Synthesis of 1,5 and 2,5-disubstituted tetrazoles and their evaluation as antimicrobial agents

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ABSTRACT
Treatments of tetrazolate salts, malononitrile and sodium azide using NiO nanoparticles with benzyl bromide gave the corresponding 1,5- and 2,5-disubstituted tetrazoles. Reaction of tetrazolate salts with 2,4′-dibromoacetophenone by NiO nanoparticles provided 2,5-disubstituted derivatives as an only isomer. The structures of tetrazoles were characterized by 1H and 13C NMR spectra, FT-IR spectra, MS and elemental analysis. Use of simple and readily accessible starting materials, excellent yields, reusability of the catalyst, short reaction times, low amount of catalyst are some advantages of this protocol. Their antimicrobial activity has been tested in vitro against Gram positive bacteria; Gram negative bacteria and fungi. Four compounds have only moderate growth inhibitory effects against Gram positive bacteria. The antimicrobial screening suggests that compounds 5h; 6h; 5g and 6g have only medium growth inhibitory effects against Gram positive bacteria. Among the newly synthesized compounds; good antimicrobial activity was observed for compound 6g against Staphylococcus epidermidis (MIC value 125 µg/ml).

INTRODUCTION
The family of tetrazoles is known as a highly main moiety in organic; organometallic; medicinal chemistry [1-6], and in diverse materials science including propellants [7], and inflammables [8], 1,5- and 2,5-disubstituted tetrazoles are also significant as NAD(P)H oxidase inhibitors [9], potential TNF-α inhibitors [10], hepatitis C virus (HCV) serine protease NS3 inhibitors [11], selective cyclooxygenase-2 (COX-2) inhibitors [12], calcitonin gene-related peptide receptor antagonists, antimigraine [13], antiviral and antiangiogenic activity [14, 15]. Several procedures have been developed in literature for the preparation of tetrazoles [16,17]. To prepare these compounds, the alklylation of tetrazole anions are utilized. However; owing to the ambient nature of the anions 1a↔1b, the metal salt products of 5-substituted tetrazoles undergo to alkylation with electrophiles in a vast range of solvents afforded mixture of 5-substituted 1N- and 2N-alkyl tetrazoles (Scheme 1) [18]. For instance; the reaction of 5-substituted tetrazoles with epoxy compounds [19] dialkyl sulfates [20], benzyl bromide [21], and alkyl halides [22], using base; or with diazomethane, [23] afford a mixture of 1,5 and 2,5-disubstituted tetrazoles and the ratio of the regiosomers are affected by the electronegativity and size of the 5-substituent. Even by obstructing the N(2)-position with tri-n-butyltin before alkylation; the 2,5-isomer was formed about a 10% yield [24].

Nelson et al prepared a series of complexes including of the 1,5- disubstituted tetrazoles individually by obstructing the 2-position with cobalt complexes [25]. Kondo et al obtained 2,5-diarylsulfonyl tetrazoles from benzyl 2-sulfonyl hydrazones of aromatic aldehydes and arnediazonium salts [26]. Yamamoto and co-workers were prepared 2,5-disubstituted tetrazoles...
Multi-component reactions (MCRs) are efficient and rapid methods for the preparation of single reactive intermediate or products from several starting materials [28,29]. Transition metal-catalyzed multi-component systems have recently increased substantial interest [30,31]. Lately, nickel-based nanoparticles and especially NiO nanoparticles have been utilized as effective heterogeneous catalysts for organic reactions [32-35]. Our interest on the preparation of heterocyclic compounds and the development of efficient methods for MCRs which were catalyzed by nanoparticles [36-38], led us to the generation of regioselected 2,5-disubstituted tetrazoles 4a-h by Knoevenagel condensation/1,3-dipolar cycloaddition reaction of aldehydes, sodium azide, malononitrile, and 2,4′-dibromoacetophenone using nano-NiO as a catalyst.

The catalyst were prepared according to the procedure presented by Zhang et al from the
reaction of NaCl and Ni(OH)₂ [39-41].

Recently we reported an efficient method for the synthesis of 2-(1H-tetrazol-5-yl) acrylonitrile derivatives (3) starting from aldehydes (1); malononitrile (2) and sodium azide continued by acidic hydrolysis [35]. The best result was achieved at 70°C with 6 mol % nanocrystalline in DMF.

