Synthesis and *in vitro* Biological Activity of New 4,6-Disubstituted 3(2*H*)-Pyridazinone-acetohydrazide Derivatives

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New 3(2*H*)-pyridazinone derivatives containing a *N*'-benzyliden-acetohydrazide moiety at position 2 were synthesized. The structures of these newly synthesized compounds were confirmed by IR, ^{1}H NMR, and MS data. These compounds were tested for their antibacterial, antifungal, antimycobacterial, and cytotoxic activities. The compounds 2-[4-(4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl]-*N*'-(4-*tert*-butylbenzyliden)acetohydrazide and 2-[4-(4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl]-*N*'-(4-chlorobenzyliden) acetohydrazide exhibited activity against both Gram-positive and Gram-negative bacteria. Most of the compounds were active against *E. coli* ATCC 35218. The preliminary results of this study revealed that some target compounds exhibited promising antimicrobial activities.

Key words: Antimicrobial Activity, Benzyliden-acetohydrazide, Pyridazinone

Introduction

For several years the emergence of multidrug-resistant bacteria and also -resistant fungi has been reported worldwide. Rapid development of multidrug-resistant microbial pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *S. aureus* (VRSA), and vancomycin-resistant Enterococci (VRE) and the lack of effective treatments have made the treatment of infectious diseases as escalating problem (He *et al.*, 2003; Aksoy and Unal, 2008). Fungal infections are not limited to superficial tissues; in fact, advanced age, major surgery, immunosuppressive therapy, acquired immunodeficiency syndrome (AIDS), cancer treatment, and solid-organ and hematopoietic stem cell transplantation increase the risk of life-threatening systemic fungal infections (Sundriyal *et al.*, 2006). Therefore novel effective antimicrobial drugs are urgently required.

Pyridazinone derivatives have been reported to exhibit diverse pharmacological activities such as vasodilatory (Bansal *et al.*, 2009), antihypertensive (Demirayak *et al.*, 2004; Siddiqui *et al.*, 2010), antiplatelet (Cherng *et al.*, 2006), analgesic and anti-inflammatory (Gokce *et al.*, 2009), anticonvulsant (Rubat *et al.*, 1990), antibacterial (Longo *et al.*, 1993; Sönmez *et al.*, 2006; Dogruer *et al.*, 2008), anti-HIV (Livermone *et al.*, 1993), and anticancer (Malinka *et al.*, 2004). Previously, we reported the analgesic and anti-inflammatory activity of novel series of 3(2*H*)-pyridazinones (Sukuroglu *et al.*, 2006; Dogruer *et al.*, 2003). Also, pyridazinones offer a valuable ring system to researchers because of easy functionalization at various ring positions. Also, *N*-acylhydrazone (NAH) compounds have been pointed out as promising antibacterial, antifungal (Suresh Kumar *et al.*, 2010; Mallikarjuna *et al.*, 2009), antimycobacterial (Sriram *et al.*, 2006), and antioxidant (Liu *et al.*, 2009) agents. Based on all above mentioned considerations and our interest in chemical and pharmacological properties of pyridazinones, we hereby report the synthesis and biological activities of novel 3(2*H*)-pyridazinones containing the *N*'-benzyliden-acetohydrazide moiety.

Material and Methods

Chemistry

Melting points were measured on an SMP-II digital melting point apparatus (Schorpp Gerätetechnik, Überlingen, Germany). Infrared (IR) spectra were recorded on a Perkin Elmer

Spectrum 400 FT-IR/FT-NR spectrophotometer (Waltham, MA, USA).

All chemicals used for the synthesis of the compounds were purchased from Aldrich Chemicals (Sigma-Aldrich, Steinheim, Germany) and Merck (Darmstadt, Germany). The ^{1}H NMR spectra were recorded on a Varian Mercury 300 FT-NMR spectrometer (Varian Inc., Palo Alto, CA, USA). Chemical shifts (δ) are reported in parts per million (ppm) downfield from tetramethylsilane (TMS) as internal standard. DMSO-d_6 was used as solvent. High-resolution mass spectroscopy (HRMS) was done on a Micromass LCT Premier XE mass spectrometer (Waters, Milford, MA, USA) using an electrospray ion (ESI) source.

Synthesis of compounds **1** and **2** was accomplished according to the previously reported procedures (Sukuroglu *et al.*, 2006). Compounds **3a**, **3b**, and **4a**–**4r** were prepared for the first time in this study.

