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Synthesis and Biological Evaluation of *N*-Acylhydrazones as Inhibitors of MurC and MurD Ligases

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Dedicated to Professor Slavko Pečar on the occasion of his 60th birthday

The Mur ligases have an essential role in the intracellular biosynthesis of bacterial peptidoglycan, and they represent attractive targets for the design of novel antibacterials. A series of compounds with an N*-acylhydrazone scaffold were synthesized and screened for inhibition of the MurC and MurD enzymes from* Escherichia coli*. Compounds with micromolar inhibitory activities against both MurC and MurD were identified, and some of them also showed antibacterial activity.*

Introduction

Multidrug-resistant (MDR) bacteria have been observed for several human pathogens, such as *Staphylococcus aureus*, *Enterococcus faecium*, and *Pseudomonas aeruginosa*. Therefore, there is an urgent need to improve the existing compounds and to develop new antimicrobial drugs in the battle against infectious diseases.[1] Cell wall biosynthesis is an essential process that is exclusive to bacteria and thus it remains an attractive target for antimicrobial action. The early cytoplasmic steps of peptidoglycan precursor synthesis, which are catalyzed by the enzymes MurA to MurF, are still relatively underexploited. Only MurA is inhibited by a known antibiotic, fosfomycin.[2]

The Mur ligases constitute a series of four ATP-dependent enzymes (MurC to MurF) that are responsible for the stepwise addition of the pentapeptide side chain onto the D-lactoyl group of the uridine diphosphate-*N*-acetylmuramic acid (UDP-MurNAc) initially formed via MurA and MurB. In *Escherichia coli*, the first amino acid of the growing peptide stem is L-Ala (MurC), followed by D-Glu (MurD), *meso*-diaminopimelic acid (MurE), and finally the D-Ala-D-Ala dipeptide (MurF). All of the Mur ligases catalyze the formation of an amide or peptide bond through the same reaction mechanism (Figure 1). First, the carboxyl group of the UDP precursor is activated by ATP, generating an acyl phosphate intermediate and ADP. Second, the nucleophilic attack of the amino group of the condensing amino acid (or dipeptide) leads to the formation of a high-energy tetrahedral intermediate, which eventually breaks down into an amide or peptide and P_i. A divalent cation, such as Mg^{2+} or Mn^{2+}, is essential for the reaction. All of the Mur ligases have similar three-dimensional structures, where the N-terminal domain is involved in the binding of the UDP precursor, the central domain in the binding of ATP, and the C-terminal domain in the binding of the incoming amino acid or dipeptide.[3,4]

There have been several attempts to inhibit MurC to MurF using substrate or transition-state analogues[5] and structure-based design,[6–10] as reviewed by El Zoeiby et al.,[11] Kotnik et al.,[12] and Barreteau et al.[4] Recently, the first crystal structures of MurD and MurF as small-molecule inhibitor complexes have been published.[13,14] However, although many inhibitors have been described, few have good antibacterial activities.

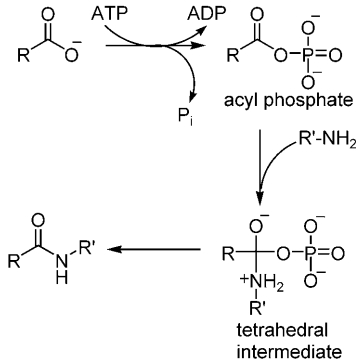

Figure 1. Reaction mechanism of the Mur ligases. First, the reaction of the carboxyl group of the UDP precursor (R-COO^-) with ATP generates an acyl phosphate intermediate and ADP. Then the acyl phosphate undergoes nucleophilic attack of the amino group of the amino acid or dipeptide (R′-NH_2) to form a tetrahedral transition state, which eventually breaks down into amide or peptide (R-CO-NH-R′) and P_i.

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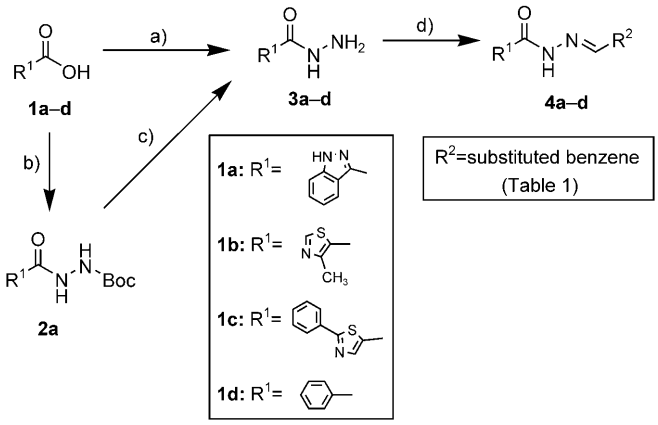
As a part of our efforts towards the discovery of new small-molecule inhibitors of the early steps of peptidoglycan biosynthesis, we screened our in-house bank of compounds for inhibitors of the MurC and MurD enzymes from *E. coli*. Some *N*-acylhydrazones were observed to inhibit these enzymes. Additionally, a literature search revealed that many *N*-acylhydrazones have antibacterial activity[15] and they have recently been identified as privileged structures in medicinal chemistry.[16] These observations prompted us to synthesize a small focused library of *N*-acylhydrazones and to evaluate their inhibition of MurC and MurD, along with the testing of their in vitro antibacterial activities.

Chemistry

We synthesized the target compounds **4a–d** according to known methods for the synthesis of *N*-acylhydrazones[15] with some modifications, as outlined in Scheme 1. The reactions between the starting carboxylic acids **1a–d** with hydrazine hydrate or *tert*-butyl 1-hydrazine carboxylate with the subsequent Boc deprotection produced the corresponding hydrazide derivatives **3a–d**. The coupling reactions were performed using *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC) and 1-hydroxybenzotriazole (HOBt) in dry *N*,*N*-dimethylformamide. Four series of hydrazides **3a–d** containing different R^1 substituents were prepared: 1*H*-indazole-3-carbohydrazides (**3a**), 4-methyl-1,3-thiazole-5-carbohydrazides (**3b**), 2-phenyl-1,3-thiazole-5-carbohydrazides (**3c**), and benzohydrazides (**3d**). Finally, condensation of hydrazides **3a–d** with variously substituted benzaldehydes gave the target *N*-acylhydrazones (**4a–d**, compounds **5**–**37** in Table 1).



Scheme 1. Synthetic route for the target *N*-acylhydrazones: a) $NH_2NH_2{\cdot}H_2O$, EDC, HOBt, *N*-methylmorpholine, DMF, room temperature; b) NH_2NH_2-Boc, EDC, HOBt, *N*-methylmorpholine, DMF, room temperature; c) CF_3COOH, CH_2Cl_2; d) substituted benzaldehydes, EtOH, reflux.

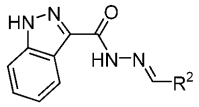
According to the literature, the *N*-acylhydrazones can exist as *Z*/*E* geometrical isomers around C=N double bonds and as *cis*/*trans* conformers around the amide bond. It has been shown that hydrazones derived from aldehydes and substituted hydrazides are present in solution in the *E* form.[17] When hydrazones are dissolved in dimethyl sulfoxide the *E* geometrical isomer of these compounds undergo a rapid *cis*/*trans* amide equilibrium, in which the *cis*-amide conformer predominates.[17] An investigation of ^{1}H NMR spectra of our target compounds demonstrated that these *N*-acylhydrazones behave similarly in [D_6]DMSO solution as far as their geometrical isomerism is concerned, and that no signals belonging to the *Z* isomer are observed. However, for most of the compounds, only the signals for *trans*-amide bond conformers were seen. The only exceptions herein were for the 4-methyl-1,3-thiazole-5-carbohydrazide-based compounds (**19**–**27**), where the ^{1}H NMR spectra showed two sets of signals between 7.96 and 8.38 ppm on the one hand, and between 8.18 and 8.51 ppm on the other, which belong to the =CH- group of the *cis*- and *trans*-amide conformers, respectively. In these compounds, the *cis*-configuration predominates. In addition, molecular energy minimization studies using Sybyl 8.0[18] indicated that compounds bearing the *E* configurations at the C=N double bond and the *trans* conformations around the amide bond have the lowest energy.

