Synthesis, Characterization and Study of Antimicrobial Activities of Mannich Bases Incorporating 1,2,4-Triazole Nucleus

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Highlights
- Some new Mannich bases incorporating 1,2,4-triazole nucleus were synthesized.
- Mannich bases were characterized by spectral techniques & antimicrobial activities studied.
- Mannich bases were much more effective than Schiff base against microbial strains.
- Antimicrobial activities influenced by the bulkiness of substituent & electron density at N1 of triazole nucleus.

Abstract
Heterocyclic compounds containing triazole moiety have great importance in the field of medicine, pharmaceuticals, biochemistry, biology, therapeutics, environmental science, and industry. Triazoles and their derivatives have been extensively used in the development of new drugs. Biological activities of Schiff bases are highly investigated, but Mannich bases are on the verge of their development, and they are being synthesized in large number nowadays. In this work, Mannich bases are synthesized by incorporation 1,2,4-triazole moiety through Schiff base using different amines. Mannich bases are found to exhibit highly effective antibacterial and antifungal activities. The formation of synthesized compounds - 1,2,4-triazole-5-thione, Schiff base (4) and Mannich bases (5a and 5b) - are confirmed and characterized by spectroscopic techniques like UV, FTIR, 1H-NMR and 13C-NMR. The activity of the synthesized compounds was tested against bacterial and fungal strain.

Keywords: 1,2,4-triazole, Schiff base, Mannich base, Antibacterial, Antifungal

Introduction
The increasing drug resistant capacity of microbes demands the development of new drugs with multisite mechanism to deprive the pathogenicity of microbes. Heterocyclic chemistry has been chosen as the most useful branch to design the required composition [1-3]. Heterocyclic chemistry has its own pattern in the synthesis of drugs [4], pesticides [5] and detergents [6] by using reagents and synthetic methods. It is widely used in the fields of pharmacology, medicinal chemistry, biochemistry [7], polymers [8, 9], dyes [10, 11] and material science [12]. Nowadays, the nucleus of triazole is used as the main structure for synthesizing much known marketed drugs. On comparison to other nuclei, it is found that triazoles are less susceptible to metabolic degradation and have excellent target specificity with a wide range of activities [13, 14].

1,2,4-triazole derivatives possess biological activities including antibacterial [15, 16], antifungal [17-19], antitubercular [20], antiviral [21], antimalarial [22], anti-inflammatory [23], anti-convulsant [24], anticancer [25, 26], analgesic [27], antioxidant [28], antimigraine [29] and potassium channel activators [30]. Along with these, it has agricultural, industrial, environmental activities. Some of the known drugs having 1,2,4-triazole nucleus are Triazolam [31], Alprazolam [32], Fluconazole, Voriconazole [33, 34], Furacilyin [35], Etizolam [36], etc.
The incorporation of a heterocyclic ring is one of the most investigated reactions in the synthesis of new drugs. One of the most important organic reaction which uses heterocyclic compounds is Mannich reaction. It involves aminoalkylation of an acidic proton next to a carbonyl functional group with formaldehyde and ammonia or any primary amine or secondary amine to yield β-aminoketone also called as Mannich base. Mannich bases can be easily converted to numerous other compounds by changing the functional group. Mannich bases have pharmacological activities like analgesic, anti-inflammatory, anaesthetic and antimicrobial activities and are used as intermediates in drug synthesis as well [37, 38].

Mannich bases were synthesized through Mannich reaction using diphenylamine and piperazine with a 1,2,4-triazole moiety having Schiff base.

Materials and Methods

Starting Materials

The precursors for the synthesis of triazole viz. Hydrazine monohydrate and formaldehyde (Qualigens), carbon disulphide and diphenylamine (Merck), ethanol (Changshu Honsgsheng Fine Chemical), anisaldehyde and methyl salicylate (Fischer scientific), piperazine (Loba Chemie) were purchased from commercial sources and were used as received.