*Synthesis of 2-[4-(phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-acetohydrazide (**3a, 3b**)*

Ethyl 2-[4-(phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl] acetate (**2a**, **2b**) (0.01 mol) was refluxed with hydrazine hydrate (0.02 mol) in ethanol (20 mL) for 4 h. At the end of the period, the reaction mixture was cooled. The precipitate was filtered, dried, and crystallized from methanol.

*Synthesis of 2-[4-(phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin)-2-yl]-N'-benzyliden-acetohydrazide derivatives (**4a**–**4r**)*

2-[4-(Phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl]acetohydrazide (**3a**, **3b**) (0.01 mol), appropriate benzaldehyde derivatives (0.011 mol), and acetic acid (1–2 drops) were heated at reflux in ethanol for 3 h. The precipitated compound was filtered. The residue was washed with hot ethanol and dried.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]acetohydrazide (**3a**): IR: ν = 1764 (C=O, CONH), 1671 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.23 (4H, t), 3.67 (4H, t), 4.23, 4.53 (2H, s), 7.49 (2H, d), 7.66 (1H, s), 7.86 (2H, d), 9.17 (1H, s). – HRMS (ESI$^+$): calcd. 364.1176 [M+H$^+$], found 364.1175.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]acetohydrazide (**3b**): IR: ν = 1763 (C=O, CONH), 1671 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.20–3.21 (4H, m), 3.67 (4H, t), 4.20, 4.50 (2H, s), 7.38–7.39 (2H, m), 7.57 (1H, s), 7.75–7.77 (3H, m), 9.14 (1H, s). – HRMS (ESI$^+$): calcd. 330.1566 [M+H$^+$], found 330.1552.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-benzyliden-acetohydrazide (**4a**): IR: ν = 3203 (NH), 1687 (C=O, CONH), 1651 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.22–3.28 (4H, m), 3.68 (4H, t), 4.71, 5.11 (2H, s), 7.41 (4H, m), 7.50 (2H, d), 7.68–7.71 (3H, m), 7.89 (2H, d), 7.99, 8.19 (1H, s), 11.67 (1H, s). – HRMS (ESI$^+$): calcd. 452.1489 [M+H$^+$], found 452.1491.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-methylbenzyliden)acetohydrazide (**4b**) : IR: ν = 3182 (NH), 1679 (C=O, CONH), 1644 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 2.32 (3H, s), 3.20–3.29 (4H, m), 3.69 (4H, t), 4.70, 5.10 (2H, s), 7.24 (2H, d), 7.51 (2H, d), 7.58 (2H, d), 7.72 (1H, s), 7.89 (2H, d), 11.60 (1H, s). – HRMS (ESI$^+$): calcd. 466.1646 [M+H$^+$], found 466.1658.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-tert-butyl benzyliden)acetohydrazide (**4c**): IR: ν = 3195 (NH), 1676 (C=O, CONH), 1649 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 1.26 (9H, s), 3.22–3.28 (4H, m), 3.68 (4H, t), 4.70, 5.09 (2H, s), 7.41 (2H, d), 7.50 (2H,d), 7.71 (1H, s), 7.89 (2H, d), 7.96, 8.15 (1H, s), 11.61 (1H, s). – HRMS (ESI$^+$): calcd. 508.2115 [M+H$^+$], found 508.2112.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-methoxybenzyliden)acetohydrazide (**4d**): IR: ν = 3195 (NH), 1682 (C=O, CONH), 1643 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.21–3.28 (4H, m), 3.69 (4H, t), 3.78 (3H, s), 4.69, 5.09 (2H, s), 6.97 (2H, d), 7.51 (2H, d), 7.62 (2H, d), 7.71 (1H, s), 7.89 (2H, d), 7.93, 8.13 (1H, s), 11.53 (1H, s). – HRMS (ESI$^+$): calcd. 482.1595 [M+H$^+$], found 482.1615.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-ethoxybenzyliden)-acetohydrazide (**4e**): IR: ν = 3201 (NH), 1684 (C=O, CONH), 1643 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 1.31 (3H, t), 3.22–3.28 (4H, m), 3.68 (4H, t), 4.01–4.59 (2H, q), 4.68, 5.08 (2H, s),