Results and Discussion

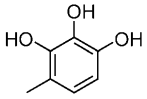
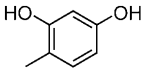
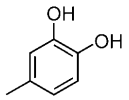
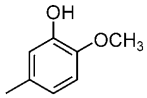
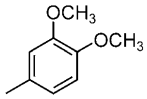
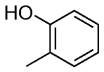
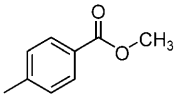
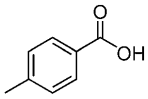
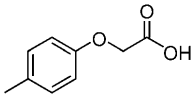
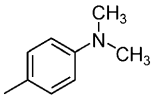
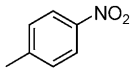
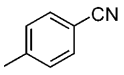
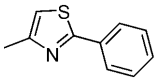
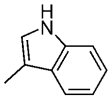
The target compounds were tested for inhibition of the MurC and MurD enzymes from *E. coli*[19] using the Malachite green assay for detection of orthophosphate generated during the reaction.[20] To exclude possible nonspecific (promiscuous) inhibition, all of the compounds were tested in the presence of detergent (Triton X-114, 0.005%).[21] All of the positive results were further verified using a secondary, radioactivity assay.[22] The results are presented as residual activities (RAs) of the enzymes in the presence of 500 μM of each compound (Table 1). For the more active compounds, IC_{50} values were also determined. Poorly soluble compounds were tested at either 250 μM or 100 μM (see Table 1).

With the systematic variation of substituents R^1 and R^2 (Scheme 1), some structure–activity relationships were deduced. We synthesized four series of potential inhibitors with variations in substituent R^1 (Table 1): 1*H*-indazole-3-carbohydrazide- (**4a**, **5**–**8**), 4-methyl-1,3-thiazole-5-carbohydrazide- (**4b**, **19**–**27**), 2-phenyl-1,3-thiazole-5-carbohydrazide- (**4c**, **28**), and benzohydrazide- (**4d**, **29**–**37**) based compounds. In the set of 1*H*-indazole-3-carbohydrazides, promising activities were obtained when the R^2 substituent is hydroxy-substituted phenyl ring (compounds **5**–**8**). The most active inhibitor is compound **5**, where R^2 is a 2,3,4-trihydroxyphenyl group, with IC_{50} values for MurC and MurD of 123 μM and 230 μM, respectively. When one hydroxy group is omitted or replaced with a methoxy group (compounds **6**–**8**), the inhibitory activities on MurC decrease and those for MurD are virtually lost. The only active compound without a hydroxy-substituted phenyl ring in this series is compound **17**, which contains the 2-phenyl-1,3-thiazole moiety. When we compared the inhibitory activities of 1*H*-indazole-3-carbohydrazides **4a** with the series **4b**–**d**, there was the same trend: the most active compounds are those where R^2 is the 2,3,4-trihydroxyphenyl ring. The combination of the 2,3,4-trihydroxyphenyl ring and the 2-phenyl-1,3-thiazole-5-car-

Table 1. Inhibitory activities of *N*-acylhydrazones toward MurC and MurD.



4a

Compd	R^2	MurC RA [%][a]	MurD RA [%][a]	MIC [μg mL^{-1}] *E. coli* 1411	*E. coli* SM1411	*S. aureus* 8325-4
5		42[b] IC_{50} = 123 μM	40[b] IC_{50} = 230 μM	128	128	128
6		74[b]	95[b]	>128	64	128
7		36 IC_{50} = 332 μM	99	>128	>128	>128
8		67[b]	86[b]	>128	>128	>128
9		100	100	>128	>128	>128
10		100[b]	100[b]	>128	>128	>128
11		100	100	>128	>128	>128
12		100	100	>128	>128	>128
13		100	100	>128	>128	>128
14		95[b]	100[b]	>128	>128	>128
15		91	100	>128	>128	>128
16		100[b]	94[b]	>128	>128	>128
17		78[c]	62[c]	>128	>128	>128
18		100[c]	100[c]	>128	>128	>128

bohydrazide moiety results in the most active inhibitor **28**, with an IC_{50} value for MurC of 32 μM.

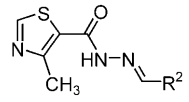
To investigate the possible binding mode of these synthesized hydrazones, the representative inhibitors **5**, **7**, **11**, and **28** were docked into the MurC and MurD active sites (PDB codes 1P3D and 3UAG, respectively), using the GOLD docking program.[23] Here, the inhibitors predominantly occupy the ATP-binding site; in addition, some of them partly occupy the binding site for the UDP precursor (Figure 2).

All of the compounds were tested for their in vitro antimicrobial activities (Table 1). The minimal inhibitory concentrations (MICs) of each compound were determined against *S. aureus* 8325-4, *E. coli* 1411, and *E. coli* SM1411 (an AcrAB-deficient derivative of 1411 that has increased susceptibility to a range of antimicrobial agents). There is a correlation between the MurC and MurD inhibitory activities and these in vitro antimicrobial activities. With the exception of **7**, all of the compounds that had substantial enzyme inhibitory activities prevented the growth of bacteria. In contrast, the inactive compounds did not prevent the growth of any of the bacterial strains under investigation, with the exception of compound **30**. Compound **6** and the best inhibitor of MurC, compound **28**, had MICs of 64 μg mL^{-1}. In addition, all of the compounds bearing the 2,3,4-trihydroxyphenyl group prevented the growth of *S. aureus*, which represents one of the major pathogenic species.

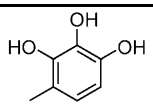
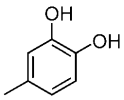
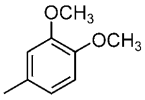
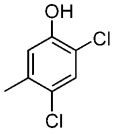
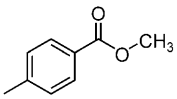
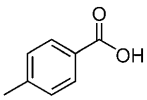
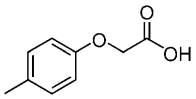
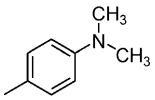
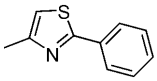
Conclusions

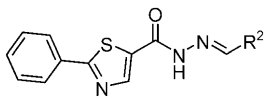
To conclude, we report herein the synthesis and inhibitory activities of a series of *N*-acylhydrazone-based compounds as a new class of MurC and MurD inhibitors. Some of these compounds were inhibitors of MurC and/or MurD with IC_{50} values in the 100–300 μM range. Compound **28** was a potent and selective inhibitor of MurC, with an IC_{50} value of 32 μM. Compound **5** significantly inhibited both of these enzymes. Additionally, a correlation between the MurC and MurD inhibitory activities and the in vitro antimicrobial activities was observed. The majority of the compounds that have significant enzyme inhibitory activities prevented the growth of bacteria. As the

Table 1. (Continued)

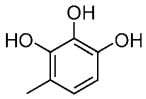


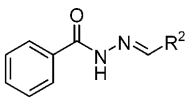
4b

19		26	76	>128	>128	128
20		95	100	>128	>128	>128
21		100	95	>128	>128	>128
22		43[b]	100[b]	>128	>128	>128
23		95	96	>128	>128	>128
24		94	95	>128	>128	>128
25		100	96	>128	>128	>128
26		97[b]	91[b]	>128	>128	>128
27		100	100	>128	>128	>128

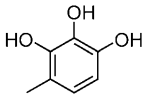
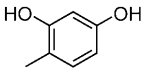
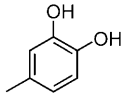


4c

28		18[b] $IC_{50}=32\ \mu M$	100[b]	>128	>128	64



4d

29		64[c]	86[c]	128	128	128
30		100	100	128	64	>128
31		100	100	>128	>128	>128

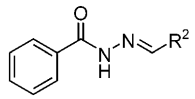
lack of antibacterial activity is the major restriction in the development of Mur ligase inhibitors, although the antimicrobial activities of our compounds are moderate, they represent promising starting points for further development.