We continued working to expand the groups of tetrazole heterocyclic compounds by beginning from this effective reagent. The first outcome of this project is the preparation of 1,5-disubstituted and 2,5-disubstituted tetrazoles from benzyl bromide and tetrazolate salts by an expeditious approach. Then we obtained the regioselected compounds of a series of new 2,5-disubstituted tetrazoles through treatment of tetrazolate salts with 2,4'-dibromoacetophenone (Scheme 2). The antimicrobial screening results against Gram negative bacteria; Gram positive bacteria and fungi are presented for compounds 4a, 4b, 4f, 5g, 5h, 6g and 6h.

**EXPERIMENTAL SECTION**

**General procedure for the preparation of disubstituted tetrazoles**

Nano NiO (6 mol %) is added to a mixture of aromatic aldehyde (1.0 mmol); malononitrile (1.0 mmol) and NaN₃ (1.0 mmol) in DMF (5 mL) and stirred at 70 °C. Then, the mixture was cooled to room temperature. Afterwards, benzyl bromide (1.0 mmol) or 2,4'-dibromoacetophenone (1.0 mmol) was added. The contents were further stirred at 70 °C until completion (monitoring by TLC). After completion; the nanocatalyst was separated off by centrifugation and rinsed with acetone (3 times). Water was added to precipitate. The precipitate was filtered and dried. Most of the compounds were obtained in pure form after easy trituration with ethyl acetate and hexane. Other compounds were purified by column chromatography (CHCl₃/CH₂OH 9.5:0.5). In a representative case (Entry 1; Table 1); the recovered catalyst was reused in three successive runs without any considerable reduce in the product yields.

**Determination of Antimicrobial activity**

**Microbial strains**

Products of 4a, 4b, 4f, 5g, 5h, 6g and 6h were appraised in vitro against for antibacterial activities against *Pseudomonas aeruginosa* (ATCC 27853); *Escherichia Coli* (ATCC 10536); *Klebsiella pneumonia* (ATCC 10031); *Shigella dysenteriae* (PTCC 1188); *Proteus vulgaris* (PTCC 1182) and *Salmonella paratyphi-A* serotype (ATCC 5702) as examples of Gram negative bacteria; *Bacillus subtilis* (ATCC 6633); *Staphylococcus aureus* (ATCC 29737) and *Staphylococcus epidermidis* (ATCC 12228) as examples of Gram positive bacteria. They were appraised in vitro for their antifungal activities against *Candida albicans* (ATCC 10231); *Aspergillus niger* (ATCC 16404) and *Aspergillus brasiliensis* (ATCC 16404) as examples of fungal strains.

**Agar diffusion assay**

Agar diffusion technique was utilized for determining preparatory antibacterial and antifungal activities [43]. Each of the test compounds was dissolved in DMSO as solvent to final concentration of 30 µg/ml and filtered by 0.45 µm Millipore filters for sterilization. One-hundred microliters of suspension including 10⁴ CFU/ml of bacteria; 10⁶ CFU/ml of yeast and 10⁴ spore/ml of fungi spread on the nutritious agar; sabouraud dextrose agar and potato dextrose agar medium; respectively. Uniform wells (6 diameters) were punched on the media plates and filled with 10 µl of the test compounds. Streptomycin (10µg/ well) was utilized as positive control for bacteria and Nystatine (100 IU/well) for fungi. DMSO was applied as a negative control. The inoculated plates were incubated for at 37°C for 24 h bacterial strains and 48 h and 72 h at 30°C for yeast and mold isolated, respectively. The results were noted for each tested compound as average diameter of inhibition zones of bacterial and fungal around the wells in mm and each test was repeated twice.

**Micro-well dilution assay**

Bacterial strains sensitive to the compounds in agar diffusion assay were investigated for their minimum inhibitory concentration (MIC) values using micro-well dilution assay procedure [44]. The inocula of microbial strains were provided from 12 h broth cultures and suspensions were modified to 0.5 McFarland standard turbidity. The compounds were dissolved in 10% DMSO as solvent and diluted to the highest concentration (2000 µg/ml) to be tested and then serial twofold dilutions were prepared in a concentration range from 31.25 to 2000 µg/ml in 10 ml sterile tubes including brain heart infusion (BHI) broth. The 96-well plates were provided by dispensing 95 µl of the cultures media and 5 µl of the inoculums into
<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R-Br</th>
<th>Product</th>
<th>Time (min)</th>
<th>Mp (°C)</th>
<th>Yield%&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
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<td>CHO</td>
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<td>188-190</td>
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<td>89&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Br-&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4b</td>
<td>120</td>
<td>173-174</td>
<td>85</td>
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<td>4c</td>
<td>123</td>
<td>189-190</td>
<td>88</td>
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<td>4d</td>
<td>120</td>
<td>216-218</td>
<td>87</td>
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</table>