6.94 (2H, d), 7.50 (2H, d), 7.60 (2H, d), 7.71 (1H, s), 7.89 (2H, d), 7.93, 8.12 (1H, s), 11.52 (1H, s). – HRMS (ESI$^+$): calcd. 496.1752 [M+H$^+$], found 496.1743.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-chlorobenzyliden)-acetohydrazide (**4f**): IR: ν = 3203 (NH), 1687 (C=O, CONH), 1651 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.21–3.29 (4H, m), 3.69 (4H, t), 4.71, 5.12 (2H, s), 7.46–7.53 (4H, m), 7.72 (3H, m), 7.89 (2H, d), 7.99, 8.19 (1H, s), 11.72 (1H, s). – HRMS (ESI$^+$): calcd. 486.1100 [M+H$^+$], found 486.1086.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-fluorobenzyliden)-acetohydrazide (**4g**): IR: ν = 3190 (NH), 1683 (C=O, CONH), 1644 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.24–3.29 (4H, m), 3.69 (4H, t), 4.71, 5.11 (2H, s), 7.23–7.28 (2H, m), 7.51 (2H, d), 7.72–7.85 (3H, m), 7.89 (2H, d), 7.99, 8.19 (1H, s), 11,68 (1H, s). – HRMS (ESI$^+$): calcd. 470.1395 [M+H$^+$], found 470.1395.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-trifluoromethylbenzyliden)acetohydrazide (**4h**): IR: ν = 3198 (NH), 1684 (C=O, CONH), 1646 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.21–3.28 (4H, m), 3.68 (4H, t), 4.73, 5.14 (2H, s), 7.49–7.52 (2H, m), 7.72–7.90 (7H, m), 8.06, 8.26 (1H, s), 11.86 (1H, s). – HRMS (ESI$^+$): calcd. 520.1363 [M+H$^+$], found 520.1364.

2-[4-(4-Chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(2-fluorobenzyliden)-acetohydrazide (**4i**): IR: ν = 3193 (NH), 1685 (C=O, CONH), 1644 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.22–3.29 (4H, m), 3.69 (4H, t), 4.71, 5.12 (2H, s), 7.22–7.31 (2H, m), 7.46–7.52 (3H, m), 7.72 (1H, s), 7.88–7.95 (3H, m), 8.20, 8.42 (1H, s), 11.78 (1H, s). – HRMS (ESI$^+$): calcd. 470.1395 [M+H$^+$], found 470.1389.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-benzyliden-acetohydrazide (**4j**): IR: ν = 3198 (NH), 1685 (C=O, CONH), 1649 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.67 (4H, t), 4.69, 5.10 (2H, s), 7.35–8.16 (11H, m), 11.69 (1H, s). – HRMS (ESI$^+$): calcd. 418.1879 [M+H$^+$], found 418.1876.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-methylbenzyliden)acetohydrazide (**4k**): IR: γ = 3201 (NH), 1684 (C=O, CONH), 1646 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 2.28 (3H, s), 3.23 (4H, t), 3.66 (4H, t), 4.67, 5.07 (2H, s), 7.18–7.80 (10H, m), 7.93, 8.12 (1H, s), 11.57 (1H, s). – HRMS (ESI$^+$): calcd. 432.2036 [M+H$^+$], found 432.2036.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-tert-butylbenzyliden)acetohydrazide (**4l**): IR: ν = 3195 (NH), 1685 (C=O, CONH), 1646 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 1.24 (9H, s), 3.24–3.26 (4H, m), 3.66 (4H, t), 4.67, 5.07 (2H, s), 7.60–8.84 (10H, m), 7.93, 8.14 (1H, s), 11.57 (1H, s). – HRMS (ESI$^+$): calcd. 474.2505 [M+H$^+$], found 474.2508.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-methoxybenzyliden)acetohydrazide (**4m**): IR: ν = 3201 (NH), 1680 (C=O, CONH), 1642 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.24 (4H, t), 3.66 (4H, t), 3.75 (3H, s), 4.67, 5.07 (2H, s), 6.92–6.97 (2H, m), 7.40–7.41 (3H, m), 7.59 – 7.80 (5H, m), 8.03, 8.24 (1H, s), 11.82 (1H, s). – HRMS (ESI$^+$): calcd. 448.1985 [M+H$^+$], found 448.1978.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-ethoxybenzyliden)acetohydrazide (**4n**): IR: ν = 3195 (NH), 1683 (C=O, CONH), 1644 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 1.27 (3H, t), 3.26 (4H, t), 3.65 (4H, t), 3.97–4.03 (2H, q), 4.65, 5.05 (2H, s), 6.89–6.94 (2H, m), 7.39–7.79 (8H, m), 7.89, 8.09 (1H, s), 11.48 (1H, s). – HRMS (ESI$^+$): calcd. 462.2141 [M+H$^+$], found 462.2142.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-chlorobenzyliden)acetohydrazide (**4o**): IR: ν = 3195 (NH), 1685 (C=O, CONH), 1648 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.20–3.23 (4H, m), 3.66 (4H, t), 4.68, 5.09 (2H, s), 7.40 – 8.16 (11H, m), 11.69 (1H, s). – HRMS (ESI$^+$): calcd. 452.1489 [M+H$^+$], found 452.1483.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-fluorobenzyliden)acetohydrazide (**4p**): IR: ν = 3206 (NH), 1685 (C=O, CONH), 1647 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.20–3.23 (4H, m), 3.65 (4H, t), 4.67, 5.08 (2H, s), 7.18–7.78 (10H, m), 7.59, 8.16 (1H, s), 11,63 (1H, s). – HRMS (ESI$^+$): calcd. 436.1785 [M+H$^+$], found 436.1788.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(4-trifluoromethylbenzyliden)-acetohydrazide (**4q**): IR: ν = 3208 (NH), 1687 (C=O, CONH), 1647 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.23–3.28 (4H, m), 3.65 (4H, t), 4.70, 5.11 (2H, s), 7.39–7.40 (3H, m), 7.63–7.89 (7H, m), 8.03, 8.24 (1H, s), 11.82 (1H, s). – HRMS (ESI$^+$): calcd. 486.1753 [M+H$^+$], found 486.1742.

