of DCS (0.63 mM) after 30 min. All flask were cooled in an ice/water bath after incubation and three 10 mL-aliquots of each were centrifuged at 4 °C for 10 min at 4000 rpm. Cell pellet were then washed/re-pelleted three times with 400 μL of M9 minimal medium (Na_2HPO_4 30 g/L, KH_2PO_4 15 g/L, NH_4Cl 5 g/L and NaCl 2.5 g/L) and finally suspended in 100 μL of M9 minimal medium. 400 μL of ice-cold lysis solvent (MeOH/H_2O/formic acid, 80:20:0.1 v/v) were then added and this solution was occasionally vortexed on ice during 5–10 min before centrifugation. Supernatants (~500 μL) were then collected on ice and subsequently derivatized in triplicate (45 μL-samples) with Marfey's reagent, according to a procedure described in the literature [54].

Author contributions

Conceived and designed the experiments: AA, LP, RF, FVB. Compounds synthesis and characterization: AA, EY. Enzymatic assays: AA, BES. Biological activities: AA, GW. UHPLC-MS/MS experiments: AA, LP. X-ray structure determination: JW. Manuscript preparation: AA, RF, FVB. All authors have given approval to the final version of the manuscript.

Declaration of competing interestCOI

The authors declare no competing financial interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112444.

ABBREVIATIONS

EtOAc	ethyl acetate
Alr	alanine racemase
CAMHB	cation-adjusted Muller-Hinton broth
CA-MRSA	community-acquired MRSA
CFU	colony-forming unit
CIP	ciprofloxacin
DCS	D-cycloserine
Ddl	D-alanyl-D-alanine ligase
DD-ligases	D-Ala-D-Ala, D-Ala-D-Lac and D-Ala-D-Ser ligases
GlcNAc	N-acetylglucosamine
HA-MRSA	hospital-acquired MRSA
LZD	linezolid

MurNAc	N-acetylmuramic acid
NMDA	N-methyl-D-aspartate
Ph	Phenyl
PE	petroleum ether
S	solubility
SAR	structure-activity relationship
StaDDl	*S. aureus* Ddl
TPSA	topological polar surface area
TSA	tryptic soy agar
VAN	vancomycin
VISA	vancomycin intermediate resistant *S. aureus*
VRE	vancomycin resistant enterococcus
VRSA	vancomycin resistant *S. aureus*

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Supporting Information

Pharmacomodulations of the benzoyl-thiosemicarbazide scaffold revealed antimicrobial agents inhibiting D-Ala-D-Ala ligase *in bacterio*.

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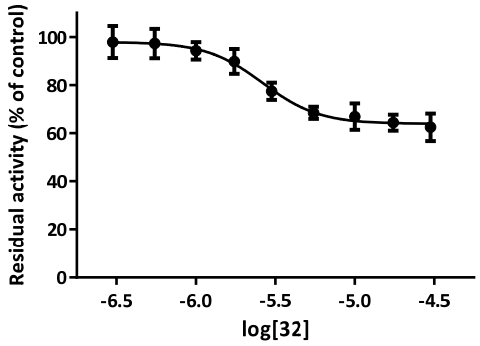
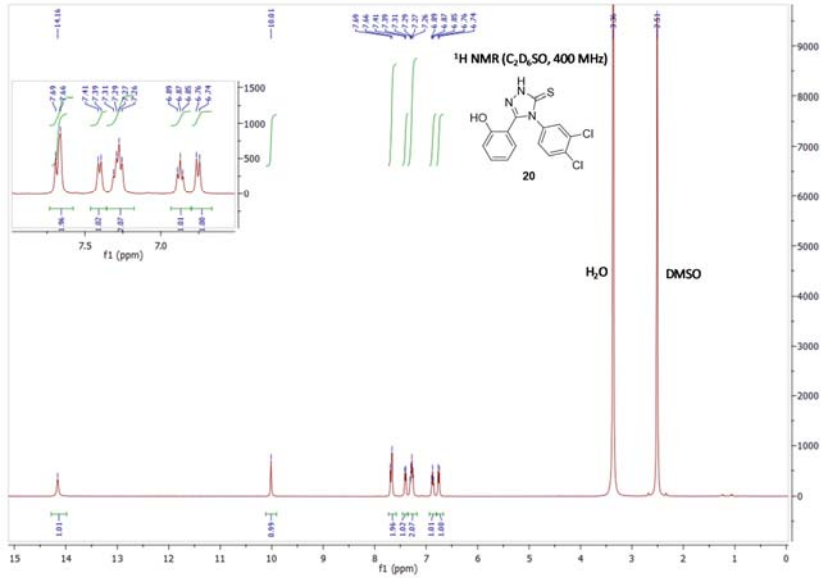
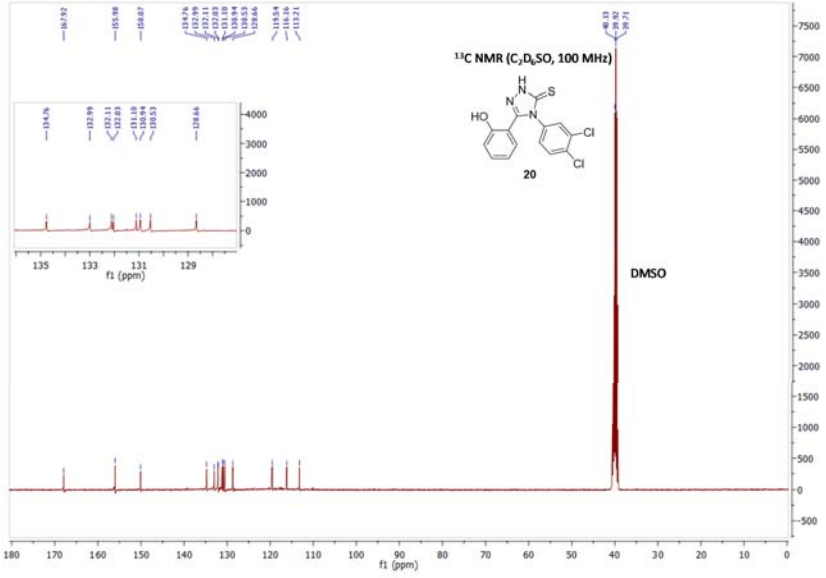
Dose-response curve of phenylthiosemicarbazone **32** illustrating the incomplete inhibition of this compound. This experiment was performed in triplicate, n=2. The other compounds that exhibited no complete inhibition of the enzyme had a similar profile, with minimal residual activity of about 60 %.

