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Synthesis and in vitro anti-*Helicobacter pylori* activity of 2-(substituted benzylthio)-5-(5-nitro-2- furyl)-1, 3, 4-thiadiazole derivatives

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Starting from (5-nitrofuran-2-yl)methylene diacetate, a new series of 2-[(substituted benzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazoles (**6a-n**) were synthesized and the structures of the compounds were determined using spectroscopic methods including mass spectrometry, ^{1}H-nuclear magnetic resonance, infrared spectroscopy, and elemental analysis.

The in vitro anti-*Helicobacter pylori* activity of the synthesized compounds was evaluated by the disk diffusion method against the clinical isolates of *Helicobacter pylori*. The results indicated that most of the synthesized compounds exhibited significant inhibitory activity against *H. pylori* with respect to standard drug metronidazole. Compound **6l**, containing the 2-chloro-6-fluorobenzylthio moiety, was the most potent compound tested.

Key Words: 1,3,4-Thiadiazole, nitrofuran, anti-*Helicobacter pylori* activity.

Introduction

Nitro-heterocyclic compounds such as nitrofurans are being extensively used in therapy against amoebic and anaerobic infections.[1] Although metronidazole has been frequently used in treatment regimens for *H. pylori*

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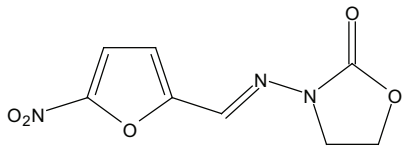
infection, other nitro-heterocyclic drugs such as furazolidone (Figure 1) have been used in place of metronidazole to treat *H. pylori* with varying degrees of success.[2] Moreover, the antimicrobial property of 1,3,4-thiadiazole derivatives is well documented and the synthesis of several new 5-membered heterocycles including alkyl(phenyl)amino-1,3,4-thidazoles as potential antimicrobial agents has been reported.[3–7]

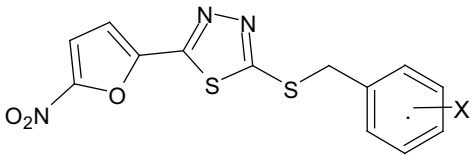
Recently, several compounds from 1,3,4-thiadiazole series that exhibited significant anti-*H. pylori* activity have been reported by our group.[8–10] There are also several reports regarding the antimicrobial properties of 1,3,4-thiadiazoles with different substitutions at 2 and 5 positions.[11] Furthermore, the synthesis and antimicrobial activity of some pyridyl and naphthyl substituted 1,2,4-triazole and 1,3,4-thiadiazole derivatives containing a benzylthio group have also been reported.[12] Moreover, the synthesis of 2-acylamino, 2-aroylamino, and ethoxycarbonyl imino-1,3,4-thiadiazoles as antitumor agents is reported.[13]

Recently, as a part of a research program to find a new and potent drug candidate for treatment of *H. pylori* infection, we have reported the synthesis and anti-*H. pylori* activity of a series of 2-[(chlorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazoles, several of which have shown potent anti-*H. pylori* activity.[14] In view of the potent biological activity of this group of compounds and to find the structure-activity relationship (SAR) of this series of compounds, herein we report the synthesis and anti-*H. pylori* activity of some novel 5-(5-nitro-2-furyl)-1,3,4-thiadiazole derivatives containing fluoro, nitro, methyl, and methoxy benzylthio groups (**6a-n**, Figure 2).



Furazolidone

Figure 1.



X= 2-F, 3-F, 4-F, 2-Me, 3-Me, 4-Me,
2-NO_2, 3-NO_2, 4-NO_2, 3-OCH_3, 4-OCH_3,
2,5-DiF, 2,4-DiF, 2-Cl-6-F.

Figure 2.