2-[4-Phenyl-6-(morpholin-4-yl)-3-oxo-(2H)-pyridazin-2-yl]-N'-(2-fluorobenzyliden)acetohydrazide (**4r**): IR: ν = 3185 (NH), 1684 (C=O, CONH), 1646 cm^{-1} (C=O, ring). – ^{1}H NMR (DMSO-d_6): δ = 3.22–3.26 (4H, m), 3.66 (4H, t), 4.68, 5.09 (2H, s), 7.19–7.41 (6H, m), 7.61–7.92 (4H, m), 8.18, 8.40 (1H, s), 11.74 (1H, s). – HRMS (ESI$^+$): calcd. 436.1785 [M+H$^+$], found 436.1785.

Antibacterial assay

The synthesized compounds were evaluated for their antibacterial activities against the Gram-negative bacteria *Escherichia coli* ATCC 35218, *E. coli* clinical isolate (ESBL), *Pseudomonas aeruginosa* ATCC 27853, *P. aeruginosa* clinical isolate, and the Gram-positive bacteria *Staphylococcus aureus* ATCC 29213, *S. aureus* clinical isolate (MRSA), *Enterococcus faecalis* ATCC 29212, *E. faecalis* clinical isolate. The tested isolates were provided by Gazi University, Faculty of Pharmacy, Department of Pharmaceutical Microbiology, Ankara, Turkey. Resistance in clinical isolates was determined by the disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006). All organisms were tested in triplicate in each run of the experiments.

All bacterial isolates were subcultured on Mueller-Hinton agar (MHA; Merck) and incubated overnight at 37 °C. The stock solutions of the newly synthesized compounds and standard drugs were diluted with liquid medium stepwise by half from 2048 down to 0.0625 μg/mL in the wells of microplates. The bacterial susceptibility test was performed according to the guidelines of CLSI M100-S18 (CLSI, 2008a). The bacterial suspensions used for inoculation were prepared at 10^5 CFU/mL by diluting fresh cultures at a McFarland density of 0.5 (10^7 CFU/mL), and the wells were inoculated at 10^4 CFU/mL. Mueller-Hinton broth (MHB; Merck) was used for all dilutions. Dimethyl sulfoxide (DMSO; Sigma Aldrich, Poole, UK), phosphate-buffered saline (PBS), pure microorganisms, and pure media were used in control wells. A 10-μL bacterial inoculum was added to each well. The trays were incubated at 37 °C, and minimum inhibitory concentration (MIC) endpoints were read after 24 h of incubation.

Antifungal assay

The newly synthesized compounds were evaluated for their antifungal activity against *Candida albicans* ATCC 10231 and *Candida krusei* ATCC 6258. All organisms were tested in triplicate in each run of the experiments.

Candida was subcultured on Sabouraud dextrose agar (SDA; Merck) and incubated at 35 °C for 24–48 h. Susceptibility testing was performed in RPMI-1640 medium supplemented with L-glutamine (Sigma) and buffered with 3-(*N*-morpholino)propanesulfonic acid (MOPS, pH 7; Sigma), and culture suspensions were prepared according to the guideline of CLSI M27-A3 (CLSI, 2008b). Yeast suspensions were prepared at a McFarland density of 0.5, and a working suspension was prepared by 1:100 dilution followed by 1:20 dilution of the stock suspension ($2.5 \cdot 10^3$ CFU/mL). A 10-μL yeast inoculum was added to each well of the microdilution trays. The trays were incubated at 35 °C, and MIC endpoints were read after 48 h of incubation.