Physical Measurements

Optics technology melting apparatus was used to determine the melting points of synthesized compounds. TLC of synthesized compounds was done by using silica gel coated plates, using n-hexane: ethyl acetate solvent system and the spots were visualized by using iodine vapours in an iodine chamber. The UV-visible spectrophotometer of Labtronics (Model LT-2802) was used to record the visible absorption spectra in DMSO of the synthesized compounds in the region 1100-200 nm. FT-IR spectra were measured in the range of (4000-400) cm⁻¹. The measurement was done in IR prestige-21, Shimadzu, Japan. ¹H-NMR and ¹³C-NMR spectroscopy of synthesized compounds were recorded on Bruker AV III 500MHz NMR spectrometer using DMSO as the solvent, and chemical shifts are expressed in δ ppm.

Synthesis and Analysis

![Scheme 1: Synthetic route for Mannich base of 1,2,4-triazole](image_url)

Synthesis of 2-hydroxybenzohydrazide (1)

A mixture of methyl salicylate (0.06 mol) and 99% hydrazine monohydrate (0.09 mol) was refluxed for 6 hours. Total volume was reduced to half by evaporating the excess solvent and was cooled. The solid was separated by suction filtration, washed with cold ethanol, recrystallized from absolute ethanol and dried in an air oven at 50-60 °C. Yield: 80 % (7.325 g, 0.048 mol); white shining crystalline solid; m.p.:147-150 °C; R₇; 0.66 (n-hexane: ethyl acetate, 8:2).
**Synthesis of Potassium-2-(2-hydroxybenzoyl)hydrazinecarbodithioate (2)**

2-hydroxybenzohydrazide (I) (0.03 mol) was added to 20 mL ethanolic solution of potassium hydroxide (0.03 mol) at ice-cold condition. Carbon disulphide (0.03 mol) was added dropwise, and the reaction mixture was stirred for 21 h at room temperature. The mixture was diluted with 20 mL of anhydrous diethyl ether, the crude solid was washed twice with anhydrous diethyl ether and dried in a desiccator. Yield: 64 % (5.103 g, 0.0191 mol); white crystalline solid; m.p.: 240 °C; Rf : 0.58 (n-hexane : ethyl acetate, 8:2).

**Synthesis of 4-amino-3-(2-hydroxyphenyl)-1H-1,2,4-triazole-5-thione (3)**

A suspension containing potassium-2-(2-hydroxybenzoyl) hydrazinecarbodithioate (2) (0.015 mol) in 5 mL distilled water and 1.48 mL of hydrazine monohydrate was refluxed till hydrogen sulphide gas was ceased. The reaction mixture was cooled, diluted with 100 mL of water containing some crushed ice and acidified with conc. hydrochloric acid. The solid was filtered, washed and recrystallized with absolute ethanol. Yield: 81 % (2.541 g, 0.0122 mol); white crystalline solid; m.p.: 168 ℃; Rf : 0.72 (n-hexane : ethyl acetate, 6:4).

**Synthesis of Schiff base 3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-1H-1,2,4-triazole-5-thione (4)**

Anisaldehyde (0.01 mol) and conc. sulphuric acid (5 drops) were added to a hot ethanol solution of triazole thione (3) (0.01 mol), and refluxed for 5 hours. The mixture was filtered, under suction, washed with cold ethanol and recrystallized with hot ethanol. Yield: 62 % (2.051 g, 0.006 mol); yellowish white crystalline solid; m.p.: 180 ℃; Rf : 0.35 (n-hexane : ethyl acetate, 8:2). UV–Visible spectrum (λmax) nm = 302, 309, 331, 353. IR spectrum cm–1 = 3287, 3186, 3063, 1612, 1590, 1543, 1489, 1296, 1011, 946, 741. 1H NMR (500MHz, DMSO-d6) δ ppm = 13.86 (br s, 1H, Triazole NH), 9.38 (d, J = 8.2 Hz, 1H, Ar-H), 8.37 (d, J = 8.2 Hz, 1H, Ar-H), 7.66-7.67 (m, 2H, Ar-H), 7.30–7.51 (m, 2H, Ar-H), 7.04 (m, 1H, Ar-H), 6.93–7.02 (m, 2H, Ar-H), 6.91 (d, J = 7.57 Hz, 1H, Ar-H), 6.91 (d, J = 7.57 Hz, 1H, Ar-H), 6.85–6.93 (m, 1H, Ar-H), 5.14 (br s, 2H, N-CH2-N), 3.87 (br s, 4H, Piperazine-CH2), 3.79–3.85 (m, 7H, Piperazine-CH2). **13C NMR (100MHz, DMSO-d6) δ ppm = 165.54(Triazole-C5), 156.53(Ar-C), 149(Triazole-C3), 132.60(Ar-C), 119.53(Ar-C), 116.67(Ar-C), 113.52 (Ar-C).

