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Click Chemistry Approach for Bis-Chromenyl Triazole Hybrids and Their Antitubercular Activity

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1,4-Disubstituted bis-chromenyl triazole hybrids 5a–m have been synthesized in a three-step reaction sequence from 4-(bromomethyl)-2*H*-chromen-2-ones 3a–m. The intermediate azides 4a–m underwent a regioselective 1,3-dipolar cycloaddition with a 2*H*-chromen-2-one linked acetylenic dipolarophile in the presence of Cu (II)/ascorbate/water/n-butanol reaction medium. Three compounds 5h–j exhibited 6.25 μg/mL MIC against *M. tuberculosis*. Among the compounds screened for antifungal activity, lowest MIC of 6.25 μg/mL was observed for 5c against *A. niger* that also exhibited DNA cleavage observed by agarose gel electrophoresis. All the compounds were moderately active against both Gram-positive and Gram-negative bacterial strains. The cytotoxic effect of potent compounds on normal cells (V79 and HBL100) was assessed by MTT assay.

Key words: 1,2,3-triazole, 2*H*-chromen-2-one, antibacterial, antifungal, antitubercular, click reaction, cytotoxic

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Designing drugs for chemotherapy of tuberculosis continues to be an active area of medicinal chemistry in view of the rapid emergence of multidrug-resistant strains. Molecular and structural diversity associated with heterocycles have made them the most promising candidates for antitubercular activity (1). Pharmacophoric moieties like hydrazone, guanidine, β-amino ethanol, β-amino glycoside, etc. have been incorporated in quinoline (2), triazole (3), 2*H*-chromen-2-one (4), benzofuran (5), thiophene (6), etc., which have resulted in potent antitubercular compounds exhibiting low minimum inhibitory concentrations (Figure 1). In view of their extensive natural occurrence and biocompatibility, 2*H*-chromen-2-ones have been found to exhibit variety of biological activities (7). 2*H*-chromen-2-one derivatives possessing hydrazone moiety, akin to naturally occurring calanolide (8), have recently been screened against *M. tuberculosis* (9). Biodegradation of 2*H*-chromen-2-ones leads to *in situ* generation of carboxylic and phenolic -OH groups that might facilitate the penetration through the cell wall of the bacterial species (10). 1,2,3-Triazoles have been the nuclei of choice in recent years because of their excellent pharmacokinetic characteristics, favourable safety profile, latent ability for the formation of hydrogen bonds, moderate dipole character, rigidity and stability under *in vivo* conditions (11). Further, they have also been found to be inhibitors of thymidine monophosphate kinase (TMP Kinase), which catalyses the phosphorylation of deoxythymidine monophosphate (dTMP) to deoxythymidine diphosphate (dTDP) utilizing ATP as a phosphoryl donor (12).

'Click' chemistry has emerged as a new trend for constructing 1,2,3-triazoles through exergonic Huisgen 1,3-dipolar cycloaddition of alkynes to azides (13). Classical Huisgen 1,3-dipolar cycloaddition with unsymmetrical dipolarophiles leads to a mixture of 1,4- and 1,5-regioisomers. Innovations in click chemistry have demonstrated the use of copper and ruthenium salts under aqueous conditions for the regioselective synthesis of 1,4 and 1,5-disubstituted triazoles, respectively, which cannot be achieved in the conventional Huisgen approaches. 1,4-Substituted triazoles were synthesized by copper-catalyzed azide alkyne cycloaddition reaction using sodium ascorbate as reducing agent (14), and synthesis of the same compound with 1,5-substitution was achieved by using ruthenium catalyst (15). For 1,4-substituted triazoles, the origin of the observed regioselectivity lies in the inherent ability of Cu(I) to form an *in situ* covalent bond with the acetylenic dipolariphile and coordinate exclusively with the nitrogen that is linked to the R-group in the azide. The nucleophilic attack of the anionic nitrogen on the other actylenic carbon is followed by ring closure, which is the rate-determining step in this stepwise mechanism. This pathway was predicted by DFT calculations (16) and has been supported by a real-time infrared analysis (17). Unusual anisotropic effects observed in the DMAD adducts of 4-azido methyl 2*H*-chromen-2-ones have been reported by our group (18). In the light of the above observations, it was thought of considerable interest to undertake a regioselective synthesis of 2*H*-chromen-2-one triazole hybrids as potential antitubercular and antimicrobial agents using click chemistry conditions.