Experimental Section

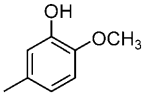
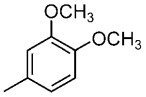
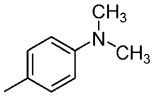
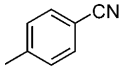
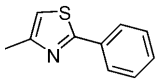
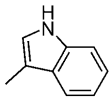
General synthesis remarks: All reagents were purchased from commercial suppliers and used without further purification, unless otherwise noted. Solvents were RP grade unless otherwise indicated. Yields refer to purified products and were not optimized. Analytical thin-layer chromatography (TLC) was performed using silica gel $60F_{254}$ pre-coated plates (0.2 mm thick) with a fluorescent indicator from Merck (Germany). Chromatographic separations were performed on silica gel columns (Kieselgel 0.063–0.200 mm; Merck, Germany). Chromatographic eluents are given as volume-to-volume ratios (*v*/*v*). Melting points were determined on a Polytherm A Heintsch apparatus and are uncorrected. The structures of the compounds were confirmed by routine spectrometric analyses. The IR spectra were recorded on a Perkin–Elmer 1600 FT IR spectrometer. ^{1}H NMR spectra were recorded on a Bruker Advance DPX_{300} NMR spectrometer at 300 MHz, and were referenced to TMS. Some assignments were supported by 2D COSY. EI and ESI analyses were carried out on a Varian-MAT311 A mass spectrometer. Elemental analyses were within $\pm 0.4\%$ of theoretical values, and were performed on a Perkin–Elmer 2400 analyzer. Only analyses for compounds not previously described are given.

***tert*-butyl 2-(1*H*-indazol-3-ylcarbonyl)-1-hydrazinecarboxylate (2a):** 1*H*-indazole-3-carboxylic acid (**1a**) (2.49 g, 15.3 mmol) and *tert*-butyl 1-hydrazinecarboxylate (2.03 g, 15.3 mmol) were dissolved in dry *N*,*N*-dimethylformamide (9.0 mL). HOBt (2.31 g, 17.1 mmol), and, after adjusting to pH 8 with *N*-methylmorpholine, EDC (3.23 g, 16.9 mmol) were added. The reac-

Table 1. (Continued)



4d

32		92	100	>128	>128	>128
33		97	95	>128	>128	>128
34		100[b]	97[b]	>128	>128	>128
35		100[b]	93[b]	>128	>128	>128
36		70[b]	90[b]	>128	>128	>128
37		93	98	>128	>128	>128

[a] RA: residual activity of the enzyme in the presence of the defined concentration of inhibitor; inhibitor concentration = 500 μM. [b] Inhibitor concentration = 250 μM. [c] Inhibitor concentration = 100 μM.

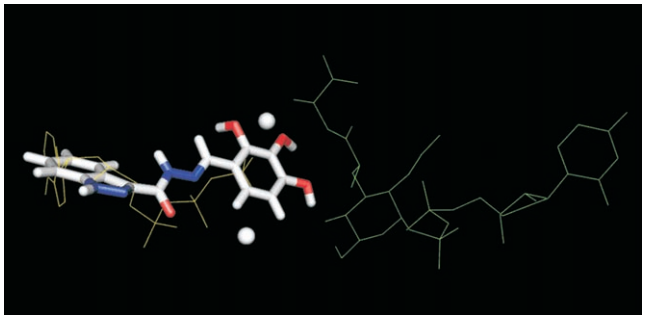


Figure 2. Superposition of the highest ranked solution of inhibitor **5**, as calculated by GOLD v 3.2, and phosphoaminophosphonic acid adenylate ester (ANP, a non-hydrolysable analogue of ATP) (in yellow), UDP-Mur*N*Ac-L-Ala (in green), and Mn^{2+} (in white), from the X-ray crystal structure of MurC (image generated with Pymol[24]).

tion mixture was stirred for 1 h at room temperature, and then held at reflux overnight. The solvent was evaporated under reduced pressure, and the residue was then partitioned between CH_2Cl_2 and saturated aqueous $NaHCO_3$. The organic layer was washed with brine, dried over Na_2SO_4, and filtered, and the solvent removed under vacuum. The crude product was washed with ethyl ether and EtOAc to give 1.40 g (74%) of a white powder; mp: 189–190 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 1.44 (s, 9H), 7.26 (ddd, J_1 = J_2 = 7.3 Hz, 1H), 7.43 (ddd, J_1 = J_2 = 7.2 Hz, 1H), 7.64 (dd, J = 8.4 Hz, 1H), 8.14 (dd, J = 8.1 Hz, 1H), 8.85 (s, 1H), 10.04 (s, 1H), 13.66 ppm (s, 1H); IR (KBr): $\tilde{\nu}$ = 3411 (NH), 1711 (C=O), 1668 cm^{-1} (C=O); FAB-MS *m/z*: 277 $[M+H]^+$; anal. calcd for $C_{13}H_{16}N_4O_3 \cdot {}^1/_2\,H_2O$ (276.29): C 54.73, H 6.01, N 19.64; found: C 54.41, H 5.58, N 20.13.

1*H*-indazole-3-carbohydrazide (3a): The Boc deprotection of compound **2a** (1.40 g, 5.07 mmol) was carried out by treatment with TFA (12 mL) in CH_2Cl_2 (9 mL) for 90 min. The solvent and acid were evaporated under reduced pressure, and the residue was then washed with ethyl ether. Water was added to the residue, the pH was adjusted to 9 with 2 M NaOH, and the residue extracted with EtOAc. The organic phases were dried (Na_2SO_4) and concentrated to give white crystals (811 mg, 90.8%); mp: 205–206 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 4.47 (s, 2H), 7.23 (ddd, J = 6.8, 7.8 Hz, 1H), 7.41 (ddd, J = 6.8, 8.3 Hz, 1H), 7.60 (dd, J = 8.4 Hz, 1H), 8.15 (dd, J = 8.2 Hz, 1H), 9.53 (s, 1H), 13.49 ppm (s, 1H); IR (KBr): $\tilde{\nu}$ = 3319 (NH), 1632 (C=O), 1543 cm^{-1} (C=O); FAB-MS *m/z*: 176 $[M+H]^+$.

4-methyl-1,3-thiazole-5-carbohydrazide (3b): 4-methyl-1,3-thiazole-5-carboxylic acid (**1b**) (0.50 g, 3.49 mmol) was dissolved in dry *N*,*N*-dimethylformamide (10 mL) and cooled to 0 °C. Hydrazine hydrate (0.32 mL, 6.60 mmol) and HOBt (0.57 mg, 4.19 mmol) were added to the solution, the pH was adjusted to pH 8 with *N*-methylmorpholine, and EDC (0.87 g, 4.54 mmol) was then added. The reaction mixture was stirred under an argon atmosphere at 0 °C for 3 h. The solvent was evaporated under reduced pressure. Purification of the residue by column chromatography (CH_2Cl_2/MeOH = 15/1) gave white crystals (0.49 g, 89%): mp: 160–162 °C (lit: 166 °C[25]); ^{1}H NMR ([D$_6$]DMSO): δ = 2.56 (s, 3H), 4.51 (s, 2H), 9.02 (s, 1H), 9.50 ppm (s, 1H); IR (KBr): $\tilde{\nu}$ = 3310 (NH), 1654 (C=O), 1623 cm^{-1} (C=O); FAB-MS *m/z*: 158 $[M+H]^+$.