**Synthesis of Mannich bases**

40% formaldehyde (0.2 mL) and desired amine (0.003 mol) were added to a hot ethanolic solution of triazole thione (3) (0.01 mol), and refluxed for 5 hours. An excess amount of distilled water was added and left overnight. The solid was filtered under suction, washed with cold ethanol and recrystallized from absolute ethanol.

*2-(diphenylamino)methyl-3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-1H-1,2,4-triazole-5-thione (5a):* Yield: 60 % (0.916 g, 0.0018 mol); yellowish white crystalline solid; m.p.:122 °C; Rf : 0.72 (n-hexane : ethyl acetate, 8:2). UV–Visible spectrum (λmax) nm = 302, 309, 336, 379. IR spectrum cm–1 = 3286(m), 3249(m), 3061(m), 2916(m), 1597(m), 1551(m), 1504(m), 1481(s), 1304(m), 1234(s) 1026(m), 825(m). 1H NMR (500 MHz, DMSO-d6) δ ppm = 10.09 (s, 1H, Triazole NH), 10.90 (br s, 1H, OH), 9.37 (br s, 1H, N=CH), 7.94 (dd, J = 8.20 Hz, 1H, Ar-H), 7.66-7.78 (dd, J = 8.20, 7.57 Hz, 1H, Ar-H), 7.30–7.51 (m, 2H, Ar-H), 7.04 (m, 1H, Ar-H), 6.93-7.02 (m, 2H, Ar-H), 6.91 (d, J = 7.57 Hz, 1H, Ar-H), 6.91 (d, J = 7.57 Hz, 1H, Ar-H), 6.85–6.93 (m, 1H, Ar-H), 5.62 (br s, 2H, NH2). 13C–NMR δ, ppm = 166.29(Ar-C), 163.30(Triazole-C5), 159.00(Triazole-C3), 156.61(Ar-C), 148.67(Ar-C), 134.55(Ar-C), 131.57(Ar-C), 131.05(Ar-C), 129.30(Ar-C), 119.71(Ar-C), 117.73(Ar-C), 115.41(Ar-C), 115.06(Ar-C), 56.00(OCH3).

*3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-2-(piperazin-1-ylmethyl)-1H-1,2,4-triazole-5-thione (5b):* Yield: 65 % (0.829 g, 0.0020 mol); Greyish white crystalline solid; m.p.: 132 °C; Rf : 0.89 (n-hexane : ethyl acetate, 8:2). UV–Visible spectrum (λmax) nm = 302, 309, 339, 379. IR spectrum cm–1 = 3248, 3148, 3070, 2831, 1628, 1597, 1489, 1450, 1304, 1242, 1026, 825, 741. 1H NMR (500MHz, DMSO-d6) δ ppm = 10.11 (br s, 1H, OH), 9.30 (s, 1H, N=CH), 7.88 (d, J = 8.83 Hz, 1H, Ar-H), 7.76 (d, J = 8.83 Hz, 1H, Ar-H), 7.71 (d, J = 8.20 Hz, 2H, Ar-H), 7.41-7.48 (m, 1H, Ar-H), 7.14 (d, J = 8.2 Hz, 2H, Ar-H), 6.90-7.01 (d, J = 8.20 Hz, 1H, Ar-H), 5.14 (br s, 2H, N-CH2-N), 3.87 (br s, 4H, Piperazine-CH2), 3.79–3.85 (m, 7H, Piperazine-CH2 & OCH3), 2.80 (s, 1H, Piperazine-NH). 13C–NMR (500MHz, DMSO-d6) δ ppm = 191.80(Triazole-C5), 165.13(Ar-C), 156.69(Ar-C), 146.66(Triazole-C5), 134.57(Ar-C), 131.51(Ar-C), 129.59(Ar-C), 129.28(Ar-C), 124.88(Ar-C), 123.01(Ar-C), 121.92(Ar-C), 120.11(Ar-C), 117.74(Ar-C), 115.10(Ar-C), 115.00(Ar-C), 64.93(N-CH2-N), 56.03(OCH3).
Antimicrobial Screening