Experimental Section

Materials and methods

The melting points were determined by open capillary method and are uncorrected. The IR spectra (KBr disc) were recorded on a Nico-

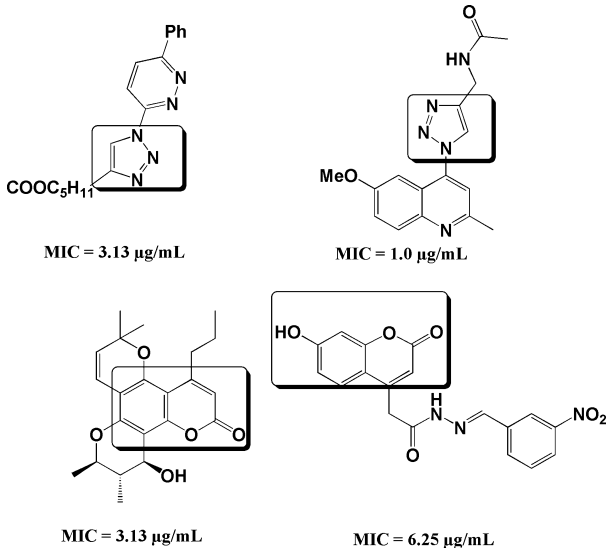


Figure 1: Antimycobacterial agents possessing triazole and 2*H*-chromen-2-one moieties.

let-5700 FT-IR spectrophotometer. ^{1}H-NMR spectra were recorded on Bruker 300 MHz spectrometer using $CDCl_3$ and DMSO-d_6 as solvents and TMS as an internal standard (Figure S1). The chemical shifts are expressed in δ ppm. The mass spectra were recorded using Agilent-single Quartz GC-MS. The elemental analysis was carried out using Heraus CHN rapid analyzer. The purity of the compound was checked by T.L.C. All the chemicals purchased were of analytical grade and were used without further purification unless otherwise stated.

Procedure for the synthesis of 4-methyl-7-(prop-2-ynyloxy)-2H-chromen-2-one (2)

To a solution of 7-hydroxy-4-methyl-2*H*-chromen-2-one **1** (0.01 mol equiv) in dry acetone, anhydrous potassium carbonate (0.01 mol equiv) and 3-bromoprop-1-yne (0.01 mol equiv) were added. The resultant mixture was stirred at 50 °C for 18 h, cooled and the solvent was removed under reduced pressure. The residue was treated with 50 mL of cold water, and the so-obtained white solid was filtered and washed with water (19). The crude product was purified by crystallization from ethyl alcohol. White solid; yield, 95%; mp 132–134 °C, IR (KBr) per cm 1726 (C=O), 3304 (C≡C–H); ^{1}H-NMR ($CDCl_3$, 300 MHz, TMS): δ ppm 2.71 (s, 3H, CH_3), 2.59 (s, 1H, C≡C–H), 4.77 (s, 2H, –CH_2O–), 6.16 (s, 1H, C_3-H), 6.95–6.93 (m, 2H, C_8-H & C_6-H), 7.55–7.52 (m, 1H, C_5-H). MS m/z 214. Anal. Calcd for $C_{13}H_{10}O_3$ (%): Calcd. C, 72.89; H, 4.71; Found: C, 72.76; H, 4.78.

General procedure for the synthesis of 4-(bromomethyl)-2H-chromen-2-ones (3a–m)

The required 4-(bromomethyl)-2*H*-chromen-2-ones (20,21) have been synthesized by the Pechmann cyclization of substituted phenols with ethyl 4-bromoacetoacetate (22).

General procedure for the synthesis of 4-(azidomethyl)-2H-chromen-2-ones (4a–m)

4-(Bromomethyl)-2*H*-chromen-2-ones **3a**–**m** (0.01 mol equiv) was taken in 20 mL of acetone in a round-bottom flask. To this, sodium azide (0.012 mol equiv) in 3 mL of water was added drop wise with stirring, which was continued for 10 h (reaction was monitored by TLC). The reaction mixture was then poured in to ice-cold water. Separated solid was filtered and recrystallized using ethanol (18,23). Among azides, four azides (**4e, 4f, 4l, 4m**) are newly synthesized.