2-phenyl-1,3-thiazole-5-carbohydrazide (3c): Compound **3c** was synthesized (31%) using the same procedure described for **3b**, starting from 2-phenyl-1,3-thiazole-5-carboxylic acid (**1c**) and hydrazine hydrate. Beige solid; mp: 128–130 °C (lit: 137–138 °C[26]); ^{1}H NMR ([D$_6$]DMSO): δ = 4.56 (s, 2H), 7.51–5.56 (m, 3H), 8.04–8.10 (m, 2H), 8.27 (s, 1H), 9.74 ppm (s, 1H); IR (KBr): $\tilde{\nu}$ = 3416 (NH), 1677 (C=O), 1618 cm^{-1} (C=O); FAB-MS *m/z*: 219 $[M+H]^+$.

General procedure for the synthesis of compounds 4a–d. A solution of 1 mol of carbohydrazide (**3a**–**d**) and an equimolar amount of the appropriate aldehyde in EtOH was heated under reflux for 1–2 h. The precipitate obtained was filtered off, washed with water, and then washed twice with cold EtOH.

***N*′-[(*E*)-(2,3,4-trihydroxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (5):** Compound **5** was synthesized (59%) using the general procedure, starting from **3a** and 2,3,4-trihydroxybenzaldehyde. Yellow solid: mp: 271–273 °C; ^{1}H NMR ([D$_6$]DMSO): δ = 6.40 (d, J = 8.4 Hz, 1H), 6.72 (d, J = 8.5 Hz, 1H), 7.30 (ddd, J = 7.4 Hz, 1H), 7.47 (ddd, J = 7.3 Hz, 1H), 7.67 (dd, J = 8.4 Hz, 1H), 8.22 (dd, J = 8.1 Hz, 1H), 8.45 (s, 1H), 8.58 (s, 1H), 9.42 (s, 1H), 11.69 (s, 1H), 12.15 (s, 1H), 13.80 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO): δ = 108.5,

111.7, 111,8, 122.1, 122.3, 122.8, 123.3, 127.7, 133.6, 137.8, 141.9, 148.4, 149.5, 151.1, 159.1 ppm; IR (KBr): $\tilde{\nu}=3440$ (NH), 3260 (OH), 1632 cm^{-1} (C=O); EI-MS *m/z*: 312 $[M]^+$; anal. calcd for $C_{15}H_{12}N_4O_4 \cdot {}^1/_3$EtOH (312.28): C 56.63, H 4.04, N 16.86; found: C 56.48, H 4.24, N 16.72.

***N'*-[(*E*)-(2,4-dihydroxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (6)**: Compound **6** was synthesized (73%) using the general procedure, starting from **3a** and 2,4-diihydroxybenzaldehyde. Yellow solid: mp: 283–285 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=6.33$ (d, $J=$ 2.0 Hz, 1H), 6.37 (dd, $J=2.2$, 8.4 Hz, 1H), 7.23 (dd, $J=8.4$ Hz, 1H), 7.30 (ddd, $J=7.5$ Hz, 1H), 7.46 (ddd, $J=7.6$ Hz, 1H), 7.67 (dd, $J=$ 8.4 Hz, 1H), 8.22 (dd, $J=8.1$ Hz, 1H), 8.62 (s, 1H), 9.94 (s, 1H), 11.66 (s, 1H), 12.12 (s, 1H), 13.79 ppm (s, 1H); ^{13}C NMR ([D$_6$]DMSO): $\delta=$ 111.7, 113.6, 116.5, 121.3, 122.3, 122.8, 123.2, 126.9, 127.6, 138.4, 141.9, 146.6, 148.7, 148.9, 156.3 ppm; IR (KBr): $\tilde{\nu}=3249$ (NH, OH), 1606 cm^{-1} (C=O); EI-MS *m/z*: 296 $[M]^+$; anal. calcd for $C_{15}H_{12}N_4O_3 \cdot {}^1/_4$ EtOH (296.28): C 60.48, H 4.42, N 18.20; found: C 60.24, H 4.03, N 18.31.

***N'*-[(*E*)-(3,4-dihydroxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (7):** Compound **7** was synthesized (78%) using the general procedure, starting from **3a** and 3,4-dihydroxybenzaldehyde. White solid: mp: 283–286 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=6.79$(d, J=8.1 Hz, 1H), 6.92 (dd, $J=1.8$, 8.1 Hz, 1H), 7.25 (d, $J=1.6$ Hz, 1H), 7.29 (ddd, $J=7.6$ Hz, 1H), 7.45 (ddd, $J=7.2$ Hz, 1H), 7.66 (dd, $J=$ 8.4 Hz, 1H), 8.21 (dd, $J=8.1$ Hz, 1H), 8.38 (s, 1H), 9.30 (s, 2H), 11.68 (s, 1H), 12.12 (s, 1H), 13.72 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3490$ (NH), 3249 (OH), 1634 cm^{-1} (C=O); EI-MS *m/z*: 296 $[M]^+$; anal. calcd for $C_{15}H_{12}N_4O_3 \cdot {}^1/_5$EtOH (296.28): C 60.55, H 4.36, N 18.34; found: C 60.41, H 3.93, N 18.31.

***N'*-[(*E*)-(3-hydroxy-4-methoxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (8):** Compound **8** was synthesized (69%) using the general procedure, starting from **3a** and 3-hydroxy-4-methoxybenzaldehyde. White solid: mp: 280–283 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.82$ (s, 3H), 6.98 (d, $J=8.4$ Hz, 1H), 7.04 (dd, $J=7.9$ Hz, 1H), 7.24–7.32 (m, 2H), 7.46 (ddd, $J=7.6$ Hz, 1H), 7.66 (dd, $J=8.4$ Hz, 1H), 8.21 (dd, $J=8.1$ Hz, 1H), 8.41 (s, 1H),), 9.28 (s, 1H), 11.75 (s, 1H), 13.73 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3214$ (NH, OH), 1662 cm^{-1} (C=O); EI-MS *m/z*: 310 $[M]^+$; anal. calcd for $C_{16}H_{14}N_4O_3$ (310.31): C 61.93, H 4.55, N 18.06; found: C 61.83, H 4.48, N 17.72.

***N'*-[(*E*)-(3,4-dimethoxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (9):** Compound **9** was synthesized (85%) using the general procedure, starting from **3a** and 3,4-dimethoxybenzaldehyde. White solid: mp: 184–186 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.82$ (s, 3H), 3.84 (s, 3H), 7.04 (d, $J=8.3$ Hz, 1H), 7.18 (d, $J=8.3$ Hz, 1H), 7.30 (ddd, $J=7.5$, 1H), 7.35 (s, 1H), 7.46 (ddd, $J=7.0$, 8.3 Hz, 1H), 7.66 (dd, $J=8.3$ Hz, 1H), 8.21 (dd, $J=8.1$ Hz, 1H), 8.49 (s, 1H),), 11.83 (s, 1H), 13.74 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3460$ (NH), 3256 (OH), 1664 cm^{-1} (C=O); EI-MS *m/z*: 324 $[M]^+$; anal. calcd for $C_{17}H_{16}N_4O_3$ (324.33): C 62.95, H 4.97, N 17.27; found: C 63.30, H 5.21, N 17.06.

***N'*-[(*E*)-(2-hydroxyphenyl)methylidene]-1*H*-indazole-3-carbohydrazide (10):** Compound **10** was synthesized (74%) using the general procedure, starting from **3a** and 2-hydroxybenzaldehyde. White solid: mp: 161–165 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=6.94$ (ddd $J=$ 8.4 Hz, 2H), 7.42–7.52 (m, 2H), 7.67 (dd, $J=8.4$ Hz, 1H), 8.23 (dd, $J=8.1$ Hz, 1H), 8.75 (s, 1H), 11.49 (s, 1H), 12.34 (s, 1H), 13.85 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3158$ (NH, OH), 1664 cm^{-1} (C=O); EI-MS *m/z*: 280 $[M]^+$; anal. calcd for $C_{15}H_{12}N_4O_2$ (280.28): C 64.28, H 4.32, N 19.99; found: C 64.24, H 4.17, N 19.76.

methyl4-{[(*E*)-2-(1*H*-indazol-3-ylcarbonyl)hydrazono]methyl} benzoate (11): Compound **11** was synthesized (74%) using the general procedure, starting from **3a** and methyl 4-formylbenzoate. White solid: mp: 314–316 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=3.89$ (s, 3H), 7.31 (ddd, $J=7.5$, 7.8 Hz, 1H), 7.48 (ddd, $J=7.6$, 6.9 Hz, 1H) 7.68 (dd $J=$ 8.3 Hz, 1H), 7.86 (dd, $J=7.92$ Hz, 1H), 8.05 (dd, $J=7.4$ Hz, 2H), 8.22 (dd, $J=8.08$ Hz, 1H), 8.64 (s, 1H), 12.16 (s, 1H), 13.85 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3307$ (NH), 1710 (C=O), 1674 cm^{-1} (C=O); ESI-MS *m/z*: 323 $[M+H]^+$; anal. calcd for $C_{17}H_{14}N_4O_3$ (322.32): C 63.35, H 4.38, N 17.38; found: C 63.22, H 4.47, N 17.44.