Antimycobacterial assay

The *in vitro* activity of the synthesized compounds against *Mycobacterium tuberculosis* H37RV ATCC 27294 was determined by the microplate alamar blue assay (MABA) (Franzblau *et al.*, 1998). All organisms were tested in triplicate in each run of the experiments.

Mycobacterium tuberculosis H37RV ATCC 27294 was subcultured on Middlebrook 7H11 agar (Becton Dickinson, Franklin Lakes, NJ, USA). Culture suspensions were prepared in 0.04% (v/v) Tween 80/0.2% bovine serum albumin (Sigma) at a McFarland density of 1. Suspensions were then diluted 1:25 in 7H9GC broth (Difco, Le Pont de Claix, France), 20 mL of 10% (v/v) glycerol, 1 g of Bacto Casitone (Difco), 880 mL of distilled water, and 100 mL of Middlebrook OADC Growth Supplement (Sigma). The stock solutions of the newly synthesized compounds and standard drugs were diluted with liquid medium stepwise by half from 2048 down to 0.0625 μg/mL in the wells of microplates in the liquid media. The plates were sealed with parafilm and incubated at 37 °C for 5 d. Fif-

ty μL of a freshly prepared 1:1 mixture of 10X alamar blue (AbD Serotec, Oxford, UK) reagent and 10% Tween 80 were added to the control well. The plates were incubated at 37 °C for 24 h. The control well turned pink, and the reagent mixture was added to all wells in the microplate. The microplates were resealed with parafilm and incubated for 24 h at 37 °C, and the colours of all wells were recorded. A blue colour in the well was scored as no growth and a pink colour as growth, respectively. The MIC was defined as the lowest drug concentration which prevented a colour change from blue to pink.

Cytotoxicity assay

The level of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) reduction was quantified as previously described in the literature with small modifications (Mossmann, 1983; Keiser *et al.*, 2000).

Cell culture and drug treatment

NIH/3T3 cells were obtained from the American Type Culture Collection (ATCC, CRL-1658). The cells were incubated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco, Paisley, Scotland), 100 IU/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Exponentially growing cells were plated at $2 \cdot 10^4$ cells/mL into 96-well microtiter tissue culture plates (Nunc, Roskilde, Denmark) and incubated for 24 h and 48 h before the addition of the drugs (the optimum cell number for cytotoxicity assays was determined in preliminary experiments). Stock solutions of compounds were prepared in DMSO (Sigma Aldrich), and further dilutions were made with fresh culture medium (the content of DMSO in the final culture medium was <0.1% which had no effect on cell viability).

MTT assay for cytotoxicity tests of the compounds

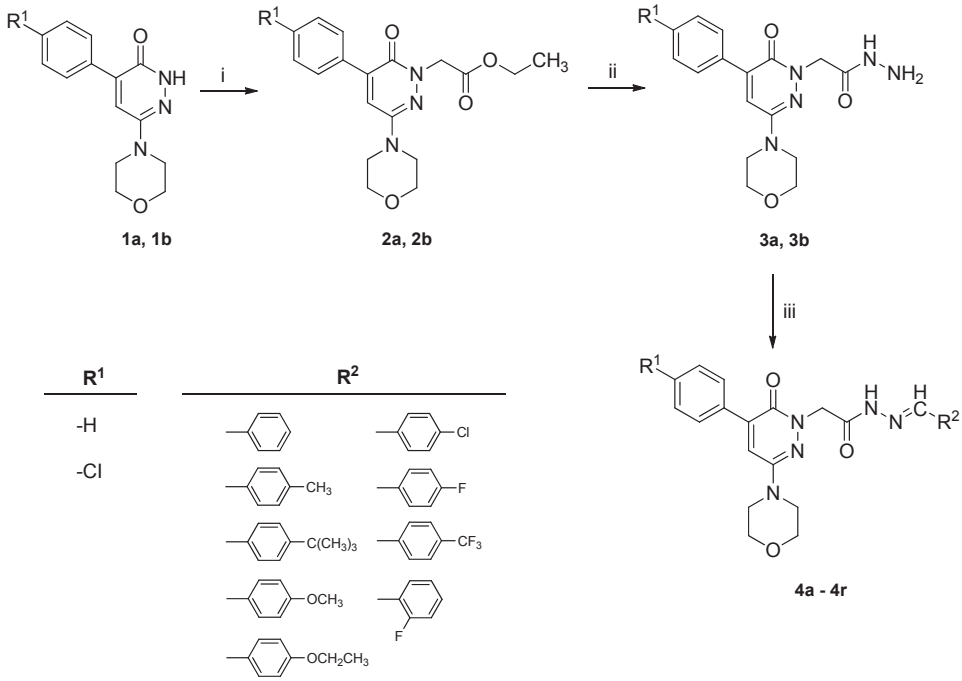
After 24 h or 48 h of preincubation, the synthetized compounds were added to give final concentrations in the range 0.5–512 μM, and the cells were incubated for 24 h. At the end of this period, MTT was added to a final concentration of 0.5 mg/mL, and the cells were incubated for 4 h at 37 °C. After the medium was removed, the formazan crystals formed were solubilized by addition of 200 mL DMSO to each well, and absorbance was read at 540 nm with a microtitre plate spectrophotometer (Bio-Tek plate reader; Winooski, VT, USA). Every concentration was repeated in three wells, and IC_{50} values were defined as the drug concentrations that reduced absorbance to 50% of control values.