Experimental

Melting points were determined on a Kofler hot stage apparatus and are uncorrected (C. Reichert, Vienna, Austria). ^{1}H-NMR spectra were recorded using a Bruker AC-80 or Bruker 500 MHz spectrometer (Brucker, Rheinstetten, Germany) , and chemical shifts are expressed as δ (ppm) with tetramethylsilane as internal standard. The mass spectra were run on a Finnigan TSQ-70 spectrometer (Finnigan, USA) at 70 eV. Elemental

analyses were carried out on a CHN-O-rapid elemental analyzer (Foss-Heraeus GmbH, Germany) for C, H, and N, and the results are within ± 0.4% of the theoretical values. The purity of the synthesized compounds was confirmed by thin-layer chromatography (TLC) using various solvents of different polarities. Merck silica gel 60 F254 plates were applied for analytical TLC.

General method for the synthesis of 2-[(substitutedbenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazoles **6a-n**

To a mixture of 5-(5-nitofuran-2-yl)-1,3,4-thiadaizole-2-thiol[14] (5) (229 mg, 1.0 mmol) and substituted benzyl chloride (1.0 mmol) in ethanol (15 mL), KOH (66 mg in 5 mL of H_2O) was added dropwise and the mixture was stirred at room temperature overnight. Then water was added, and the separated solid was filtered off, washed with H_2O, and crystallized from EtOH-H_2O (90-10). The following compounds were prepared according to the general procedure.

2-[(2-Fluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6a)

Mp 122-123 °C; IR (KBr) ν: 1342, 1541 (NO_2), 1587 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$)δ: 4.67 (s, 2H, CH_2), 7.10-7.42 (m, 6H, aromatic). MS: m/z (%) 337 (M^+, 9), 325(8), 190(7), 166(15), 132(7), 108(100), 81(30). Anal. calcd. for $C_{13}H_8FN_3O_3S_2$: C, 46.28; H, 2.39; N, 12.46. Found: C, 46.08; H, 2.17; N, 12.66.

2-[(3-Fluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6b)

Mp 107-108 °C; IR (KBr) ν: 1347, 1501 (NO_2), 1585 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 4.61 (s, 2H, CH_2), 7.00-7.49 (m, 6H, aromatic). MS: m/z (%) 337 (M^+, 7), 325(15), 236(8), 191(8), 109(43), 134(15), 131(30), 110(42), 109(12), 98(7), 68(100). Anal. calcd. for $C_{13}H_8FN_3O_3S_2$: C, 46.28; H, 2.39; N, 12.46. Found: C, 46.51; H, 2.49; N, 12.22.

2-[(4-Fluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6c)

Mp 141-143 °C; IR (KBr) ν: 1347, 1501 (NO_2), 1590 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 4.59 (s, 2H, CH_2), 6.90-7.41 (m, 6H, aromatic). MS: m/z (%) 337 (M^+, 5), 325(8), 190(16), 136(8), 132(16), 109(28), 83(60), 57(97), 55(100). Anal. calcd. for $C_{13}H_8FN_3O_3S_2$: C, 46.28; H, 2.39; N, 12.46. Found: C, 46.33; H, 2.30; N, 12.46.

2-[(2-Methylbenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6d)

Mp 104-106 °C; IR (KBr) ν: 1352, 1536 (NO_2), 1580 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 2.44 (s, 3H, CH_3), 4.66 (s, 2H, CH_2), 7.21-7.50 (m, 6H, aromatic), MS: m/z (%) 333 (M^+, 5), 300(5), 162(14), 135(5), 105(100), 80(15). Anal. calcd. for $C_{14}H_{11}N_3O_3S_2$: C, 50.44; H, 3.33; N, 12.60. Found: C, 50.24; H, 3.33; N, 12.90.

2-[(3-Methylbenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6e)

Mp 134-135 °C; IR (KBr) ν: 1352, 1526 (NO_2), 1585 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 2.35 (s, 3H, CH_3), 4.56 (s, 2H, CH_2), 7.21-7.50 (m, 6H, aromatic), MS: m/z (%) 333 (M^+, 5), 300(7), 162(11),

105(100), 79(22). Anal. calcd. for $C_{14}H_{11}N_3O_3S_2$: C, 50.44; H, 3.33; N, 12.60. Found: C, 50.65; H, 3.14; N, 12.42.