The antimicrobial activity of the newly synthesized Mannich bases was screened against gram-positive bacterial strain - *Bacillus subtilis*, *Enterococcus faecalis*, *Staphylococcus aureus*; gram-negative bacterial strain - *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella dysenteriae*; and fungal strain- *Candida albicans*, *Saccharomyces cerevisiae*. Required numbers of colonies of freshly cultured (within 18–24 hours) test organisms were inoculated aseptically to a tube containing 5 mL of sterilized nutrient broth and homogenized by vortexing. The synthesized compounds were screened for antimicrobial activity using agar well diffusion methods as described by Perez [39]. Swabbing was done by using the appropriate medium Muller-Hinton Agar (MHA) for bacteria and Muller-Hinton Agar with Glucose and Methylene Blue (MHA, GMB) for fungi. The inoculated plates were then incubated in an inverted position at a suitable temperature (35±2 °C for bacteria and 25±2 °C for fungi), and zone of inhibition (ZOI) were examined around the well, which was suggested by a clear area with no growth of organisms. Activity of compounds were compared with Chloramphenicol as standard (Positive Control) for bacteria while Clotrimazole was used as standard (Positive Control) to analyze fungi.

Results and Discussion

Chemistry

Mannich bases 5a and 5b were synthesized from Schiff’s base having 1,2,4-triazole-5-thione moiety as shown in scheme 1. Hydrazine hydrate and ethanol were mixed to the methyl salicylate and refluxed to obtain acid hydrazide (1), which on reaction with alc. potassium hydroxide and carbon disulphide gave dithiocarbazinate (2). Ring closure of compound (2) in the presence of excess hydrazine hydrate in ethanol results 4-amino-3-(2-hydroxyphenyl)-1H-1,2,4-triazole-5-thione (3). 3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-1H-1,2,4-triazole-5-thione (4) is formed by the condensation of 1,2,4-triazole (3) with anisaldehyde on refluxing ethanolic solution in the presence of a catalytic amount of conc. sulphuric acid. Finally, Mannich base (5a) was obtained by refluxing formaldehyde, ethanolic solution of Schiff base (4) and diphenylamine, whereas (5b) was obtained by refluxing formaldehyde, ethanolic solution of Schiff base (4) and piperazine.

Spectroscopic Studies

The spectral techniques UV, FT-IR, 1H NMR, 13C NMR were used to confirm the structure of synthesized compounds (3), (4), (5a) and (5b).

UV-Visible analysis

In UV-spectra triazole thione (3) exhibited four bands while Schiff base (4) and Mannich bases (5a&5b) exhibited five bands. The first two bands observed at 302 nm and 309 nm are attributed to aromatic C=C and azomethine C=N (π→π*) of triazole ring, respectively. The third band at 331 nm (in compound 3), 336 nm (in Schiff base 4), 338 nm (in Mannich base 5a) and 339 nm (in Mannich base 5b) is due to non-bonding electron pair of triazole nitrogen atoms and sulphur atom of thione group C=S (n→π*). The fourth band at 353 nm (in compound 3) and 355 nm (in Schiff base and Mannich bases) is due to(n→π*) transition of the α-hydroxy group. The fifth band at 378 (5a) and 379 nm (4 & 5b), indicates azomethine group C=N [40].