Results and Discussion

We synthesized twenty new compounds, the synthetic route of which is outlined in Scheme 1. Procedures for the synthesis of compounds **1a**, **1b** and **2a**, **2b** have been reported by us in our previous study (Sukuroglu *et al.*, 2006). Compounds **3a**, **3b** and **4a**–**4r** were prepared for the first time in this study. 2-[4-(Phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl]-acetohydrazide (**3a**, **3b**) was obtained by the reaction of ethyl 2-[4-(phenyl/4-chlorophenyl)-6-(morpholin-4-yl)-3-oxo-(2*H*)-pyridazin-2-yl] acetate with hydrazine hydrate in ethanol. The new compounds **4a**–**4r** were synthesized, in good yields, by condensing compounds **3a** and **3b** with the respective aromatic aldehydes in ethanol (Table I).

The structures of these stable and crystalline compounds were fully characterized by the usual methods (IR, ^{1}H NMR). In the IR spectra, N-H, C=O (acylhydrazone), and C=O (pyridazinone ring) peaks were seen at 3208–3183 cm^{-1}, 1764–1676 cm^{-1}, and 1671–1642 cm^{-1}, respectively. In the ^{1}H NMR spectra of these compounds, signals due to N=CH and -CH_2CO- groups appeared as two separate singlets. It is well known that *N*-acylhydrazones may exist as two geometrical isomers, *E*/*Z*. On the other hand, the reason for the existence of two different singlets of the -CH_2CO- group may be due to two rotamers. This is, because the N-H group may form a hydrogen bond with the ring carbonyl group and thus form a pseudoring, which restricts the rotation. This suggestion may also be supported by the relative weakness of the ring carbonyl group peak in the IR spectra of the compounds (CLSI, 2006; CLSI, 2008b). High-resolution mass spectra confirmed the molecular masses and empirical formulas of compounds **3a**, **3b**, and **4a**–**4r**, with less than 5 ppm bias between calculated and experimental *m/z* values of the molecular ions.

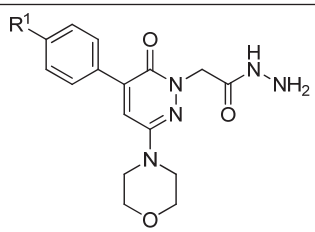
The synthesized 3(2*H*)-pyridazinone derivatives **3a**, **3b**, and **4a**–**4r** were screened for their antibacterial, antifungal, and antimycobacterial activities. Twelve strains were used as test micro-



Scheme 1. Synthetic route towards 4,6-disubstituted 3(2*H*)-pyridazinone-acetohydrazide derivatives **4a**–**4r**. i, ethyl bromoacetate/K_2CO_3/DMF; ii, NH_2NH_2/C_2H_5OH; iii, substituted benzaldehyde derivatives/HAc/C_2H_5OH.

organisms including ten bacterial and two fungal species, respectively. Ampicillin, gentamicin, amoxicillin, clavulanic acid, fluconazole, and amphotericin B for antibacterial activity, and ethambutol for antimycobacterial activity were used as reference drugs. The minimal inhibitory concentration (MIC) values of the 3(2*H*)-pyridazinone derivatives are reported in Table II and Table III along with those of the standard drugs. The results revealed that the majority of the synthesized

Table I. Characteristics of compounds **3a**, **3b**, and **4a**–**4r**.