2-[(4-Methylbenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6f)

Mp 163-164 °C; IR (KBr) ν: 1352, 1536 (NO_2), 1587 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 2.34 (s, 3H, CH_3), 4.58 (s, 2H, CH_2), 7.10-7.50 (m, 6H, aromatic), MS: m/z (%) 333 (M^+, 21), 162(6), 105(100),79(5). Anal. calcd. for $C_{14}H_{11}N_3O_3S_2$: C, 50.44; H, 3.33; N, 12.60. Found: C, 50.60; H, 3.40; N, 12.61.

2-[(2-Nitrobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6g)

Mp 184-186 °C; IR(KBr) ν: 1342, 1511 (NO_2), 1590 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 4.99 (s, 2H, CH_2), 7.27-8.21 (m, 6H, aromatic), MS: m/z (%) 365(M^+, 5), 318(37), 316(5), 231(9), 229(36), 214(7), 156(8), 137(30), 92(23). Anal. calcd. for $C_{13}H_8N_4O_5S_2$: C, 42.85; H, 2.21; N, 15.38. Found: C, 43.00; H, 1.98; N, 15.18.

2-[(3-Nitrobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6h)

Mp 124-126 °C; IR(KBr) ν: 1344, 1526 (NO_2), 1588 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 4.27 (s, 2H, CH_2), 7.27-8.40 (m, 6H, aromatic), MS: m/z (%) 365 (M^+, 5), 347 (15), 317 (5), 273 (14), 209(14), 193 (100), 158 (15), 136 (100), 90 (43), 78 (27). Anal. calcd. for $C_{13}H_8N_4O_5S_2$: C, 42.85; H, 2.21; N, 15.38. Found: C, 42.75; H, 2.00; N, 15.49.

2-[(4-Nitrobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thidiazole (6i)

Mp 171-172 °C; IR (KBr) ν: 1342, 1511 (NO_2), 1588 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 4.69 (s, 2H, CH_2), 7.25-8.25 (m, 6H, aromatic), MS: m/z (%) 364 (M^+, 65), 286(8), 93(100), 156(16), 136(28), 90(28), 78(43). Anal. calcd. for $C_{13}H_8N_4O_5S_2$: C, 42.85; H, 2.21; N, 15.38. Found: C, 43.15; H, 2.39; N, 15.21.

2-[(2,5-Difluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6j)

Mp 114-115 °C; IR (KBr) ν: 1357, 1547 (NO_2), 1590 cm^{-1} (C=N); ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 4.64 (s, 2H, CH_2), 6.96-7.00 (m, 1H, benzyl), 7.04-7.08 (m, 1H, benzyl), 7.26-7.30 (m, 1H, benzyl), 7.33 (d, 1H, furyl, J= 4 Hz), 7.47 (d, 1H, furyl, J= 4 Hz). MS: m/z (%) 355 (M^+, 20), 237(6), 184(12), 135(12), 127(100), 105(12), 81(6). Anal. calcd. for $C_{13}H_7F_2N_3O_3S_2$: C, 43.94; H, 1.99; N, 11.83. Found: C, 44.21; H, 2.19; N, 11.83.

2-[(2,4-Difluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6k)

Mp 120-121 °C; IR(KBr) ν: 1362, 1536 (NO_2), 1590 cm^{-1} (C=N); ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 4.61 (s, 2H, CH_2), 6.83-6.88 (m, 2H, benzyl), 7.33 (d, 1H, furyl, J= 4.0 Hz), 7.46 (d, 1H, furyl, J= 4 Hz), 7.51-7.56 (m, 1H, benzyl). MS: m/z (%) 355 (M^+, 18), 184(18), 156(5), 127(100), 82(12), 77(5). Anal. calcd. for $C_{13}H_7F_2N_3O_3S_2$: C, 43.94; H, 1.99; N, 11.83. Found: C, 44.14; H, 2.13; N, 12.01.