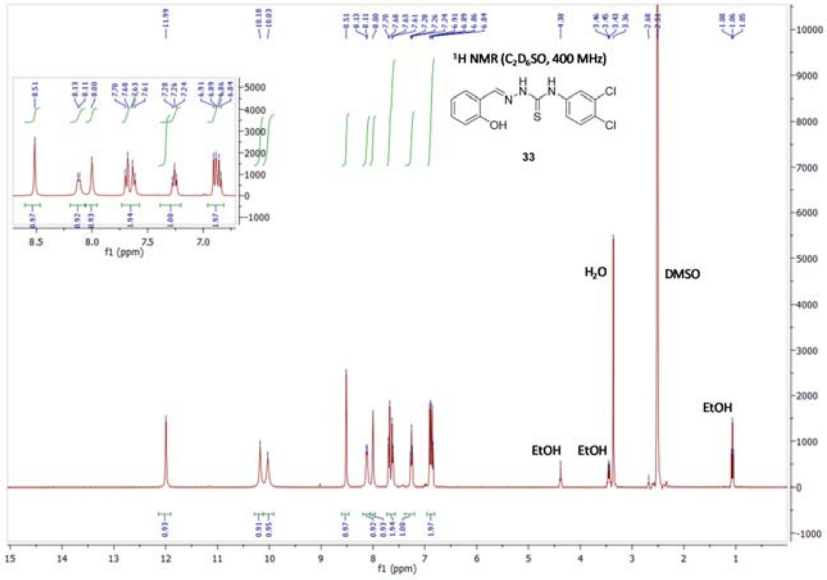


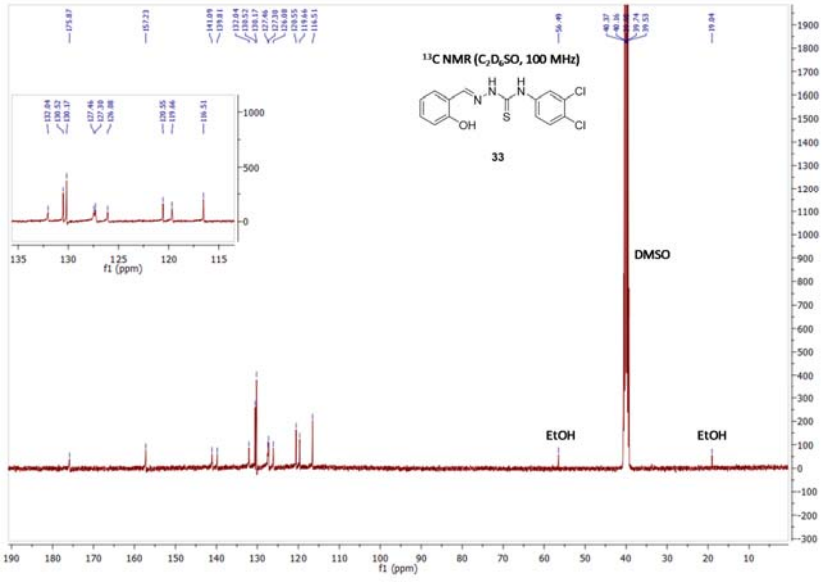
^{1}H and ^{13}C NMR spectra of the representative thiotriazole **20**.





^{1}H and ^{13}C NMR spectra of the representative phenylthiosemicarbazone **33**.





Experimental data of benzohydrazides 2-3.

*2-Hydroxybenzohydrazide (**2**).*[1] This compound was synthesized according to the general procedure described above using commercial methyl-2-hydroxybenzoate (1 equiv, 1.52 g, 10.0 mmol), hydrazine hydrate (5 equiv, 2.50 g, 50.0 mmol) in ethanol (10.0 mL). Reaction mixture was heated under reflux and stirred overnight. The reaction progress was followed up by TLC. After 22 h this mixture was evaporated under reduce pressure to afford a brown oil which precipitates after trituration with brine. A white solid was collected after filtration (1.02 g, 67 %). The title compound was used for the following syntheses without any further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 4.68 (brs, 2H, NH_2), 6.83-6.93 (m, 2H, ArH), 7.34-7.41 (m, 1H, ArH), 7.81 (dd, J = 1.6 Hz, J = 8.0 Hz, 1H, ArH), 10.10 (s, 1H, NH), 12.48 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 115.6 (Ar), 118.0 (Ar), 118.3 (Ar), 128.1 (Ar), 133.5 (Ar), 160.7 (Ar), 168.0 (C=O). HRMS (APCI$^+$): *m/z* calcd for $C_7H_9N_2O_2$ $(M+H)^+$ 153.06585, found 153.06545.

*2-Methoxybenzohydrazide (**3**).*[1] This compound was synthesized according to the general procedure described above using commercial methyl-2-methoxybenzoate (5.00 g, 30.0 mmol), hydrazine hydrate (7.53 g, 150 mmol) in ethanol (11.0 mL) except that no precipitate was formed after cooling the reaction mixture. After 2.5 h this mixture was evaporated under reduce pressure to afford a yellow oil which was extracted 3 times with Et_2O/AcOEt. The organic layers were dried over Na_2SO_4 and evaporated under reduce pressure. White crystals were obtained overnight from the resulting oil (4.03 g, 81 %). The title compound was used for the following syntheses without any further purification. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 3.86 (s, 3H, OCH_3), 4.51 (brs, 2H, NH_2), 7.02 (td, J = 0.8 Hz, J = 7.5 Hz, 1H, ArH), 7.11 (d, J = 8.3 Hz, 1H, ArH), 7.45 (ddd, J = 1.0 Hz, J = 1.8 Hz, J = 8.5 Hz, 1H, ArH), 7.68 (dd, J = 1.8 Hz, J = 7.6 Hz, 1H, ArH), 9.20 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 55.7 (OCH_3), 111.8 (Ar), 120.4 (Ar), 122.3 (Ar), 130.1 (Ar), 132.0 (Ar), 156.8 (Ar), 164.7 (C=O).

Experimental data of thiosemicarbazides 6-16.

*1-Benzoyl-4-phenyl-3-thiosemicarbazide (**6**).*[2] This compound was synthesized according to the general procedure using commercial benzohydrazide **1** (0.54 g, 4.00 mmol) and phenyl isothiocyanate (0.54 g, 4.00 mmol) in methanol (20.0 mL). After 17 h of reaction, the pure product was collected as white needles (0.82 g, 76 %). R_f 0.30 (PE/EtOAc 1:1). Mp: 162.6-163.1°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 7.17 (dd, *J* = 7.3 Hz, 1H, ArH), 7.34 (dd, *J* = 7.7 Hz, 2H, ArH), 7.45 (m, 2H, ArH), 7.51 (dd, *J* = 7.5 Hz, 2H, ArH), 7.59 (dd, *J* = 7.3 Hz, 1H, ArH), 7.97 (d, *J* = 7.4 Hz, 2H, ArH), 9.73 (s, 1H, NH), 9.83 (brs, 1H, NH), 10.56 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 125.2 (Ar), 125.8 (Ar), 128.0 (Ar), 128.3 (Ar), 131.9 (Ar), 132.6 (Ar), 139.1 (Ar), 166.0 (C=O), 181.1 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{14}N_3OS$ $(M+H)^+$ 272.08521, found 272.08499.