Compound	R^1	Molecular formula	M.p. [°C]	Yield (%)
3a	Cl	$C_{16}H_{18}N_5O_3Cl$	162	85
3b	H	$C_{16}H_{19}N_5O_3$	151–152	82

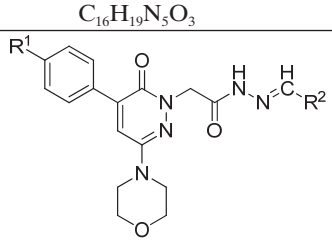


Table I continued.

Compound	R^1	R^2	Molecular formula	M.p. [°C]	Yield (%)
4a	Cl	C_6H_5	$C_{23}H_{22}N_5O_3Cl$	242–243	73
4b	Cl	4-$CH_3C_6H_4$	$C_{24}H_{24}N_5O_3Cl$	241–242	91
4c	Cl	4-$C(CH_3)_3C_6H_4$	$C_{27}H_{30}N_5O_3Cl$	260–261	86
4d	Cl	4-$CH_3OC_6H_4$	$C_{24}H_{24}N_5O_4Cl$	247–248	90
4e	Cl	4-$C_2H_5OC_6H_4$	$C_{25}H_{26}N_5O_4Cl$	258–259	88
4f	Cl	4-ClC_6H_4	$C_{23}H_{21}N_5O_3Cl_2$	240	64
4g	Cl	4-FC_6H_4	$C_{23}H_{21}N_5O_3ClF$	219	87
4h	Cl	4-$CF_3C_6H_4$	$C_{24}H_{21}N_5O_3ClF_3$	253	84
4i	Cl	2-FC_6H_4	$C_{23}H_{21}N_5O_3ClF$	249–250	87
4j	H	C_6H_5	$C_{23}H_{23}N_5O_3$	212–213	82
4k	H	4-$CH_3C_6H_4$	$C_{24}H_{25}N_5O_3$	235–236	78
4l	H	4-$C(CH_3)_3C_6H_4$	$C_{27}H_{31}N_5O_3$	255	89
4m	H	4-$CH_3OC_6H_4$	$C_{24}H_{25}N_5O_4$	233	87
4n	H	4-$C_2H_5OC_6H_4$	$C_{25}H_{27}N_5O_4$	251	87
4o	H	4-ClC_6H_4	$C_{23}H_{22}N_5O_3Cl$	251–252	90
4p	H	4-FC_6H_4	$C_{23}H_{22}N_5O_3F$	229	88
4q	H	4-$CF_3C_6H_4$	$C_{24}H_{22}N_5O_3F_3$	239–240	81
4r	H	2-FC_6H_4	$C_{23}H_{22}N_5O_3F$	205	86

Table II. MIC values of 4,6-disubstituted 3(2*H*)-pyridazinone-acetohydrazide derivatives against bacterial and fungal strains.

Compound	Minimum inhibitory concentration [μg/mL]									
	Gram-positive bacteria				Gram-negative bacteria				Fungi	
	S. aureus ATCC 29213	*S. aureus* isolate	*E. faecalis* ATCC 29212	*E. faecalis* isolate	*E. coli* ATCC 35218	*E. coli* isolate	*P. aeruginosa* ATCC 27853	*P. aeruginosa* isolate	*C. albicans* ATCC 10231	*C. krusei* ATCC 6258
3a	256	128	256	256	64	256	256	256	128	128
3b	256	128	256	256	64	256	256	256	128	128
4a	128	128	128	128	128	256	256	256	128	128
4b	256	128	128	128	16	256	256	256	128	128
4c	256	128	64	128	32	256	256	256	128	128
4d	256	128	256	128	128	256	256	256	128	128
4e	256	128	8	256	64	256	256	256	128	128
4f	128	64	64	64	64	256	256	128	128	128
4g	256	128	256	256	128	256	256	256	128	128
4h	256	128	128	128	32	256	256	256	64	128
4i	256	128	128	128	64	256	256	256	128	128
4j	256	128	256	256	64	256	256	256	128	128
4k	256	128	256	256	64	256	128	128	128	128
4l	256	128	256	256	64	256	256	256	128	128
4m	256	128	256	256	64	256	256	256	128	128
4n	256	128	256	256	64	256	256	256	128	128
4o	256	128	128	128	128	256	256	256	128	128
4p	256	128	128	256	64	256	128	128	128	128
4q	256	128	64	128	32	256	128	256	128	128
4r	256	128	256	256	64	256	256	256	128	128
Ampicillin	0.5	-	2	0.5	-	>1024	-	-	-	-
Gentamicin	0.5	16	16	32	-	1024	1	256	-	-
Amoxicillin/ clavulanic acid (2:1)	0.125	8	0.25	0.5	8	4	-	-	-	-
Fluconazole	-	-	-	-					1	32
Amphotericin B	-	-	-	-	-	-	-	-	0.125	0.5

compounds showed varying degrees of inhibition against the selected test microorganisms.