2-[(2-Chloro-6-fluorobenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6l)

Mp 144-145 °C; IR(KBr) ν: 1347, 1501 (NO_2), 1587 cm^{-1} (C=N); ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 4.84 (d, 2H, CH_2, $J_{H,F} = 1.5$ Hz), 7.03-7.07 (m, 1H, benzyl), 7.24-7.30 (m, 2H, benzyl), 7.35 (d, 1H, furyl, $J = 4.0$ Hz), 7.47 (d, 1H, furyl, $J = 4.0$ Hz) MS: m/z (%) 371(M^+, 6), 339(10), 336(7), 200(10), 156(10), 143(100), 107(18), 82(7). Anal. calcd. for $C_{13}H_7ClFN_3O_3S_2$: C, 42.00; H, 1.90; N, 11.30. Found: C, 42.22; H, 1.78; N, 11.20.

2-[(3-Methoxybenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6m)

Mp 106-107 °C; IR (KBr) ν: 1347, 1531 (NO_2), 1582 cm^{-1} (C=N); ^{1}H-NMR 80 MHz ($CDCl_3$) δ: 3.61 (s, 3H, OCH_3), 4.60 (s, 2H, CH_2), 6.54-7.50 (m, 6H, aromatic). MS: m/z (%) 349 (M^+, 7), 316(5), 178(9), 152(15), 122(100), 92(30), 78(15). Anal. calcd. for $C_{14}H_{11}N_3O_4S_2$: C, 48.13; H, 3.17; N, 12.03. Found: C, 48.01; H, 2.95; N, 11.78.

2-[(4-Methoxybenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazole (6n)

Mp 139-140 °C; IR (KBr) ν: 1347, 1536 (NO_2), 1585 cm^{-1} (C=N); ^{1}H-NMR 500 MHz ($CDCl_3$) δ: 3.79 (s, 3H, OCH_3), 4.57 (s, 2H, CH_2), 6.85 (d, 2H, benzyl, $J = 8.7$ Hz), 7.22-7.41 (m, 4H, furyl-benyl). MS: m/z (%) 349 (M^+, 9), 178(6), 152(12), 122(100), 77(7). Anal. calcd. for $C_{14}H_{11}N_3O_4S_2$: C, 48.13; H, 3.17; N, 12.03. Found: C, 48.17; H, 3.37; N, 12.01.

Biological activity

Bacterial isolates and culture conditions

Clinical *H. pylori* isolates from gastric biopsy specimens were obtained from the Shariati hospital (Tehran, Iran). Primary isolation was performed on selective blood agar base no. 2 (Oxoid, Basingstoke, Hants, UK) supplemented with horse blood 5% (v/v), and 1 selectatab tablet 500 mg (Mast Diagnostic, Merseyside, UK). Following primary selective isolation, *H. pylori* bacterial cells were identified according to colony morphology, gram staining, microaerophilic growth (at 37 °C), oxidase+, catalase+, urease+, nitrate$^-$, H_2S^-, and hippurate hydrolysis$^-$. Growth of *H. pylori* was maintained at 37 °C for 3-5 days in an atmosphere of 5% O_2, 15% CO_2, and 80% N_2 in an anaerobic chamber (Hirayama, Tokyo, Japan). Bacterial strains were stored at -70 °C in brain heart infusion broth (BHIB) (Difco, East Molesey, UK) containing 10% (v/v) fetal calf serum (FCS) and 15% (v/v) glycerol. Frozen clinical isolates were thawed and inoculated on Mueller-Hinton agar (MHA) plates (Oxoid) supplemented with 10% horse blood and incubated under microaerophilic conditions. Given the importance of inoculum homogeneity, cellular viability was controlled microscopically by morphological observation with gram staining, in order to check the proportions of coccoid cells in cultures. Cultures were always used after 48 h of incubation, when they generally did not present coccoid forms. Suspensions were prepared in sterile distilled water to opacity of 2 McFarland standards (107-108 CFU/mL).