*1-Benzoyl-4-(3,4-dichlorophenyl)-3-thiosemicarbazide (**7**).*[3] This compound was synthesized according to the general procedure using commercial benzohydrazide **1** (0.48 g, 3.50 mmol) and 3,4-dichlorophenyl isothiocyanate (0.72 g, 3.50 mmol) in methanol (30.0 mL). After 4 h of reaction, the pure product was collected as white powder (0.88 g, 73 %). R_f 0.62 (PE/EtOAc 1:1). Mp: 198.5-199.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 7.48-7.56 (m, 3H, ArH), 7.57-7.62 (m, 2H, ArH), 7.82 (s, 1H), 7.96 (d, *J* = 7.6 Hz, 2H), 9.92 (brs, 1H, NH), 9.99 (brs, 1H, NH), 10.60 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ_C (ppm) 125.8 (Ar), 127.0 (Ar), 128.0 (Ar), 128.4 (Ar), 129.8 (Ar), 130.1 (Ar), 132.1 (Ar), 132.4 (Ar), 139.5 (Ar), 166.1 (C=O), 181.0 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{12}Cl_2N_3OS$ $(M+H)^+$ 340.00726, found 340.00731.

*1-(2-Hydroxybenzoyl)-4-phenyl-3-thiosemicarbazide (**8**).*[4] This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.29 g, 1.93 mmol) and phenyl isothiocyanate (0.26 g, 1.93 mmol) in methanol (20.0 mL). After 7 h of reaction, the pure product was collected as a white powder (0.16 g, 28 %). R_f 0.75 (PE/EtOAc

4:6). Mp: 187.8-190.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.92-7.01 (m, 2H, Ar), 7.17 (dd, *J* = 7.4 Hz, 1H, ArH), 7.37 (dd, *J* = 7.8 Hz, 2H, ArH), 7.44-7.56 (m, 3H, Ar), 7.91 (d, *J* = 5.6 Hz, 1H, Ar), 9.7-10.5 (m, 1.7H and 0.3H, NH), 10.6-11.5 (brs, 0.7H and 0.3H, NH), 11.91 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ_C (ppm) 115.1 (Ar), 117.1 (Ar), 118.9 (Ar), 125.1 (Ar), 125.8 (Ar), 128.1 (Ar), 128.8 (Ar), 134.0 (Ar), 139.1 (Ar), 159.4 (Ar-OH), 168.9 (C=O), 180.9 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{14}N_3O_2S$ $(M+H)^+$ 288.08012, found 288.08008.

*4-(3,4-Dichlorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide (**9**).*[5] This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.43 g, 2.55 mmol) and 3,4-dichlorophenyl isothiocyanate (0.52 g, 2.55 mmol) in methanol (10.0 mL). After 24 h of reaction, the pure product was collected as white needles (0.56 g, 63 %). R_f 0.19 (PE/EtOAc 4:6). Mp: 200.0-201.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.91-7.02 (m, 2H, ArH), 7.47 (ddd, *J* = 7.6 Hz, *J* = 0.8 Hz, 1H, ArH), 7.54 (dd, *J* = 8.8 Hz, *J* = 2.1 Hz, 1H, ArH), 7.60 (d, *J* = 9.2 Hz 1H, ArH), 7.80-7.96 (m, 2H, ArH), 9.90-10.30 (m, 2H, NH), 10.5-11.5 (brs, 1H, NH), 11.88 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-*d*$_6$): δ_C (ppm) 120.1 (Ar), 122.5 (Ar), 124.1 (Ar), 130.7 (Ar), 132.0 (Ar), 132.1 (Ar), 134.0 (Ar), 135.1 (Ar), 135.3 (Ar), 139.5 (Ar), 144.6 (Ar), 164.7 (Ar-OH), 174.0 (C=O), 186.1 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{12}Cl_2N_3O_2S$ $(M+H)^+$ 356.00218, found 356.00235.

*4-(4-Fluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide (**10**).*[6] This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.40 g, 2.38 mmol) and 4-fluorophenyl isothiocyanate (0.36 g, 2.38 mmol) in methanol (10.0 mL). After 5 h 30 of reaction, the pure product was collected as white powder without recrystallization (0.46 g, 63 %). R_f 0.25 (PE/EtOAc 1:1). Mp: 184.6-185.1°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.92-7.01 (m, 2H, ArH), 7.18 (dd, *J* = 8.8 Hz, 2H, ArH), 7.37-7.53

(m, 3H, ArH), 7.91 (d, J = 8.4 Hz, 1H, ArH), 9.89 (m, 2H, NH), 10.6-11.1 (m, 1H, NH), 11.92 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 114.7 (d, J = 25.0 Hz, Ar), 117.2 (Ar), 118.9 (Ar), 127.2 (Ar), 127.9 (Ar), 128.8 (Ar), 134.1 (Ar), 135.4 (Ar), 159.4 (d, J = 242.0 Hz, Ar C-F), 159.5 (Ar-OH), 168.9 (C=O), 181.3 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{13}FN_3O_2S$ $(M+H)^+$ 306.07070, found 306.07074.

*4-(3,4-Difluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide (**11**).* This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.50 g, 3.30 mmol) and 3,4-difluorophenyl isothiocyanate (0.57 g, 3.30 mmol) in methanol (10.0 mL). After 17 h of reaction, the pure product was collected as white powder (0.36 g, 34 %). R_f 0.15 (PE/EtOAc 1:1). ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.86-7.05 (m, 1H, ArH), 7.26 (d, J = 8.7 Hz, 1H, ArH), 7.31-7.43 (m, 1H, ArH), 7.46 (dd, J = 7.2 Hz, 1H, ArH), 7.65 (brs, 1H, ArH), 7.89 (d, J = 3.4 Hz, 1H , ArH), 9.73-10.23 (m, 1.7H, NH), 10.49 (s, 0.3H, NH), 10.72 (s, 0.7H, NH), 11.19 (s, 0.3H, NH), 11.88 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm): 115.4 (Ar), 117.7 (Ar), 119.4 (dd, J = 8.7, 3.1 Hz, Ar), 122.8 (Ar), 129.3 (Ar), 134.6 (Ar), 136.5 (dd, J = 8.7, 3.0 Hz, Ar), 136.7 (Ar), 147.2 (d, J = 236.6 Hz, Ar-F), 148.9 (d, J = 253.7 Hz, Ar-F), 159.9 (Ar-OH), 169.4 (C=O), 181.4 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{12}F_2N_3O_2S$ $(M+H)^+$ 324.06128, found 324.06073.