Among the tested compounds, compound **4b** was most active (MIC 16 μg/mL), with a 50% higher rate than amoxicillin/clavulanic acid, against *E. coli* ATCC 35218. While the antibacterial activities of compounds **4c**, **4h**, and **4q** (MIC 32 μg/mL) were found to be 25% that of the reference drug, compounds **3a**, **3b**, **4e**, **4f**, **4i**, **4j**, **4k**, **4l**, **4 m**, **4n**, **4p**, and **4r** exhibited antibacterial activity at 64 μg/mL against *E. coli* ATCC 35218. Among the synthesized compounds **4e**, having an MIC value of 8 μg/mL, was found to be the most active derivative against the Gram-positive bacterium *E. faecalis* ATCC 29212. Compounds **4c**, **4f**, and **4q** had lower MIC values (64 μg/mL) than compounds **4a**, **4b**, **4h**, **4i**, **4o**, and **4p** (128 μg/mL) against *E. faecalis* ATCC 29212. Furthermore, the compounds were inactive against *P. aeruginosa* ATCC 27853. The results given in Table II indicate that among the new compounds tested, only compound **4h** showed a somewhat higher antifungal activity against *C. albicans* ATCC 10231 than the others.

Table III. MIC values of 4,6-disubstituted 3(2*H*)-pyridazinone-acetohydrazide derivatives against *M. tuberculosis*.

Compound	Minimum inhibitory concentration [μg/mL]	
	M. tuberculosis H37RV ATCC 27294	*M. tuberculosis* isolate
3a	256	128
3b	256	128
4a	512	128
4b	512	128
4c	512	128
4d	512	128
4e	512	128
4f	256	128
4g	512	128
4h	512	128
4i	512	128
4j	512	128
4k	256	128
4l	256	128
4m	256	128
4n	256	128
4o	256	128
4p	256	128
4q	256	128
4r	256	256
Ethambutol	4	1

In comparison to their antibacterial activity, none of the compounds had promising antifungal activity against *C. albicans* and *C. krusei*. The acylhydrazones **4a**–**4r** inhibited *M. tuberculosis* with MIC values ranging from 256 to 512 μg/mL, and were less effective than the reference drug (Table III).

In general, the inhibitory activities of the compounds tested against the Gram-negative bacteria were higher than those against the Gram-positive bacteria. Compounds **4c**, **4f**, and **4q** exhibited both Gram-positive and Gram-negative antibacterial activity. Therefore, one can state that the presence of electron-donating or -withdrawing properties, respectively, of the groups on the phenyl ring at the side chain do not play a crucial role in the activity of the synthesized compounds.

Cytotoxic effects of the most active compounds were evaluated as well. The toxicity of all studied compounds increased in a dose-dependent manner, compounds **4e** and **4q** being the most toxic ones. Also, toxicity of **4q** increased with increasing incubation time. But toxicity of the other compounds decreased with increasing incubation time and their IC_{50} values were higher (Table IV).

In conclusion, a series of new 3(2*H*)-pyridazinone derivatives, **3a**, **3b**, and **4a**–**4r**, were synthesized in an effort to obtain new antimicrobial agents. All compounds were examined for their *in vitro* antimicrobial and antimycobacterial activities. The results of the preliminary activity tests of this class of compounds might lead to the development of better candidates with potent antimicrobial activities.

Acknowledgements

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Table IV. Cytotoxicity of compounds to the mouse fibroblast cell line NIH/3T3 after incubation for 24 h and 48 h, respectively. IC_{50} is the drug concentration required to inhibit the cell growth by 50%. The values represent means ± standard deviations of triplicate determinations.

Compound	IC_{50} [μM]	
	24 h	48 h
4b	288.3 ± 24.6	350.0 ± 17.3
4c	236.7 ± 32.5	320 ± 10
4e	29.3 ± 4.04	38 ± 3.46
4h	243.3 ± 11.5	365 ± 21.8
4f	257.7 ± 6.8	306.7 ± 115
4q	29.2 ± 1.44	27.7 ± 0.6

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