Bacterial growth inhibition assay (disk diffusion method)

Growth inhibition was performed by the filter paper disk diffusion method on Mueller-Hinton agar with 7% of defibrinated horse blood under microaerophilic conditions at 37 °C.[15,16] The samples were tested using

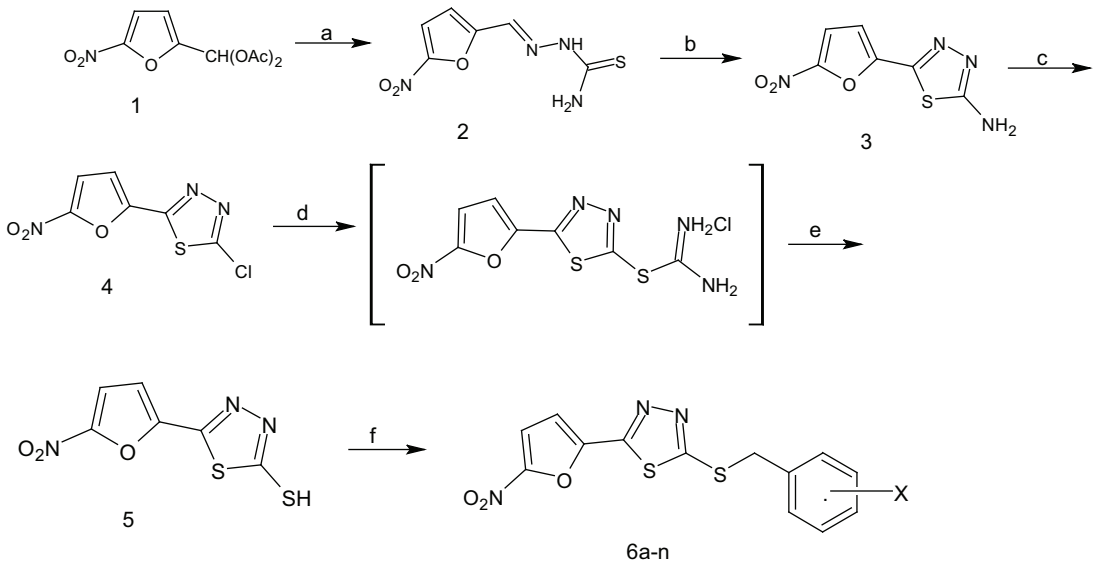
different amounts of the synthesized compounds. A sample in 40 μL of methanol was applied by a microsyringe to the paper disks (6 mm diameter). After drying in a fume hood, the disks were placed on the agar surface inoculated with *Helicobacter pylori*. Following incubation for 3-5 days at 37 °C, the inhibition zone around each disk (average diameter), if any, was recorded. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by the title compounds.

Results and discussion

Chemistry

The synthetic pathway for the target compounds is outlined in the Scheme. Reaction of 5-nitrofuran-2-carboxaldehyde diacetate (**1)** with thiosemicarbazide in refluxing ethanol yielded thiosemicarbazone **2**. In the next step, oxidative cyclization of **2** in the presence of ammonium ferric sulfate afforded amino-1,3,4-thiadiazole **3**. Diazotization of **3** in hydrochloric acid and in the presence of copper powder yielded chloro-1,3,4-thiadiazole **4**, whereby its further reaction with thiourea in refluxing ethanol and subsequent hydrolysis with hydrochloric acid gave 5-(5-nitro-2-furyl)-1,3,4-thiadiazole-2-thiol **5**.[14] Reaction of compound **5** with benzyl chlorides gave the corresponding 2-[(substitutedbenzyl) thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazoles (**6a-n**) in high yield.

The structures of compounds **6a-n** were determined using spectroscopic methods including mass spectrometry, ^{1}H-nuclear magnetic resonance (NMR), infrared (IR) spectroscopy, and elemental analysis.



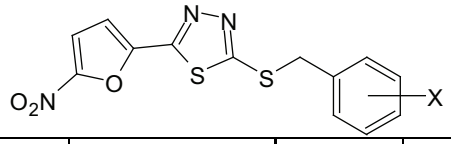
X= 2-F, 3-F, 4-F, 2-Me, 3-Me, 4-Me, 2-NO_2,
3-NO_2, 4-NO_2, 3-OCH_3,
4-OCH_3, 2,5-DiF, 2,4-DiF, 2-Cl-6-F

Scheme. Reagent and condition: a) thiosemicarbazide, EtOH, HCl, reflux, 1.5 h; b) $NH_4Fe(SO_4)_2.12H_2O$,H_2O, reflux, 25 h; c) $NaNO_2$, HCl, Cu, 0° → rt, 3 h; d) thiourea, EtOH, reflux, 1.5 h ; e) HCl f) appropriate benzyl chloride derivative, NaOH, EtOH, rt.