4-{[(*E*)-2-(1*H*-indazol-3-ylcarbonyl)hydrazono]methyl}benzoic acid (12): 1 M NaOH (3 mL) was added to a solution of **11** (0.10 g, 0.31 mmol) in dioxane (3 mL). After completion of the hydrolysis, as monitored by TLC, the solvent was evaporated under reduced pressure, the residue was diluted with water, and the mixture was washed with EtOAc. The aqueous phase was acidified to pH 1 with 1 M HCl and extracted with EtOAc. The organic phases were dried (Na_2SO_4) and concentrated to give a white solid (73%); mp: 355–356 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=7.34$ (ddd, $J=7.5$, 7.8 Hz, 1H), 7.48 (ddd, $J=7.95$, 8.73 Hz, 1H) 7.68 (dd, $J=8.4$ Hz, 2H), 7.8 (dd, $J=$ 7.92 Hz, 2H), 8.05 (dd, $J=8.3$ Hz, 1H), 8.22 (dd, $J=8.1$ Hz, 1H), 8.64 (s, 1H), 12.13 (s, 1H), 13.05 (s, 1H) 13.85 ppm (s, 1H); IR (KBr): $\tilde{\nu}=$ 3289 (NH, OH), 1677 (C=O),1534 (C=O); ESI-MS *m/z*: 309 $[M+H]^+$; anal. calcd for $C_{16}H_{12}N_4O_3$ (308.29): C 62.33, H 3.92, N 18.17; found: C 62.31, H 4.01, N 18.20.

2-(4-{[(*E*)-2-(1*H*-indazol-3-ylcarbonyl)hydrazono]methyl} phenoxy) acetic acid (13): Compound **13** was synthesized (80%) using the general procedure, starting from **3a** and 2-(4-formylphenoxy)-acetic acid. White solid: mp: 306–309 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=$ 4.75 (s, 2H), 7.01 (dd, $J=7.67$ Hz, 2H), 7.30 (ddd, $J=7.49$, 7.80 Hz, 1H) 7.48 (ddd $J=7.64$, 6.96 Hz, 1H), 7.66 (dd, $J=8.43$ Hz, 3H), 8.21 (dd, $J=8.21$ Hz, 1H), 8.51 (s,1H), 11.82 (s, 1H), 12.95 (br s, 1H), 13.74 (s, 1H), 13.85 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3307$ (NH), 3239 (OH), 1735 (C=O),1663 cm^{-1} (C=O); ESI-MS *m/z*: 339 $[M+H]^+$; anal. calcd for $C_{17}H_{14}N_4O_4$ (338.32): C 60.35, H 4.17, N 16.56; found: C 60.22, H 4.25, N 16.71.

***N'*-{(*E*)-[4-(dimethylamino)phenyl]methylidene}-1*H*-indazole-3-carbohydrazide (14):** Compound **14** was synthesized (24%) using the general procedure, starting from **3a** and 4-(dimethylamino)-benzaldehyde. Purification of the crude product by chromatography (CH_2Cl_2/MeOH, 40/1) gave a yellow solid; mp: 268–270 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=2.98$ (s, 6H), 6.77 (d, $J=8.90$ Hz, 2H), 7.29 (ddd, $J=7.49$, 7.80 Hz, 1H) 7.45 (ddd $J=7.20$ Hz, 1H), 7.54 (d, $J=$ 8.80 Hz, 2H), 8.21 (dd, $J=8.10$ Hz, 1H), 8.42 (s,1H), 11.62 (s, 1H), 13.74 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3321$ (NH), 3191, 3239 (OH), 1672 (C=O), 1601 cm^{-1} (C=O); EI-MS *m/z*: 307 $[M]^+$; anal. calcd for $C_{17}H_{17}N_5O \cdot {}^1/_2$ EtOH (307.35): C 65.44, H 6.10, N 21.20; found: C 65.63, H 5.76, N 21.23.

***N'*-[(*E*)-(4-nitrophenyl)methylidene]-1*H*-indazole-3-carbohydrazide (15):** Compound **15** was synthesized (81%) using the general procedure, starting from **3a** and 4-nitrobenzaldehyde. Pale yellow, mp: 310–330 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=7.68$ (ddd, $J=7.5$ Hz, 1H) 7.48 (ddd $J=7.2$ Hz, 1H), 7.69 (dd, $J=8.4$ Hz, 1H), 7.98 (d, $J=$ 8.8 Hz, 2H), 8.22 (dd, $J=8.1$ Hz, 1H), 8.32 (d, $J=8.8$ Hz, 2H), 8.69 (s,1H), 12.31 (s, 1H), 13.85 ppm (s, 1H); IR (KBr): $\tilde{\nu}=3322$ (NH), 3220 (OH), 1681 (C=O), 1594 cm^{-1} (C=O); EI-MS *m/z*: 309 $[M]^+$; anal. calcd for $C_{15}H_{11}N_5O_3 \cdot {}^1/_6$ EtOH (309.28): C 58.50, H 3.97, N 21.77; found: C 58.97, H 3.51, N 21.33.

***N'*-[(*E*)-(4-cyanophenyl)methylidene]-1*H*-indazole-3-carbohydrazide (16):** Compound **16** was synthesized (78%) using the general procedure, starting from **3a** and 4-formylbenzonitrile. White solid, mp: 332–335 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta=7.31$ (ddd, $J=7.5$ Hz, 1H) 7.48 (ddd $J=7.2$ Hz, 1H), 7.68 (dd, $J=8.5$ Hz, 1H), 7.91 (d, $J=$

8.71 Hz, 4H), 8.22 (dd, J=8.1 Hz, 1H), 8.63 (s, 1H), 12.23 (s, 1H), 13.84 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3322 (NH), 2220 (CN), 1681 (C=O), 1594 cm^{-1} (C=O); EI-MS m/z: 289 $[M]^+$; anal. calcd for $C_{16}H_{11}N_5O$ (289.29): C 66.43, H 3.83, N 24.21: found: C 66.77, H 3.68, N 23.93.

***N*′-[(*E*)-(2-phenyl-1,3-thiazol-4-yl)methylidene]-1*H*-indazole-3-carbohydrazide (17):** Compound **17** was synthesized (74%) using the general procedure, starting from **3a** and 2-phenyl-1,3-thiazole-4-carbaldehyde. White solid, mp: 264–272 °C; ^{1}H NMR ([D$_6$]DMSO): δ=7.31 (ddd, J=6.8, 7.7 Hz, 1H) 7.48 (ddd J=6.9, 8.3 Hz, 1H), 7.54 (dd, J=5.1 Hz, 3H), 7.69 (dd, J=8.4 Hz, 1H), 8.01 (dd, J=6.5, 3.1 Hz, 2H), 8.14 (s, 1H), 8.23 (dd. J=8.1 Hz, 1H), 8.74 (s, 1H), 12.23 (s, 1H), 13.81 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3066 (NH), 1657 (C=O), 1529 cm^{-1} (C=O); EI-MS m/z: 347 $[M]^+$; anal. calcd for $C_{18}H_{13}N_5OS$ (347.40): C 62.23, H 3.77, N 20.16; found: C 62.22, H 3.95, N 22.06.