*4-(2,3,4-Trifluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide (**12**).* This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.27 g, 1.77 mmol) and 2,3,4-trifluorophenyl isothiocyanate (0.33 g, 1.77 mmol) in methanol (10.0 mL). After 18 h of reaction, the pure product was collected as white powder (0.32 g, 53 %). R_f 0.70 (PE/EtOAc 1:1). Mp: 188.0-189.7°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.88-7.03 (m, 2H, ArH), 7.10-7.23 (m, 1H, ArH), 7.32 (dd, J = 17.1 Hz, 8.3 Hz, 1H, ArH), 7.46 (dd, J = 7.5 Hz, 1H, ArH), 7.90 (d, J = 7.2 Hz, 1H, ArH), 9.81 (brs, 1H, NH), 10.16 (s, 1H, NH), 10.50-11.35 (m, 1H, NH), 11.91 (s, 1H, OH). ^{13}C NMR (100 MHz,

DMSO-d_6): δ_C (ppm): 111.9 (d, J = 19.3 Hz, Ar), 115.0 (Ar), 117.7 (Ar), 119.2 (Ar), 125.5 (d, J = 10.5 Hz, Ar), 125.7 (Ar), 129.1 (Ar), 134.8 (Ar), 139.8 (d, J = 232.6 Hz, Ar-F), 147.2 (d, J = 251.7 Hz, Ar-F), 149.2 (d, J = 257.4 Hz, Ar-F), 160.2 (Ar-OH), 169.3 (C=O), 182.9 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{11}F_3N_3O_2S$ $(M+H)^+$ 342.05186, found 342.05161.

*4-(4-Trifluoromethylphenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide (**13**).* This compound was synthesized according to the general procedure using synthesized 2-hydroxybenzohydrazide **2** (0.53 g, 3.50 mmol) and 4-trifluoromethylphenyl isothiocyanate (0.71 g, 3.50 mmol) in ethanol (20.0 mL) at reflux. After 6 h of reaction, the pure product was collected as small white needles (0.67 g, 54 %). R_f 0.33 (PE/EtOAc 8:2). Mp: 186.0-188.2°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.81-7.08 (m, 2H, ArH), 7.46 (dd, J = 7.5 Hz, 1H, ArH), 7.60-8.07 (m, 5H, ArH), 9.81-10.44 (m, 1.7H, NH), 10.75 (brs, 1H, NH), 11.30 (brs, 0.3H, NH), 11.87 (brs, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm): 115.7 (d, J = 30.1 Hz, Ar), 117.7 (Ar), 119.4 (Ar), 122.4 (Ar), 123.4 (Ar), 125.6 (Ar), 126.1 (Ar), 129.3 (Ar), 134.6 (Ar), 143.4 (Ar), 159.9 (Ar-OH), 169.5 (C=O), 181.6 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{15}H_{13}F_3N_3O_2S$ $(M+H)^+$ 356.06751, found 356.06718.

*4-(3,4-Dichlorophenyl)-1-(2-methoxybenzoyl)-3-thiosemicarbazide (**14**).*[1] This compound was synthesized according to the general procedure described above using synthesized 2-methoxybenzohydrazide **3** (3.06 g, 18.4 mmol) and 3,4-dichlorophenyl isothiocyanate (3.76 g, 18.4 mmol) in ethanol (10.0 mL) at reflux. After 1 h of reaction, pure product was collected by filtration as a white solid after washing with hot ethanol (4.75 g, 70 %). R_f 0.27 (PE/EtOAc 1:3). Mp: 197.2-198.3°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 3.93 (s, 3H, OCH_3), 7.09 (dd, J = 7.4 Hz, 1H, ArH), 7.2 (d, J = 8.2 Hz, 1H, ArH), 7.47-7.64 (m, 3H, ArH), 7.79-8.12 (m, 2H, ArH), 9.71 (brs, 0.7H, NH), 9.78-10.59 (m, 2H, NH), 11.00 (brs, 0.3H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 56.5 (OCH_3), 112.7 (Ar), 121.1 (Ar), 121.2 (Ar), 125.9 (Ar), 127.1 (Ar), 130.5 (Ar), 131.5 (Ar), 133.8 (Ar), 139.9 (Ar), 157.8 (Ar-OCH_3), 166.0 (C=O),

181.4 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{15}H_{14}Cl_2N_3O_2S$ $(M+H)^+$ 370.01783, found 370.01758.

*1-((2-Hydroxy-5-methoxy)benzoyl)-4-phenyl-3-thiosemicarbazide (**15**).* This compound was synthesized according to the general procedure using commercially available 2-hydroxy-5-methoxybenzohydrazide **4** (0.45 g, 2.47 mmol) and phenyl isothiocyanate (0.33 g, 2.47 mmol) in ethanol (20.0 mL) at reflux. After 4 h 30 of reaction, the pure product was collected as white powder (0.49 g, 63 %). R_f 0.25 (PE/EtOAc 1:1). Mp: 207.1-208.9°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 3.75 (s, 3H, OCH_3), 6.91 (d, *J* = 8.9 Hz, 1H, ArH), 7.08 (dd, *J* = 8.9, 2.7 Hz, 1H, ArH), 7.17 (dd, *J* = 7.3 Hz, 1H, ArH), 7.34 (dd, *J* = 7.7 Hz, 2H, ArH), 7.39-7.66 (m, 3H, ArH), 9.67-10.07 (m, 1,7H, NH), 10.39 (brs, 0.3H, NH), 10.72 (brs, 0.7H, NH), 11.24 (brs, 0.3H, NH), 11.46 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 56.1 (OCH_3), 112.5 (Ar), 115.4 (Ar), 118.5 (Ar), 121.8 (Ar), 125.6 (Ar), 126.3 (Ar), 128.6 (Ar), 139.6 (Ar), 152.1 (Ar-OH), 153.9 (Ar-OCH_3), 169.2 (C=O), 181.4 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{15}H_{16}N_3O_3S$ $(M+H)^+$ 318.09069, found 318.09014.

*1-(2-Aminobenzoyl)-4-phenyl-3-thiosemicarbazide (**16**).*[7] This compound was synthesized according to the general procedure using commercial 2-aminobenzohydrazide **5** (0.45 g, 3.00 mmol) and phenyl isothiocyanate (0.41 g, 3.00 mmol) in ethanol (20.0 mL) at reflux. After 4 h 30 of reaction, the pure product was collected as white powder (0.58 g, 67 %). R_f 0.28 (PE/EtOAc 1:1). Mp: 158.7-160.1°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.53 (dd, *J* = 7.5 Hz, 1H, ArH), 6.63 (brs, 2H, NH_2), 6.73 (d, *J* = 8.2 Hz, 1H, ArH), 7.09-7.23 (m, 2H, ArH), 7.33 (dd, *J* = 7.7 Hz, 2H, ArH), 7.37-7.56 (m, 2H, ArH), 7.70 (d, *J* = 7.1 Hz, 1H, ArH), 9.58 (s, 1H, NH), 9.77 (m, 2H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm): 114.8 (Ar), 116.8 (Ar), 125.4 (Ar), 126.5 (Ar), 128.4 (Ar), 129.4 (Ar), 129.4 (Ar), 133.0 (Ar), 139.8 (Ar), 150.9 (Ar), 169.2 (C=O), 181.8 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{14}H_{15}N_4OS$ $(M+H)^+$ 287.09611, found 287.09551.