Anti-*Helicobacter pylori* activity

The anti-*Helicobacter pylori* activity of compounds **6a-n** along with standard drug metronidazole was evaluated by comparing the inhibition zone diameters determined by the paper disk diffusion bioassay. Various amounts of the compound were dropped on standard disks (6 mm diameter), which were placed on Muller-Hinton agar plates previously inoculated with bacterial suspension. Following incubation for 3-5 days at 37 ° C, the inhibition zone around each disk (average diameter), if any, was recorded. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition diameters (mm) produced by the title compounds against metronidazole-sensitive and metronidazole-resistant *H. pylori* strains at 3 concentrations (8, 16, and 32 μg/disk); the results are presented in the Table. The antibacterial activity was classified as follows: strong response, zone diameter >20 mm; moderate response, zone diameter 16-20 mm; weak response, zone diameter

Table. Zone of inhibition (mm) of compounds **6a-n** against metronidazole sensitive and metronidazole resistant *H. pylori* strains.[a]



Inhibition zone (mm) against Metronidazole resistant strain			Inhibition zone (mm) against Metronidazole resistant strain			X	Compound
Dose (μg/disk)			Dose (μg/disk)				
8	16	32	8	16	32		
18	15	14	19	17	14	2-F	**6a**
17	15	14	17	15	14	3-F	**6b**
19	15	14	19	17	15	4-F	**6c**
11	0	0	13	11	8	2-CH_3	**6d**
12	0	0	0	0	0	3-CH_3	**6e**
0	0	0	13	11	0	4-CH_3	**6f**
15	12	10	16	13	11	2-NO_2	**6g**
14	12	9	14	12	9	3-NO_2	**6h**
16	13	11	14	13	11	4-NO_2	**6i**
16	15	14	14	14	12	2,5-DiF	**6j**
17	16	15	18	16	15	2,4-DiF	**6k**
20	17	15	19	18	16	2-Cl,6-F	**6l**
12	10	0	12	11	9	3-OCH_3	**6m**
12	12	9	11	10	0	4-OCH_3	**6n**
13	12	10	24	21	18		Metrinidazole

[a] The anti-*Helicobacter pylori* activity was determined by the paper disk diffusion bioassay. All tests were performed in triplicate and the antibacterial activity was expressed as the mean of inhibition zone diameters (mm) produced by title compounds.

11-15 mm; and little or no response, zone diameter $\leq$10 mm.

The inhibition zone diameters indicate that compound **6l** followed by compounds **6k** and **6a-c** are superior in inhibiting the growth of *H. pylori* (inhibition zone diameter $\geq$ 14 mm), while the remaining compounds exhibited weak or no anti-*Helicobacter pylori* activity.

The averages of the inhibition zone diameters indicate that substitution of the benzylthio moiety attached to the 1,3,4-thiadiazole nucleus with different groups can modulate the anti-*H. pylori* activity profile of the basic molecule. Substitution of the phenyl group with fluorine atom (compounds **6a-c**) resulted in compounds with moderate anti-*H. pylori* activity. Furthermore, compound **6l** having 2-chloro-6-fluoro group was found to be the most active compound tested. The anti-*H. pylori* activity of the compounds reveals that the introduction of a nitro, methyl, or methoxy group reduce the biological activity of target compounds. However, this activity is dependent on the position of the pendent group on the benzylthio moiety attached to the 1,3,4-thiadiazole nucleus.

In conclusion, a new series of 2-[(substitutedbenzyl)thio]-5-(5-nitro-2-furyl)-1,3,4-thiadiazoles (**6a-n**) were synthesized and evaluated against clinical isolates of *H. pylori*. In our target compounds, this modification results in changes in the potency and anti-*H. pylori* activity profile of the synthesized compounds.

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