***N*′-[(*E*)-1*H*-indol-3-ylmethylidene]-1*H*-indazole-3-carbohydrazide (18):** Compound **18** was synthesized (19%) using the general procedure, starting from **3a** and 1*H*-indole-3-carbaldehyde. Pink solid, mp: 240–243 °C; ^{1}H NMR ([D$_6$]DMSO): δ=7.12–7.25 (m, 2H), 7.45 (dd, J=7.7 Hz, 1H), 7.47–7.62 (m, 3H), 7.82 (dd J=2.6 Hz, 1H), 7.93 (dd, J=6.6 Hz, 2H), 8.31 (dd, J=7.3 Hz, 1H), 8.63 (s, 1H), 11.50 (s, 1H), 11.57 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3207, 3066 (NH), 1607 cm^{-1} (C=O); EI-MS m/z: 263 $[M]^+$; anal. calcd for $C_{17}H_{13}N_5O$ (303.32): C 72.99, H 4.98, N 15.96; found: C 75.76, H 5.19, N 16.07.

4-methyl-*N*′-[(*E*)-(2,3,4-trihydroxyphenyl)methylidene]-1,3-thiazole-5-carbohydrazide (19): Compound **19** was synthesized (73%) using the general procedure, starting from **3b** and 2,3,4-trihydroxybenzaldehyde. Yellow solid, mp: 305–309 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.67 (s, 3H), 6.40 (d, J=8.5, 1H) 6.79 (d, J=8.3 Hz, 1H), 8.10 and 8.47(s, 1H), 9.14 (s, 1H), 9.48 (s, 1H), 11.29 (s, 1H), 11.59 (s, 1H), 11.81 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3036 (NH, OH), 1632 cm^{-1} (C=O); ESI-MS m/z: 294 $[M+H]^+$; anal. calcd for $C_{12}H_{11}N_3O_4S$ (293.30): C 49.14, H 3.78, N 14.33; found: C 49.02, H 3.80, N 14.21.

***N*′-[(*E*)-(3,4-dihydroxyphenyl)methylidene]-4-methyl-1,3-thiazole-5-carbohydrazide (20):** Compound **20** was synthesized (63%) using the general procedure, starting from **3b** and 3,4-diihydroxybenzaldehyde. White solid, mp: 308–312 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.73 (s, 3H), 6.79 (d, J=8.1, 1H) 6.95 (dd, J=7.6 Hz, 1H), 7.22 (d, J=1.7 Hz, 1H), 7.92 and 8.18 (s, 1H), 9.20 (s, 1H), 9.36 (br s, 2H), 11.57 ppm (br s, 1H); IR (KBr): $\tilde{\nu}$=3454 (NH, OH), 1646 cm^{-1} (C=O); ESI-MS m/z: 278 $[M+H]^+$; anal. calcd for $C_{12}H_{11}N_3O_3S\cdot{}^1/_5$ EtOH (277.30): C 51.31, H 4.09, N 14.96; found: C 51.40, H 4.17, N 15.21.

***N*′-[(*E*)-(3,4-dimethoxyphenyl)methylidene]-4-methyl-1,3-thiazole-5-carbohydrazide (21):** Compound **21** was synthesized (60%) using the general procedure, starting from **3b** and 3,4-dimethoxybenzaldehyde. White solid, mp: 183–185 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.74 (s, 3H), 3.81 (s, 3H), 3.83 (s, 3H), 7.03 (d, J=8.3, 1H) 7.23 (dd, J=7.8 Hz, 1H), 7.38 (d, J=1.7 Hz, 1H), 8.02 and 8.25 (s, 1H), 9.21 (s, 1H), 11.74 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3046 (NH), 1653 cm^{-1} (C=O); EI-MS m/z: 330 $[M+H]^+$; anal. calcd for $C_{14}H_{15}N_3O_3S$ (305.35): C 55.07, H 4.95, N 13.76; found: C 55.40, H 5.16, N 13.33.

***N*′-[(*E*)-(3,5-dichloro-2-hydroxyphenyl)methylidene]-4-methyl-1,3-thiazole-5-carbohydrazide (22):** Compound **22** was synthesized (78%) using the general procedure, starting from **3b** and 3,5-dichloro-2-hydroxybenzaldehyde. White solid, mp: 290–293 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.68 (s, 3H), 7.63 (s, 1H), 7.66 (s, 1H), 8.15 and 8.53 (s, 1H), 9.19 (s, 1H), 11.98 (br s, 1H), 12.30 ppm (br s, 1H); IR (KBr): $\tilde{\nu}$=3039 (NH, OH), 1653 cm^{-1} (C=O); EI-MS m/z: 330 $[M]^+$; anal. calcd for $C_{12}H_9Cl_2N_3O_2S$ (330.19): C 43.65, H 2.75, N 12.73; found: C 43.64, H 2.77, N 12.72.

methyl4-({(*E*)-2-[(4-methyl-1,3-thiazol-5-yl)carbonyl]hydrazono} methyl)benzoate (23): Compound **23** was synthesized (83%) using the general procedure, starting from **3b** and methyl 4-formylbenzoate. White solid, mp: 248–294 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.72 (s, 3H), 3.88 (s, 3H), 7.87 (dd, J=8.1 2H), 8.04 (dd, J=8.1, 2H), 8.18 and 8.31 (s, 1H), 9.22 (s, 1H), 11.98 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3155, 3058 (NH, OH), 1712 (C=O), 1653 cm^{-1} (C=O); ESI-MS m/z: 303 $[M+H]^+$; anal. calcd for $C_{14}H_{13}N_3O_3S$ (303.34): C 55.43, H 4.32, N 13.85; found: C 55.45, H 4.37, N 13.88.

4-({(*E*)-2-[(4-methyl-1,3-thiazol-5-yl)carbonyl]hydrazono} methyl) benzoic acid (24): 1 M NaOH (5 mL) was added to a solution of **23** (0.085 mg, 0.28 mmol) in dioxane (5 mL). After completion of the hydrolysis, as monitored by TLC, the solvent was evaporated under reduced pressure, the residue was diluted with water, and the mixture was washed with EtOAc. The aqueous phase was acidified to pH 1 with 1 M HCl and extracted with EtOAc. The organic phases were dried (Na_2SO_4) and concentrated to give a white solid (71%); mp: 268–282 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.73 (s, 3H), 7.85 (dd J=8.3 Hz, 2H), 8.02 (dd, J=8.3 Hz, 2H), 8.18 and 8.35 (s, 1H), 9.22 (s, 1H), 11.96 (s, 1H), 12.92 ppm (br s, 1H); IR (KBr): $\tilde{\nu}$=3440 (NH, OH), 1685 cm^{-1} (C=O); ESI-MS m/z: 290 $[M+H]^+$; anal. calcd for $C_{14}H_{13}N_3O_3S$ (289.31.29): C 53.97, H 3.83, N 14.52; found: C 54.22, H 3.91, N 14.13.

2-[4-({(*E*)-2-[(4-methyl-1,3-thiazol-5-yl)carbonyl]hydrazono} methyl)phenoxy]acetic acid (25): Compound **25** was synthesized (82%) using the general procedure, starting from **3b** and 2-(4-formylphenoxy)acetic acid. White solid, mp: 245–249 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.73 (s, 3H), 4.74 (s, 2H), 7.01 (dd, J=8.8 2H), 7.68 (dd, J=8.5, 2H), 8.10 and 8.32 (s, 1H), 9.19 (s, 1H), 11.71 (s, 1H) 12.06 ppm (br s, 1H); IR (KBr): $\tilde{\nu}$=3062 (NH, OH), 1734 (C=O), 1661 cm^{-1} (C=O); ESI-MS m/z: 320 $[M+H]^+$; anal. calcd for $C_{14}H_{13}N_3O_4S$ (319.34): C 52.66, H 4.10, N 13.16; found: C 52.56, H 4.31, N 12.80.