Experimental data of 1,2,4-thiotriazoles-5-thiones 17-25 and compound 29.

*4,5-Diphenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**17**).*[8] This compound was synthesized according to the general procedure using synthesized 1-benzoyl-4-phenyl-3-thiosemicarbazide **6** (0.50 g, 1.84 mmol). After 7 h of reaction, the pure product was collected as white needles (0.35 g, 75 %). R_f 0.72 (PE/EtOAc 1:1). Mp: 288.4-293.1°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 7.26-7.36 (m, 6H, ArH), 7.40 (ddd, *J* = 6.9 Hz, 2.5 Hz, 1H, ArH), 7.44-7.51 (m, 3H, ArH), 14.13 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 125.8 (Ar), 128.2 (Ar), 128.5 (Ar), 128.7 (Ar), 129.3 (Ar), 129.4 (Ar), 130.3 (Ar), 134.5 (Ar), 150.5 (-C=N), 168.6 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{14}H_{12}N_3S$ $(M+H)^+$ 254.07464, found 254.07381.

*4-(3,4-Dichlorophenyl)-5-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**18**).*[9] This compound was synthesized according to the general procedure using synthesized 1-benzoyl-4-(3,4-dichlorophenyl)-3-thiosemicarbazide **7** (0.72 g, 2.11 mmol). After 5 h 30 of reaction, the pure product was collected as white needles (0.46 g, 68 %). R_f 0.76 (PE/EtOAc 1:1). Mp: 255.2-257.2°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 7.32-7.47 (m, 6H, ArH), 7.77 (d, *J* = 8.6 Hz, 1H, ArH), 7.85 (d, *J* = 2.3 Hz, 1H, ArH), 14.21 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO-d_6): δ_C (ppm) 123.7 (Ar), 126.7 (Ar), 126.9 (Ar), 127.6 (Ar), 128.7 (Ar), 129.4 (Ar), 129.6 (Ar), 130.5 (Ar), 132.6 (Ar), 148.6 (-C=N), 166.7 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{14}H_{10}Cl_2N_3S$ $(M+H)^+$ 321.99670, found 321.99585.

*5-(2-hydroxyphenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**19**).*[8] This compound was synthesized according to the general procedure using synthesized 1-(2-hydroxybenzoyl)-4-phenyl-3-thiosemicarbazide **8** (0.60 g, 2.09 mmol). After 7 h of reaction, the pure product was collected as white needles (0.29 g, 52 %). R_f 0.57 (PE/EtOAc 1:1). Mp: 293.7-295.7°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.73 (d, *J* = 8.2 Hz, 1H, ArH), 6.80 (dd, *J* = 7.5 Hz, 1H, ArH), 7.20-7.29 (m, 3H, ArH), 7.29-7.41 (m, 4H, ArH), 9.93 (s, 1H, OH),

14.05 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 113.8 (Ar), 116.1 (Ar), 119.3 (Ar), 128.3 (Ar), 129.0 (Ar), 129.2 (Ar), 132.0 (Ar), 132.6 (Ar), 134.8 (Ar), 150.2 (-C=N), 156.3 (Ar-OH), 168.0 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{12}N_3OS$ $(M+H)^+$ 270.06956, found 270.06871.

4-(3,4-Dichlorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***(20)***. This compound was synthesized according to the general procedure using synthesized 4-(3,4-dichlorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **9** (1.00 g, 2.81 mmol). After 22 h of reaction, the pure product was collected as pale pink powder (0.32 g, 33 %). R_f 0.55 (PE/EtOAc 1:2). Mp: 271.9-274.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.76 (d, *J* = 8.2 Hz, 1H, ArH), 6.87 (dd, *J* = 7.4 Hz, 1H, ArH), 7.21-7.33 (m, 2H, ArH), 7.40 (d, *J* = 7.5 Hz, 1H, ArH), 7.61-7.77 (m, 2H, ArH), 10.04 (s, 1H, OH), 14.16 (brs, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 113.2 (Ar), 116.2 (Ar), 119.5 (Ar), 128.7 (Ar), 130.5 (Ar), 130.9 (Ar), 131.1 (Ar), 132.0 (Ar), 132.1 (Ar), 133.0 (Ar), 134.8 (Ar), 150.1 (-C=N), 156.0 (Ar-OH), 167.9 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}Cl_2N_3OS$ $(M+H)^+$ 337.99161, found 337.99101.

4-(4-Fluorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***(21)***.[10] This compound was synthesized according to the general procedure using synthesized 4-(4-fluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **10** (0.50 g, 1.64 mmol). After 8 h of reaction, the pure product was collected as white powder (0.37 g, 78 %). R_f 0.62 (PE/EtOAc 1:2). Mp: 255.3-257.6°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.74 (d, *J* = 8.2 Hz, 1H, ArH), 6.83 (dd, *J* = 7.5 Hz, 1H, ArH), 7.18-7.29 (m, 3H, ArH), 7.29-7.38 (m, 3H, ArH), 9.94 (s, 1H, OH), 14.06 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 113.6 (Ar), 115.9 (d, *J* = 23.1 Hz, Ar), 116.1 (Ar), 119.4 (Ar), 130.6 (d, *J* = 9.1 Hz, Ar), 131.1 (d, *J* = 2.9 Hz, Ar), 132.0 (Ar), 132.7 (Ar), 150.3 (-C=N), 156.2 (Ar-OH), 162.1 (d, *J* = 245.9 Hz, Ar-F), 168.1 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{11}FN_3OS$ $(M+H)^+$ 288.06014, found 288.05950.

4-(3,4-Difluorophenyl)-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***(22)***. This compound was synthesized according to the general procedure using synthesized 4-(3,4-difluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **11** (0.34 g, 1.04 mmol). After 4 h of reaction, the pure product was collected as white powder (0.11 g, 35 %) after column chromatography (cyclohexane/AcOEt elution gradient from 8:2 to 6:4). R_f 0.16 (PE/EtOAc 7:3). Mp: 263.8-265.5°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.75 (d, *J* = 8.2 Hz, 1H, ArH), 6.85 (dd, *J* = 7.4 Hz, 1H, ArH), 7.12 (d, *J* = 8.8 Hz, 1H, ArH), 7.28 (dd, *J* = 7.6, 1.4 Hz, 1H, ArH), 7.37 (dd, *J* = 7.4, 0.8 Hz, 1H, ArH), 7.40-7.60 (m, 2H), 9.98 (s, 1H, OH), 14.12 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 113.3 (Ar), 116.2 (Ar), 117.8 (d, *J* = 17.4 Hz, Ar), 118.5 (dd, *J* = 17.8, 2.3 Hz, Ar), 119.5 (Ar), 125.8 (dd, *J* = 6.7, 3.4 Hz, Ar), 131.5 (dd, *J* = 8.7, 3.7 Hz, Ar), 132.1 (Ar), 132.9 (Ar), 149.0 (dd, *J* = 248.1, 15.5 Hz, Ar-F), 149.8 (dd, *J* = 250.8, 14.5 Hz, Ar-F), 150.2 (-C=N), 156.1 (Ar-OH), 168.1 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}F_2N_3OS$ $(M+H)^+$ 306.05072, found 306.04999.