FT-IR analysis

The formation of triazole is confirmed by the presence of a medium band at 1296 cm⁻¹ and a strong band at 946 cm⁻¹ corresponding to thioamide II (N-C=S) and thioamide IV (C=S). Furthermore, no absorption bands were detected about 1651-1707 cm⁻¹, indicating the absence of the C=O group of the compound, which is the evidence for the conversion of dithiocarbazinate into triazoles [41]. The lack of band at 1700 cm⁻¹ clearly indicates the amino condensation and hence the formation of Schiff bases [42]. This is further supported by the absence of medium intensity bands in the region of 3500-3200 cm⁻¹ attributable to –NH, stretching [43]. Moreover, the absence of an absorption band in the region of 2300-2600 cm⁻¹ region cited for the –SH group clearly states that, in the solid-state, the compound exists predominantly in the thionic form [44, 45]. The medium absorption band found at the region of 1303 cm⁻¹ refers to the N-C=S (thioamide II) group in (5a), whereas the same group is found at the
region of 1304 cm\(^{-1}\) in (5b). For the thioamide IV group, the absorption bands are observed at 833 cm\(^{-1}\) and 825 cm\(^{-1}\) respectively for (5a) and (5b). The formation of Mannich bases (5a) and (5b) is supported by the presence of medium absorption band at 1303 cm\(^{-1}\) and weak absorption band at 1304 cm\(^{-1}\) respectively due to the N-C=S group. Besides, the absence of absorption band at 2250 cm\(^{-1}\) due to S-H structure supports the formation of N- Mannich bases but not S- Mannich bases [42].

**1\(^{1}\)HNMR**

In the \(^{1}\)HNMR spectrum of compound (3), the broad singlet at 13.86 ppm is attributed to the –NH group, which suggests the formation of thione based triazole [46]. This event is also supported by the absence of IR absorption band at 2600 cm\(^{-1}\) due to the thiol group. The Schiff base (4) contains the singlet at 14.04 ppm due to the presence of the NH proton. The singlet at 10.06 ppm is attributed to the hydrogen of the OH group. Moreover, the \(^{1}\)H – NMR spectrum of Schiff base showed a singlet at 9.37 due to the presence of –N=CH group. The confirmation of formation of the Schiff base is done by the absence of signals approximately at 5.76 ppm (NH\(_2\)) in the molecule.In compound (5a) and (5b), the peak obtained due to the –NH proton is absent. The signal due piperazine ring proton (CH\(_2\), 5b) was found to be quartets at 3.87 ppm [47] and while that due to the –NH of piperazine was found to be broad singlet at 2.80 ppm. The singlets at 6.16 ppm and 5.14 ppm in the spectra of (5a) and (5b) respectively due to the N-CH\(_2\)-N group confirms the formation of Mannich bases from the Schiff base. The absence of a peak at 11.5 ppm clearly suggests the absence of thiol group in the structure, and hence the formation of thione based Mannich base [42].

**\(^{13}\)CNMR**

The peak corresponding to resonance of triazole C5, i.e. C=S is observed at 165.54, 163.30, 191.80 and 191.80 ppm, respectively in the compounds (3), (4), (5a) & (5b)[47]. The signal of C=N is observed at 149.62, 148.67, 146.66 and 149.13 ppm for compounds (3), (4), (5a) & (5b), respectively. The signal at 156.61 ppm attributable to the azomethine carbon confirms the formation of the Schiff base [48]. Similarly, the peaks observed at 156.61 and 156.64 ppm were associated with the –N=CH group in compound (5a) & (5b), respectively. In the spectra (5b), the piperazine carbons exhibited resonance in the region of 55.08 and 56.02 ppm. The methoxy carbon in the compound (4), (5a)& (5b) are found to give peaks at 56.00, 56.03, and 56.16 ppm, respectively. The formation of Mannich bases (5a)& (5b) was confirmed by a peak at 64.93 ppm and 69.40 ppm, respectively.

**Antimicrobial Screening**

The antibacterial activity of the synthesized compounds exhibited moderate activity against the tested bacterial strains using Chloramphenicolas standard (positive control). Mannich bases (5a) and (5b) are more active than the Schiff base (4) against the tested bacterial strain. Compound (5b) showed more potent activity than (5a) against many bacterial strains. Both bases (5a) and (5b) have shown high activity against P. vulgaris in comparison to other tested bacterial strain, whereas compound (4) is ineffective with P. vulgaris. B. subtilis shows no response towards both the Mannich bases but the Schiff base (4) has some effect on it.