***N*′-{(*E*)-[4-(dimethylamino)phenyl]methylidene}-4-methyl-1,3-thiazole-5-carbohydrazide (26):** Compound **26** was synthesized (68%) using the general procedure, starting from **3b** and 4-(dimethylamino)benzaldehyde. Yellow solid, mp: 232–234 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.73 (s, 3H), 2.98 (s, 6H), 6.76 (dd, J=8.8 2H), 7.55 (dd, J=8.5, 2H), 7.96 and 8.20 (s, 1H), 9.18 (s, 1H), 11.54 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3043 (NH), 1642 cm^{-1} (C=O); ESI-MS m/z: 289 $[M+H]^+$; anal. calcd for $C_{14}H_{16}N_4OS$ (288.37): C 58.31, H 5.59, N 19.43; found: C 58.40, H 5.68, N 19.50.

4-methyl-*N*′-[(*E*)-(2-phenyl-1,3-thiazol-4-yl)methylidene]-1,3-thiazole-5-carbohydrazide (27): Compound **27** was synthesized (63%) using the general procedure, starting from **3b** and 2-phenyl-1,3-thiazole-4-carbaldehyde. White solid, mp: 210–213 °C; ^{1}H NMR ([D$_6$]DMSO): δ=2.73 (s, 3H), 7.61–7,47 (m, 3H), 7.96–8.03 (m, 2H), 8.15 and 8.51 (s, 1H), 8.26 (s, 1H), 11.92 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3050 (NH), 1642 cm^{-1} (C=O); EI-MS m/z: 328 $[M]^+$; anal. calcd for $C_{15}H_{12}N_4OS_2$ (328.41): C 54.86, H 3.68, N 17.06; found: C 54.90, H 3.73, N 17.02.

2-phenyl-*N*′-[(*E*)-(2,3,4-trihydroxyphenyl)methylidene]-1,3-thiazole-5-carbohydrazide (28): Compound **28** was synthesized (63%) using the general procedure, starting from **3c** and 2,3,4-trihydroxybenzaldehyde. Beige solid, mp: 121–128 °C; ^{1}H NMR ([D$_6$]DMSO): δ=6.42 (d, J=8.4, 1H), 6.78 (d, J=8.5, 1H), 7.54–7.60 (m, 3H), 8.09–8.16 (m, 2H), 8.50 (s, 1H), 8.60 (s, 1H), 8.67 (s, 1H), 9.47 (s, 1H), 11.92 (s, 1H), 11.93 ppm (s, 1H); IR (KBr): $\tilde{\nu}$=3119 (NH), 1664 (C=O), 1636 cm^{-1} (C=O); ESI-MS m/z: 356 $[M+H]^+$; anal. calcd for $C_{17}H_{13}N_3O_4S_2$ (355.37): C 57.46, H 3.69, N 11.82; found: C 57.22, H 3.94, N 11.32.

***N′*-[(*E*)-(2,3,4-trihydroxyphenyl)methylidene]benzohydrazide (29):** Compound **29** was synthesized (96%) using the general procedure, starting from **3d** and 2,3,4-trihydroxybenzaldehyde. Yellow solid, mp: 206–211 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 6.40$ (d, $J = 8.4$, 1H) 6.79 (d, $J = 8.5$ Hz, 1H), 7.49–7.65 (m, 3H), 7.93 (dd, $J = 7.0$ Hz, 1H), 8.47 (s, 2H), 9.46 (s, 1H), 11.53 (s, 1H), 11.95 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3417$ (NH, OH), 1615 cm^{-1} (C=O); FAB-MS *m/z*: 273 $[M+H]^+$; anal. calcd for $C_{14}H_{12}N_2O_4$ (272.26): C 61.76, H 4.44, N 10.29; found: C 61.14, H 4.62, N 9.79.

***N′*-[(*E*)-(2,4-dihydroxyphenyl)methylidene]benzohydrazide (30):** Compound **30** was synthesized (86%) using the general procedure, starting from **3d** and 1,4-dihydroxybenzaldehyde. White solid, mp: 258–262 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 6.33$ (d, $J = 2.1$ Hz, 1H), 6.37 (dd, $J = 2.2$, 8.1 Hz, 1H), 7.31 (d, $J = 8.4$ Hz, 1H), 7.46–7.64 (m, 3H), 7.92 (dd, $J = 6.8$ Hz, 2H), 8.51 (s, 1H), 9.95 (s, 1H), 11.49 (s, 1H), 11.90 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3257$ (NH, OH), 1626 cm^{-1} (C=O); EI-MS *m/z*: 256 $[M+H]^+$.

***N′*-[(*E*)-(3,4-dihydroxyphenyl)methylidene]benzohydrazide (31):** Compound **31** was synthesized (91%) using the general procedure, starting from **3d** and 3,4-dihydroxybenzaldehyde. White solid, mp: 219–222 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 6.79$ (d, $J = 8.0$ Hz, 1H), 6.94 (d, $J = 8.1$ Hz, 1H), 7.25 (s, 1H), 7.44–6.62 (m, 3H), 7.90 (dd, $J = 7.1$ Hz, 1H), 8.24 (s, 2H), 9.31 (s, 2H), 11.59 (s, 1H), 11.95 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3417$ (NH, OH), 1615 cm^{-1} (C=O); EI-MS *m/z*: 256 $[M]^+$.

***N′*-[(*E*)-(3-hydroxy-4-methoxyphenyl)methylidene] benzohydrazide (32):** Compound **32** was synthesized (95%) using the general procedure, starting from **3d** and 3-hydroxy-4-methoxybenzaldehyde. White solid, mp: 80–87 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.81$ (s, 3H), 6.98 (d, $J = 8.2$ Hz, 1H), 7.06 (d, $J = 7.7$ Hz, 1H), 7.28 (s, 1H), 7.47–7.60 (m, 3H), 7.90 (dd, $J = 7.2$ Hz, 1H), 8.31 (s, 2H), 9.29 (s, 2H), 11.65 (s, 1H), 11.95 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3386$ (NH, OH), 1648 cm^{-1} (C=O); EI-MS *m/z*: 270 $[M]^+$.

***N′*-[(*E*)-(3,4-dimethoxyphenyl)methylidene]benzohydrazide (33):** Compound **33** was synthesized (94%) using the general procedure, starting from **3d** and 3,4-dimethoxybenzaldehyde. White solid, mp: 175–177 °C (lit: 178–180 °C[27]); ^{1}H NMR ([D$_6$]DMSO): $\delta = 3.81$ (s, 3H), 3.83 (s, 3H), 6.04 (d, $J = 8.2$ Hz, 1H), 7.21 (d, $J = 8.2$ Hz, 1H), 7.35 (s, 1H), 7.49–7.62 (m, 3H), 7.90 (dd, $J = 7.3$ Hz, 1H), 8.39 (s, 1H), 11.71 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3430$ (NH), 1651 cm^{-1} (C=O); EI-MS *m/z*: 284 $[M]^+$.

***N′*-{(*E*)-[4-(dimethylamino)phenyl]methylidene}benzohydrazide (34):** Compound **34** was synthesized (62%) using the general procedure, starting from **3d** and 4-(dimethylamino)benzaldehyde. Yellow solid, mp: 211–214 °C (lit: 217 °C[28]); ^{1}H NMR ([D$_6$]DMSO): $\delta = 2.98$ (s, 6H), 6.77 (d, $J = 8.8$ Hz, 2H), 7.47–7.61 (m, 5H), 7.90 (d, $J = 6.9$ Hz, 2H), 8.32 (s, 2H), 11.53 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3444$ (NH), 1595 cm^{-1} (C=O); EI-MS *m/z*: 267 $[M]^+$.