4-(3,4-Dichlorophenyl)-5-(2-methoxyphenyl)-2,4-dihydro-3H-1,2,4-triazole-3-thione ***(23)***. This compound was synthesized according to the general procedure using synthesized 4-(3,4-dichlorophenyl)-1-(2-methoxybenzoyl)-3-thiosemicarbazide **14** (1.00 g, 2.70 mmol). After 3 h of reaction, the pure product was collected as white powder (0.71 g, 75 %). R_f 0.28 (PE/EtOAc 8:2). Mp: 246.2-248.4°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 3.46 (s, 3H, OCH_3), 6.94 (d, *J* = 8.3 Hz, 1H, ArH), 7.04 (dd, *J* = 7.4 Hz, 1H, ArH), 7.26 (d, *J* = 8.1 Hz, 1H, ArH), 7.46 (dd, *J* = 7.7 Hz, 1H, ArH), 7.52 (d, *J* = 7.3 Hz, 1H, ArH), 7.63 (s, 1H, ArH), 7.67 (d, *J* = 8.6 Hz, 1H, ArH), 14.21 (brs, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 55.5 (OCH_3), 111.8 (Ar), 114.7 (Ar), 121.1 (Ar), 128.7 (Ar), 130.5 (Ar), 130.9 (Ar), 131.2 (Ar), 132.1 (Ar), 132.2 (Ar), 133.4 (Ar), 134.7 (Ar), 149.8 (-C=N), 157.0 (OCH_3), 168.0 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{15}H_{12}Cl_2N_3OS$ $(M+H)^+$ 352.00726, found 352.00638.

*5-(2-Hydroxy-5-methoxyphenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**24**).* This compound was synthesized according to the general procedure using synthesized 1-((2-hydroxy-5-methoxy)benzoyl)-4-phenyl-3-thiosemicarbazide **15** (0.25 g, 0.78 mmol). After 15 h of reaction, the pure product was collected as white powder (0.09 g, 38 %). R_f 0.55 (PE/EtOAc 1:1). Mp: 275.5-246.7°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 3.62 (s, 3H, OCH_3), 6.65 (d, J = 8.9 Hz, 1H, ArH), 6.84 (dd, J = 8.9, 3.0 Hz, 1H, ArH), 6.90 (d, J = 2.7 Hz, 1H, ArH), 7.29 (d, J = 6.6 Hz, 2H, ArH), 7.33-7.46 (m, 3H, ArH), 9.43 (s, 1H, OH), 14.04 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 55.9 (OCH_3), 113.8 (Ar), 116.3 (Ar), 117.0 (Ar), 118.5 (Ar), 128.4 (Ar), 129.0 (Ar), 129.2 (Ar), 134.8 (Ar), 150.0 (Ar-OH), 150.1 (-C=N), 151.9 (Ar-OCH_3), 168.0 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{15}H_{14}N_3O_2S$ $(M+H)^+$ 300.08012, found 300.07953.

*5-(2-Aminophenyl)-4-phenyl-2,4-dihydro-3H-1,2,4-triazole-3-thione (**25**).*[11] This compound was synthesized according to the general procedure using synthesized 1-(2-aminobenzoyl)-4-phenyl-3-thiosemicarbazide **16** (0.50 g, 1.75 mmol). After 17 h of reaction, the pure product was collected as small white needles (0.32 g, 69 %). R_f 0.50 (PE/EtOAc 1:1). Mp: 258.0-260.2°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 5.54 (s, 2H, NH_2), 6.34 (dd, J = 7.4 Hz, 1.6 Hz, 1H, ArH), 6.61 (d, J = 8.2 Hz, 1H, ArH), 6.81 (d, J = 7.6 Hz, 1H, ArH), 7.02 (dd, J = 7.7 Hz, 1H, ArH), 7.32 (dd, J = 7.8 Hz, 1.6 Hz, 2H, ArH), 7.36-7.47 (m, 3H, ArH), 14.02 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 109.0 (Ar), 115.3 (Ar), 115.6 (Ar), 128.8 (Ar), 129.2 (Ar), 129.3 (Ar), 131.0 (Ar), 131.5 (Ar), 135.06 (Ar), 148.18 (Ar), 150.18 (-C=N), 168.25 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{14}H_{13}N_4S$ $(M+H)^+$ 269.08554, found 269.08485.

*7,8-Difluorodibenzo[b,f][1,2,4]triazolo[4,3-d][1,4]oxazepine-3(2H)-thione (**29**).* This compound was synthesized according to the general procedure using synthesized 4-(2,3,4-trifluorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **12** (0.25 g, 0.74 mmol). After 19

h of reaction, the pure product was collected as small white needles (0.07 g, 27 %). R_f 0.78 (PE/EtOAc 1:1). Mp: 326-328°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 7.40-7.64 (m, 3H, ArH), 7.70 (dd, J = 5.5 Hz, 1H, ArH), 7.87 (d, J = 5.5 Hz, 1H, ArH), 8.35 (d, J = 3.0 Hz, 1H, ArH), 14.61 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 113.8 (d, J = 18.4 Hz, ArH), 114.6 (Ar), 119.1 (Ar), 121.6 (d, J = 10.8 Hz, Ar), 125.2 (d, J = 3.4 Hz, Ar), 127.8 (Ar), 129.8 (Ar), 134.4 (Ar), 142.0 (d, J = 16.5 Hz, Ar-O), 143.2 (d, J = 239.0 Hz, Ar-F), 147.7 (-C=N), 149.9 (d, J = 249.7 Hz, Ar-F), 157.6 (Ar-O), 167.1 (C=S). HRMS (ESI^+): *m/z* calcd for $C_{14}H_9F_3N_3OS$ $(M+H)^+$ 304.03507, found 304.03486.

Experimental data of 1,3,4-thiadiazoles 26-28.

*2-(3,4-Dichlorophenylamino)-5-phenyl-2,4-dihydro-3H-1,3,4-thiadiazole (**26**).*[5] This compound was synthesized according to the general procedure using synthesized 1-benzoyl-4-(3,4-dichlorophenyl)-3-thiosemicarbazide **7** (0.25 g, 0.73 mmol). After 1 h of reaction, the pure product was collected as pale yellow powder (0.19 g, 82 %). R_f 0.49 (PE/EtOAc 1:1). Mp: 247.0-249.3°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 7.44-7.55 (m, 4H, ArH), 7.58 (d, *J* = 8.8 Hz, 1H, ArH), 7.79-7.96 (m, 2H, ArH), 8.12 (s, 1H, ArH), 10.85 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 117.7 (Ar), 118.6 (Ar), 123.2 (Ar), 126.9 (Ar), 129.3 (Ar), 130.0 (Ar), 130.5 (Ar), 130.9 (Ar), 131.3 (Ar), 140.3 (Ar), 158.6 (HN-C=N), 163.4 (Ph-C=N). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}Cl_2N_3S$ $(M+H)^+$ 321.99670, found 321.99686.