Table 1. Inhibition zones showing antimicrobial activities of Mannich bases and reference antibiotic

| Strain               | Diameter of zone of inhibition (mm) | 4 | 5a | 5b | Chloramphenicol (PC) | Clotrimazole (PC) |
|----------------------|-------------------------------------|---|----|----|----------------------|-------------------|
| Bacillus subtilis\(^{a}\) |                                     | 7.1 | 0.0 | 0.0 | 26.58                | NT                |
| Enterococcus faecalis\(^{a}\) |                                   | 0.0 | 8.49 | 8.34 | 20.86                | NT                |
| Escherichia coli\(^{b}\)    |                                     | 6.8 | 7.48 | 8.95 | 22.75                | NT                |
| Klebsiella pneumoniae\(^{b}\) |                                  | 8.86 | 7.4  | 10.22 | 12.28                | NT                |
| Proteus vulgaris\(^{b}\)   |                                     | 0.0 | 10.45 | 11.94 | 0.0                  | NT                |
| Pseudomonas aeruginosa\(^{b}\) |                              | 7.6 | 7.58 | 9.53 | 12.28                | NT                |
| Salmonella typhi\(^{b}\)   |                                     | 7.66 | 7.30 | 8.93 | 27.44                | NT                |
| Shigella dysenteriae\(^{b}\) |                                   | 7.12 | 0.0  | 8.52 | 28.99                | NT                |
| Staphylococcus aureus\(^{b}\) |                                | 7.78 | 9.82 | 12.30 | 28.40                | NT                |
| Candida albicans\(^{c}\)  |                                     | 9.0  | 15.55 | 16.45 | NT                  | 32.33             |
| Saccharomyces cerevisiae\(^{c}\) |                               | 10.02 | 11.84 | 14.32 | NT                  | 24.33             |

\(^{a}\)Gram-positive bacteria; \(^{b}\)Gram-negative bacteria; \(^{c}\)Fungal strain; NT = Not Tested
The synthesized compounds exhibited moderate activity against fungal strains using Clotrimazole as standard (positive control). Mannich bases (5a) & (5b) are effective than Schiff base (4). Against fungal strains, Mannich bases (5b) showed more potent activity than (5a). The Schiff base (4) is more effective against \textit{S. cerevisiae} than \textit{C. albicans}, whereas Mannich bases are more effective against \textit{C. albicans} than \textit{S. cerevisiae}. Compound 5b with a more basic and less bulky substituent at \textit{N}1 of triazole nucleus exhibited comparatively more potent antimicrobial activity.

![Fig 1. Antimicrobial activity of Schiff base against (a) Staphylococcus aureus (Gram +ve bacteria) (b) Klebsiella pneumoniae (Gram –ve bacteria) and (c) Candida albicans (Fungi)](image)

![Fig 2. Antimicrobial activity of Mannich base against (a) Staphylococcus aureus (Gram +ve bacteria) (b) Klebsiella pneumoniae (Gram –ve bacteria) and (c) Candida albicans (Fungi)](image)

**Conclusions**

Schiff base 3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-1H-1,2,4-triazole-5-thione (4); Mannich bases 2-(diphenylamino)methyl-3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-1,2,4-triazole-5-thione (5a) and 3-(2-hydroxyphenyl)-4-(4-methoxybenzylideneamino)-2-(piperazin-1-ylmethyl)-1,2,4-triazole-5-thione (5b) were prepared successfully in lab. The characterization of newly synthesized compounds was done by spectroscopic techniques (UV, FTIR, and NMR). The synthesized compounds showed moderate activities against bacterial strain and were found to be very effective against the fungal strains. The Mannich bases (5a & 5b) are found to be much more effective than Schiff base (4) against bacterial strains as well as fungal strains. The difference in activities of 5a and 5b suggest that antimicrobial activities of Mannich bases are influenced by the bulkiness of the substituent and electron density at \textit{N}1 of triazole nucleus.

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