***N′*-[(*E*)-(4-cyanophenyl)methylidene]benzohydrazide (35):** Compound **35** was synthesized (66%) using the general procedure, starting from **3d** and 4-formylbenzonitrile. White solid, mp: 233–236 °C (lit: 240 °C[28]); ^{1}H NMR ([D$_6$]DMSO): $\delta = 7.50$–7.65 (m, 4H), 7.92 (s, 5H), 8.52 (s, 1H), 12.08 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3557$ (NH), 2234 (CN) 1567 cm^{-1} (C=O); EI-MS *m/z*: 249 $[M]^+$.

***N′*-[(*E*)-(2-phenyl-1,3-thiazol-4-yl)methylidene]benzohydrazide (36):** Compound **36** was synthesized (98%) using the general procedure, starting from **3d** and 2-phenyl-1,3-thiazole-4-carbaldehyde. White solid, mp: 94–98 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 7.53$–7.62 (m, 6H), 7.74 (s, 1H), 7.92–8.02 (m, 5H), 11.91 ppm (s, 1H); IR (KBr): $\tilde{\nu} =$ 3183 (NH),1677 cm^{-1} (C=O); EI-MS *m/z*: 307 $[M]^+$; anal. calcd for $C_{17}H_{13}N_3OS$ (307.37): C 66.43, H 4.26, N 13.67; found: C 66.33, H 4.48, N 14.05.

***N′*-[(*E*)-1*H*-indol-3-ylmethylidene]benzohydrazide (37):** Compound **37** was synthesized (50%) using the general procedure, starting from **3d** and 2-phenyl-1,3-thiazole-4-carbaldehyde. White solid, mp: 240–243 °C; ^{1}H NMR ([D$_6$]DMSO): $\delta = 7.12$–7.25 (m, 2H), 7.45 (dd, $J = 7.7$ Hz, 1H), 7.47–7.62 (m, 3H), 7.82 (dd, $J = 2.6$ Hz, 1H), 7.93 (dd, $J = 6.6$ Hz, 2H), 8.31 (dd, $J = 7.3$ Hz, 1H), 8.63 (s, 1H), 11.50 (s, 1H), 11.57 ppm (s, 1H); IR (KBr): $\tilde{\nu} = 3207$ (NH), 1607 cm^{-1} (C=O); EI-MS *m/z*: 263 $[M]^+$; anal. calcd for $C_{16}H_{13}N_3O$ (263.29): C 72.99, H 4.98, N 15.96; found: C 72.76, H 5.19, N 16.07.

Enzyme assay: MurC and MurD enzymes were overproduced from *E. coli* JM83 cells harboring the pAM1005 or pMLD58 plasmid, respectively; they were purified by ion-exchange chromatography as described previously.[19]

Malachite green assay: The inhibitory activities were tested for the compounds against MurC (MurD) from *E. coli*, as their ability to inhibit the addition of L-Ala (D-Glu) to UDP-Mur*N*Ac (UDP-Mur*N*Ac-L-Ala). Detection of the orthophosphate generated during the reaction was based on the colorimetric Malachite green method, as described elsewhere,[20] with slight modifications: a mixture with a final volume of 50 μL was used, containing 50 mM Hepes, pH 8.0, 3.25 mM $MgCl_2$, 6.5 mM $(NH_4)_2SO_4$, 0.005% Triton X-114, 120 μM UDP-Mur*N*Ac (80 μM UDP-Mur*N*Ac-L-Ala), 120 μM L-Ala (100 μM D-Glu), 450 μM ATP (400 μM ATP), purified MurC (MurD) from *E. coli* [diluted with 20 mM Hepes, pH 7.2, 5 mM dithiothreitol], and 500 μM of the compound being tested dissolved in DMSO. Poorly soluble compounds were tested at either 250 μM or 100 μM. The final concentration of DMSO was 5% (*v/v*). The reaction mixture was incubated at 37 °C for 15 min and then quenched with 100 μL Biomol reagent. Absorbance at 650 nm was measured after 5 min. Residual activities were calculated with respect to control assays without the compounds and with DMSO. IC_{50} values were determined by measuring the residual activities at seven different compound concentrations, and represent the concentrations of the compounds where the residual activities were 50%.

Radioactivity assay: The activities of the compounds against MurC (MurD) from *E. coli* were also tested by their inhibition of the addition of labeled amino acid substrate L-[^{14}C]Ala (D-[^{14}C]Glu) to UDP-Mur*N*Ac (UDPMur*N*Ac-L-Ala) in a mixture with a final volume of 50 μL containing 0.1 M Tris-HCl, pH 8.6, 20 mM $MgCl_2$ (5 mM $MgCl_2$), 500 μM UDP-Mur*N*Ac (25 μM UDP-Mur*N*Ac-L-Ala), 1 mM L-[^{14}C]Ala (25 μM D-[^{14}C]Glu) (50000 cpm), 5% (*v/v*) DMSO, purified MurC (MurD) [diluted with 20 mM potassium phosphate, pH 7.0, 1 mM dithiothreitol, 1 mg mL^{-1} BSA], and 500 μM, 250 μM, or 100 μM of the compound being tested. All of the compounds were soluble in the assay mixture containing 5% DMSO. The mixture was incubated for 30 min at 37 °C, and the reaction was stopped by adding 10 μL glacial acetic acid. The mixture was lyophilized and taken up in the HPLC elution buffer. The radioactive substrate and product were separated by reverse-phase HPLC with a Nucleosil 5C_{18} column (150×4.6 mm) as stationary phase, and isocratic elution at a flow rate of 0.6 mL min^{-1} with 50 mM ammonium formate, pH 4.3. The compounds were detected and quantified with an LB 506 C-1 HPLC radioactivity monitor (Berthold France, Thoiry, France) using Quickszint Flow 2 scintillator (Zinsser Analytic, Maidenhead, UK) at 0.6 mL min^{-1}. The residual activities were calculated with respect to a control assay without the compounds and with DMSO.

Determination of antibacterial activity: The MICs of the compounds were determined by broth microdilution in IsoSensitest

broth (Oxoid, Basingstoke, UK) using an inoculum of 10^4 cells mL^{-1} for *E. coli* or 10^6 cells mL^{-1} for *S. aureus*. The antimicrobial agents were prepared in a twofold dilution series in 50% DMSO. Microwell plates with 96 wells (Nunc, Fisher Scientific, Loughborough, UK) containing the antimicrobial agent and bacterial suspension were incubated for 16 h at 37 °C in a Spectramax 384 plus microwell plate reader (Molecular Devices, Abingdon, UK), running the SOFTmax PRO 3.1.1 software. Optical density readings (600 nm) were taken at 10 min intervals. The plates were shaken for 60 s before each reading. The MICs were taken as the lowest concentrations of the antimicrobial agents that prevented growth.

Docking studies: *Ligand preparation*. The ligands were built and optimized using the Sybyl 8.0 program.[18] For this purpose, appropriate fragments from the Sybyl 8.0 libraries were used to build each molecule. The Tripos force field[29] was used in the calculations, and each molecule was optimized by the method of Powell[30] until the energy gradient was less than 0.005 kcal mol^{-1} $Å^{-2}$.

Enzyme set-up. Phosphoaminophosphonic acid adenylate ester (ANP; a non-hydrolysable analogue of ATP), UDP-Mur*N*Ac-L-Ala, and water molecules were deleted from the crystal structure of MurC (PDB code: 1P3D), and hydrogen atoms were added using Sybyl 8.0. ADP, UDP-Mur*N*Ac-L-Ala, and water molecules were deleted from the crystal structure of MurD (PDB code: 3UAG), and hydrogen atoms were added using Sybyl 8.0.

Docking procedure. Molecular docking calculations were performed for representative *N*-acylhydrazones. The amino acid residues within a radius of 5 Å around the physiologically bound ligands, ANP and UDP-Mur*N*Ac-L-Ala were defined as the active sites. The ligands were docked in 50 independent genetic algorithm (GA) runs using Gold v 3.2.[23] The GA default parameters were set as suggested by Gold.[23] The ten best docking structures for each compound were inspected visually.

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