*5-(2-Hydroxyphenyl)-2-phenylamino-2,4-dihydro-3H-1,3,4-thiadiazole (**27**).*[5] This compound was synthesized according to the general procedure using synthesized 1-(2-hydroxybenzoyl)-4-phenyl-3-thiosemicarbazide **8** (0.50 g, 1.74 mmol). After 45 min of reaction, the pure product was collected as pale yellow needles (0.21 g, 45 %). R_f 0.65 (PE/EtOAc 1:2). Mp: 215.6-217.9°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.89-7.11 (m, 3H, ArH), 7.27-7.43 (m, 3H, ArH), 7.67 (d, *J* = 7.2 Hz, 2H, ArH), 8.04 (d, *J* = 7.2 Hz, 1H, ArH), 10.38 (s, 1H, OH), 11.00 (brs, 1H, NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 116.9 (Ar), 117.4 (Ar), 117.8 (Ar), 120.2 (Ar), 122.2 (Ar), 127.6 (Ar), 129.6 (Ar), 131.6 (Ar), 141.2 (Ar), 154.0 (HN-C=N), 154.41 (Ar-OH), 165.4 (Ph-C=N). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{12}N_3OS$ $(M+H)^+$ 270.06956, found 270.06886.

*2-(3,4-Dichlorophenylamino)-5-(2-hydroxyphenyl)-2,4-dihydro-3H-1,3,4-thiadiazole (**28**).*[5] This compound was synthesized according to the general procedure using synthesized 4-(3,4-dichlorophenyl)-1-(2-hydroxybenzoyl)-3-thiosemicarbazide **9** (0.50 g, 1.40 mmol). After 1 h of reaction, the pure product was collected as beige powder (0.26 g, 56 %). R_f 0.45 (PE/EtOAc 1:1). Mp: 279.0-281.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.78 (dd, *J* = 7.3 Hz, 1H, ArH), 6.97 (d, *J* = 8.2 Hz, 1H, ArH), 7.24 (dd, *J* = 7.6 Hz, 1H, ArH), 7.50 (dd, *J* = 8.8 Hz, 2.4 Hz, 1H, ArH), 7.57 (d, *J* = 8.8 Hz, 1H, ArH), 8.05 (dd, *J* = 7.8 Hz, 1.3 Hz, 1H, ArH), 8.19 (d, *J* = 2.4 Hz, 1H, ArH), 11.89 – 9.89 (m, 2H, OH and NH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 117.1 (Ar), 117.2 (Ar), 117.4 (Ar), 117.5 (Ar), 118.4 (Ar), 122.4 (Ar), 126.5 (Ar), 130.8 (Ar), 131.3 (Ar), 131.3 (Ar), 141.0 (Ar), 154.6 (HN-C=N), 157.4 (Ar-OH), 164.2 (Ph-C=N). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{10}Cl_2N_3OS$ $(M+H)^+$ 337.99161, found 337.99149.

Experimental data of thiosemicarbazides precursors (30-31).

*4-Phenylthiosemicarbazide (**30**).*[12] This compound was synthesized according to the general procedure using commercial phenyl isothiocyanate. Yield 87 %. Mp: 137.0-139.2°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 4.84 (brs, 2H, NH_2), 7.14 (dd, J = 7.4 Hz, 1H, Ar), 7.34 (dd, J = 7.6 Hz, 2H, ArH), 7.69 (d, J = 6.2 Hz, 2H, ArH), 9.18 (s, 1H, NH), 9.71 (brs, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 123.5 (Ar), 124.0 (Ar), 128.0 (Ar), 139.3 (Ar), 179.3 (C=S).

*4-(3,4-Dichlorophenyl)thiosemicarbazide (**31**).*[13] This compound was synthesized according to the general procedure using commercial 3,4-dichlorophenyl isothiocyanate with a recrystallization step. Yield 76 %. Mp: 167.2-170.0°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 5.35 (brs, 2H, NH_2), 7.54 (d, J = 8.5 Hz, 1H, ArH), 7.70 (d, J = 6.8 Hz, 1H, ArH), 8.20 (s, 1H, ArH), 9.41 (s, 1H, NH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 123.5 (Ar), 124.6 (Ar), 125.5 (Ar), 129.6 (Ar), 129.9 (Ar), 139.6 (Ar), 179.1 (C=S).

Experimental data of phenylthiosemicarbazones (32-33).

*Salicylaldehyde-4(N)-phenylthiosemicarbazone (**32**).*[14] This compound was synthesized according to the general procedure using synthesized 4-phenylthiosemicarbazide **30** (0.35 g, 2.08 mmol) in EtOH (20.0 mL). After 3 h of reaction, the pure product was collected as pale yellow needles (0.27 g, 48 %). R_f 0.50 (PE/EtOAc 1:1). Mp: 183.0-184.8°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.84 (dd, J = 7.5 Hz, 1H, ArH), 6.89 (d, J = 8.1 Hz, 1H, ArH), 7.18-7.30 (m, 2H, ArH), 7.37 (dd, J = 7.7 Hz, 2H), 7.57 (d, J = 7.8 Hz, 2H), 8.10 (d, J = 7.1 Hz, 1H), 8.49 (s, 1H, CH=N), 9.98 (brs, 1H, NH), 10.06 (s, 1H, NH), 11.78 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 116.5 (Ar), 119.7 (Ar), 120.7 (Ar), 125.7 (Ar), 126.2 (Ar), 127.5 (Ar), 128.5 (Ar), 131.8 (Ar), 139.6 (HC=N), 157.1 (Ar-OH), 176.5 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{14}N_3OS$ $(M+H)^+$ 272.08521, found 272.08469.

*Salicylaldehyde-4(N)-(3,4-dichlorophenyl)thiosemicarbazone (**33**).* This compound was synthesized according to the general procedure using synthesized 4-(3,4-dichlorophenyl)thiosemicarbazide **31** (1.18 g, 5.02 mmol) in EtOH (10.0 mL). After 3 h 15 of reaction, the pure product was collected as pale yellow powder (0.59 g, 35 %). R_f 0.52 (PE/EtOAc 1:1). Mp: 183.9-185.1°C. ^{1}H NMR (400 MHz, DMSO-d_6): δ_H (ppm) 6.78-7.01 (m, 2H, ArH), 7.26 (dd, J = 7.4 Hz, 1H, ArH), 7.62 (d, J = 8.7 Hz, 1H, ArH), 7.69 (d, J = 8.7 Hz, 1H, ArH), 8.00 (s, 1H, ArH), 8.12 (d, J = 6.8 Hz, 1H, ArH), 8.51 (s, 1H, ArH), 10.03 (brs, 1H, NH), 10.18 (s, 1H, NH), 11.99 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO- d_6): δ_C (ppm) 116.5 (Ar), 119.7 (Ar), 120.6 (Ar), 126.1 (Ar), 127.3 (Ar), 127.5 (Ar), 130.2 (Ar), 130.5 (Ar), 132.0 (Ar), 139.8 (Ar), 141.1 (HC=N), 157.2 (Ar-OH), 175.9 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{14}H_{11}Cl_2N_3OSNa$ $(M+Na)^+$ 361.98921, found 361.98849.