<sup>a</sup>Yield after 70°C for 24h in DMF
Continued Table 1. Synthesis of disubstituted tetrazoles catalyzed by NiO NPs (6 mol%) at 70°C in DMF

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R-Br</th>
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<th>Time (min)</th>
<th>Mp (°C)</th>
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<td>85</td>
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<tr>
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<td>7</td>
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<td>Br-Br-Br-Br</td>
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<td>201-202</td>
<td>86</td>
</tr>
<tr>
<td>8</td>
<td>CHO-OH</td>
<td>Br-Br-Br-Br</td>
<td><img src="image4.png" alt="Image" /></td>
<td>124</td>
<td>249-251</td>
<td>85</td>
</tr>
</tbody>
</table>
Continued Table 1. Synthesis of disubstituted tetrazoles catalyzed by NiO NPs (6 mol%) at 70°C in DMF

<table>
<thead>
<tr>
<th>Entry</th>
<th>R</th>
<th>R·Br</th>
<th>Product</th>
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<th>Mp (ºC)</th>
<th>Yield%a</th>
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<td>236-238</td>
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<tr>
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<td><img src="image4" alt="Product" /></td>
<td>260</td>
<td>162-163</td>
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</table>

aYield of isolated product
b; cThese yields correspond to the second and third runs, respectively, with the recycled catalyst.
dYield of mixture products (5g and 6g)
eYield of mixture products (5h and 6h)
each well. A 100 µl aliquot from the stock solutions of the compounds was made at the concentration of 2000 µg/ml was added into the first well. Then 100 µl from their serial dilutions was transferred into six successive wells. The last well comprising 195 µl of the cultures media without the test materials and 5 µl of the inoculums on each strip was applied as the negative control. Streptomycin was utilized as standard drug for positive control in conditions similar to tests materials. Turbidity indicated growth of microorganism and the MIC were determined as the lowest concentrations of the compounds that prevented visible growth.

RESULTS AND DISCUSSION

Chemistry

The morphology of nano-NiO was determined by Scanning Electronic Microscopy (SEM). The results from SEM images clearly demonstrate that the average size of nano-NiO is about nanometers (Fig. 1).

Fig. 1

It has been reported formerly that the reaction of tetrazolate salts with halogenoalkanes gave the 1,5-disubstituted and 2,5-disubstituted tetrazoles as mixtures [21,22]. Treatment of tetrazolate salts prepared in situ with benzyl bromide offered the corresponding mixtures of 1,5-disubstituted 5 (minor isomers) and 2,5- disubstituted 6 (major isomers) derivatives (Table 1; entry 9, 10). TLC of this solution indicated them to be a mixture of two products; which was separated by silica gel column chromatography using CHCl₃/CH₃OH as eluent. In their mass spectra; both of these compounds; the low (5g, 5h) and the high (6g, 6h) moving; showed the same molecular ion peak illustrated them to be the positional isomers. In the ¹³C NMR spectra the carbon CH₂ attached to tetrazole are very obvious, appearing at ca. 51.6 ppm in isomers 5h and at ca. 56.8 ppm in isomers 6h, also vinyl carbon attached to nitrile group (88.87 ppm for isomer 5h and 93.35 ppm for isomer 6h). In ¹H NMR spectra of (E)-2- (2-benzyl-2H-tetrazol-5-yl)-3-(4-hydroxyphenyl) acrylonitrile (6h) the appearance of resonance signal for CH₂ attached to tetrazole ring at 5.96 is relatively at higher field than the resonance signals at 5.85 for CH₂ attached to tetrazole ring in (E)-2-(1-benzyl-1H-tetrazol-5-yl)-3- (4- hydroxy phenyl) acrylonitrile (5h) further supporting their assigned structures. The ratio of isolated yields of the above two isomers (ratio of 6g/5g is 8.6 and ratio of 6h/5h is 10.8) determined by ¹H NMR. These results suggested that the 6g,h isomers are the predominant ones (Table 1). Steric factors also have a vital role in the ratio for formation of isomers [40-42].

Treatment of tetrazolate salts with 2,4′-dibromoacetophenone gave the corresponding 2,5-disubstituted derivative as an only isomer. TLC of the reaction mixture showed the product to be one isomer. The best evidences for the formation of 2,5-disubstituted derivative are its less steric hindrance which is explained in suggested mechanism and the appearance of a deshielded singlet for CH₂ attached to tetrazole ring (6-7 ppm) in the ¹H NMR spectrums of 4a-4h.

Antimicrobial activity

The results of antimicrobial activity of compounds are presented in Table 2 and 3.
results demonstrated that the synthetic compounds 4a, 4b, 4f, 5g, 5h, 6g and 6h have no antimicrobial effect against Gram negative bacteria and fungi. The compounds 5h; 6h; 5g and 6g have only moderate growth inhibitory effects against Gram positive bacteria (Bacillus subtilis; Staphylococcus aureus and Staphylococcus epidermidis) (Table 2).

As displayed in Table 3; the results of the MIC values of the elected compounds in all cases were more than 500 µg/ml against Bacillus subtilis and Staphylococcus aureus. Compound 5h and 6h showed MIC values 250 µg/ml against Staphylococcus epidermidis and good antimicrobial activity was apperceived for compound 6g against Staphylococcus epidermidis (MIC value 125 µg/ml).

CONCLUSIONS
In conclusion, we have improved an efficient procedure for the preparation of disubstituted tetrazoles in the present of NiO nanoparticles. Reaction of tetrazolate salts and 2,4′-dibromoacetophenone in the presence of NiO NPs provided the regioselected products. This procedure offers several advantages; containing facile; excellent yields in short time; ease of experimental method; and environmentally friendly. The antimicrobial screening suggests that compounds 5h; 6h; 5g and 6g have only medium growth inhibitory effects against Gram positive bacteria. Among the newly synthesized compounds; good antimicrobial activity was observed for compound 6g against Staphylococcus epidermidis (MIC value 125 µg/ml).

Supporting Information
Experimental method and product characterization data: IR, 1H NMR, 13C NMR and elemental analyses of the selected compounds are presented in Supporting Information.

ACKNOWLEDGEMENT
The authors are grateful to university of Kashan for supporting this work.

CONFLICT OF INTEREST
The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

Table 2. In vitro antimicrobial activity of the prepared compounds by agar diffusion assay.

<table>
<thead>
<tr>
<th>Test microorganisms</th>
<th>Diameter of zone of inhibition in mm</th>
<th>Streptomycin</th>
<th>Nystatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. aeruginosa</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>22 NT</td>
<td></td>
</tr>
<tr>
<td>E. coli</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>25 NT</td>
<td></td>
</tr>
<tr>
<td>K. pneumonia</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>24 NT</td>
<td></td>
</tr>
<tr>
<td>S. dysenteriae</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>24 NT</td>
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<tr>
<td>P. vulgaris</td>
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<td>23 NT</td>
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<tr>
<td>S. parathyphi-A</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>28 NT</td>
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<tr>
<td>B. subtilis</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>25 NT</td>
<td></td>
</tr>
<tr>
<td>S. aureus</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>28 NT</td>
<td></td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>* * * * * * * * * * * * * * * * * *</td>
<td>22 NT</td>
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<tr>
<td>C. albicans</td>
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<td>A. brasiliensis</td>
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<td>NT 33</td>
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</table>

*Not Active.
NT: not tested.

Table 3. Minimum inhibitory concentration (MIC in µg/ml) values of the effective synthesized compounds.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>Products</th>
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<tbody>
<tr>
<td></td>
<td>6h</td>
</tr>
<tr>
<td>B. subtilis</td>
<td>2000</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>250</td>
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REFERENCES


42. Kitazaki T, Tamura N, Tasaka A, Matsushita Y, Hayashi R, Okonogi K, et al. Optically Active Antifungal Azoles. VI. Synthesis and Antifungal Activity of N-(1R,2R)-2-(2,4-Difluorophenyl)-2-hydroxy-1-methyl-3-(1H,1,2,4-triazol-1-yl)(propyl)-N’-(4-substituted phenyl)-3(2H,4H)-1,2,4-triazolones and 5(1H,4H)-tetrazolones. CHEMICAL & PHARMACEUTICAL BULLETIN. 1996;44(2):314-27.