Experimental data of diacylthiosemicarbazides (34-35).

*4-Benzoyl-1-(2-hydroxybenzoyl)-thiosemicarbazide (**34**).*[15] This compound was synthesized according to the general procedure using benzoyl chloride (0.46 g, 3.28 mmol) in acetonitrile (10.0 mL). After 3 h of reaction, salicylhydrazide was added to the filtrate and the reaction was stirred for another 5 h. The pure product was collected as white powder (0.98 g, 95 %). R_f 0.33 (PE/EtOAc 1:2). Mp: 222.3-224.3°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.87-7.24 (m, 2H, ArH), 7.42-7.53 (m, 1H, ArH), 7.53-7.63 (m, 2H, ArH), 7.63-7.83 (m, 1H, ArH), 7.86-8.24 (m, 3H, ArH), 11.52-12.36 (m, 3H, NH), 13.70 (brs, 1H, OH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 116.0 (Ar), 117.3 (Ar), 120.3 (Ar), 128.9 (Ar), 129.2 (Ar), 130.8 (Ar), 132.2 (Ar), 133.7 (Ar), 134.6 (Ar), 157.1 (C=O), 160.7 (Ar-OH), 169.0 (C=O), 171.6 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{15}H_{14}N_3O_3S$ $(M+H)^+$ 316.07504, found 316.07490.

*4-(3,4-Dichlorobenzoyl)-1-(2-hydroxybenzoyl)-thiosemicarbazide (**35**).* This compound was synthesized according to the general procedure using 3,4-dichlorobenzoyl chloride (0.68 g, 3.28 mmol) in acetonitrile (10.0 mL). After 2 h 30 of reaction, salicylhydrazide was added to the filtrate and the reaction was stirred for another 2 h. The pure product was collected as pale yellow powder (1.05 g, 83 %). R_f 0.34 (PE/EtOAc 1:2). Mp: 217.0-219.0°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.98-7.09 (m, 2H, ArH), 7.48 (ddd, *J* = 7.7, 1.2 Hz, 1H, ArH), 7.84 (d, *J* = 8.4 Hz, 1H, ArH), 7.95 (dd, *J* = 8.5, 1.8 Hz, 1H, ArH), 7.98 (dd, *J* = 7.8, 1.1 Hz, 1H, ArH), 8.28 (d, *J* = 1.8 Hz, 1H, ArH), 11.75-12.09 (m, 2H, NH), 12.15 (s, 1H, NH), 13.56 (brs, 1H, OH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 115.5 (Ar), 116.9 (Ar), 119.8 (Ar), 129.1 (Ar), 130.4 (Ar), 130.8 (Ar), 131.3 (Ar), 132.3 (Ar), 134.2 (Ar), 136.0 (Ar), 156.7 (C=O), 160.7 (Ar-OH), 166.3 (C=O), 170.8 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{15}H_{12}Cl_2N_3O_3S$ $(M+H)^+$ 383.99709, found 383.99681.

Experimental data of phenylthioureas (36-37).

*1-(2-Hydroxyphenyl)-3-phenylthiourea (**36**).*[16] This compound was synthesized according to the general procedure using phenyl isothiocyanate (1.24 g, 9.16 mmol) in methanol (25.0 mL). After 24 h of reaction, the pure product was collected as pale yellow crystals (0.92 g, 41 %). R_f 0.41 (PE/EtOAc 1:1). Mp: 137.3-138.7°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.79 (dd, *J* = 7.6 Hz, 1H, ArH), 6.90 (d, *J* = 8.0 Hz, 1H, ArH), 7.00 (ddd, *J* = 11.1, 4. 2 Hz, 1H, ArH), 7.15 (dd, *J* = 7.3 Hz, 1H, ArH), 7.35 (dd, *J* = 7.8 Hz, 2H, ArH), 7.56 (d, *J* = 7.8 Hz, 2H, ArH), 7.94 (d, *J* = 7.8 Hz, 1H, ArH), 9.12 (s, 1H, NH), 9.87 (s, 1H, NH), 9.98 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 115.8 (Ar), 118.9 (Ar), 124.1 (Ar), 124.9 (Ar), 125.0 (Ar), 125.9 (Ar), 125.9 (Ar), 127.1 (Ar), 128.9 (Ar), 139.8 (Ar), 150.1 (Ar-OH), 179.5 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{13}H_{13}N_2OS$ $(M+H)^+$ 245.07431, found 245.07381.

*3-(3,4-Dichlorophenyl)-1-(2-hydroxyphenyl)-thiourea (**37**).*[17] This compound was synthesized according to the general procedure using 3,4-dichlorophenyl isothiocyanate (1.87 g, 9.16 mmol) in methanol (25.0 mL). After 24 h of reaction, the pure product was collected as beige powder (1.50 g, 52 %). R_f 0.56 (PE/EtOAc 1:1). Mp: 144.2-146.5°C. ^{1}H NMR (400 MHz, DMSO-*d*$_6$): δ_H (ppm) 6.80 (dd, *J* = 7.5 Hz, 1H, ArH), 6.90 (d, *J* = 7.9 Hz, 1H, ArH), 7.03 (dd, *J* = 7.5 Hz, 1H, ArH), 7.49 (dd, *J* = 8.7, 2.1 Hz, 1H, ArH), 7.54-7.63 (m, 1H, ArH), 7.75 (d, *J* = 7.7 Hz, 1H, ArH), 8.04 (d, *J* = 1.8 Hz, 1H, ArH), 9.35 (s, 1H, NH), 9.89 (s, 1H, NH), 10.07 (s, 1H, OH). ^{13}C NMR (100 MHz, DMSO- *d*$_6$): δ_C (ppm) 116.0 (Ar), 119.0 (Ar), 123.8 (Ar), 125.0 (Ar), 126.7 (Ar), 126.5 (Ar), 126.6 (Ar), 126.7 (Ar), 130.6 (Ar), 130.8 (Ar), 140.3 (Ar), 150.8 (Ar-OH), 179.6 (C=S). HRMS (ESI$^+$): *m/z* calcd for $C_{13}H_{11}Cl_2N_2OS$ $(M+H)^+$ 312.99637, found 312.99634.

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