4-(Azidomethyl)-5,7-dimethyl-2H-chromen-2-one (4e)

Colourless solid (ethanol); yield, 72%; mp 156–158 °C. IR (KBr) per cm 1711 (C=O), 2110 (N_3); ^{1}H-NMR ($CDCl_3$, 400 MHz, TMS): δ ppm 2.37 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 4.65 (s, 2H, –CH_2N–), 6.45 (s, 1H, C_3-H), 6.91 (s, 1H, C_6-H), 7.02 (s, 1H, C_8-H). MS m/z 229. Anal. Calcd for $C_{12}H_{11}N_3O_2$ (%): Calcd. C, 62.87; H, 4.84; N, 18.33; Found: C, 62.73; H, 4.82; N, 18.24.

4-(Azidomethyl)-7,8-dimethyl-2H-chromen-2-one (4f)

Colourless solid (ethanol); yield, 70%; mp 130–132 °C. IR (KBr) per cm 1715 (C=O), 2117 (N_3); ^{1}H-NMR ($CDCl_3$, 400 MHz, TMS): δ ppm 2.38 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 4.54 (s, 2H, –CH_2N–), 6.46 (s, 1H, C_3-H), 7.12 (d, 1H, C_6-H, J = 8Hz), 7.27 (d, 1H, C_5-H, J = 8Hz). MS m/z 229. Anal. Calcd for $C_{12}H_{11}N_3O_2$ (%): Calcd. C, 62.87; H, 4.84; N, 18.33; Found: C, 62.82; H, 4.79; N, 18.29.

4-(Azidomethyl)-6-bromo-2H-chromen-2-one (4l)

Yield solid (ethanol); yield, 74%; mp 120–122 °C. IR (KBr) per cm 1719 (C=O), 2132(N_3); ^{1}H- NMR ($CDCl_3$, 400 MHz, TMS): δ ppm 4.55 (s, 2H, –CH_2N–), 6.56 (s, 1H, C_3-H), 7.25–7.66 (m, 3H, Ar-H). MS m/z 279. Anal. Calcd for $C_{10}H_6BrN_3O_2$ (%): Calcd. C, 42.88; H, 2.16; N, 15.00; Found: C, 42.78; H, 2.12; N, 14.98.

4-(Azidomethyl)-7-methyl-6,8-dinitro-2H-chromen-2-one (4m)

Yield solid (ethanol); yield, 74%; mp 122–124 °C. IR (KBr) per cm 1750 (C=O), 2117 (N_3); ^{1}H-NMR ($CDCl_3$, 400 MHz, TMS): δ ppm 2.61 (s, 3H, CH_3), 4.65 (s, 2H, –CH_2N–), 6.70 (s, 1H, C_3-H), 8.32 (s, 1H, C_5-H). MS m/z 305. Anal. Calcd for $C_{11}H_7N_5O_6$ (%): Calcd. C, 43.29; H, 2.31; N, 22.95; Found: C, 43.22; H, 2.30; N, 22.92.

General procedure for the synthesis of 4-methyl-7-((1-((2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-ones (5a–m)

To a solution of compound **2** (1.0 molar equiv) in tert-BuOH/H_2O 1/1(v/v), $CuSO_4$. $5H_2O$ (0.15 molar equiv) and sodium ascorbate (0.30 molar equiv) were added. The mixture was stirred at room temperature for 15 min. Then azide **4a**–**m** (1.0 molar equiv) were

added, and the resulting reaction mixture was refluxed on water bath until the starting material was consumed as judged by TLC. Then the reaction mixture was cooled, obtained solid was filtered and washed with water and recrystallized from DMF.

4-Methyl-7-((1-((6-methyl-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5a)

White solid; yield, 97%; mp 236–238 °C, IR (KBr) per cm 1741 (C=O), 1742 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.37 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 5.33 (s, 2H, –CH_2O–), 5.78 (s, 1H, C_3-H), 6.00 (s, 2H, –CH_2N), 6.23 (s, 1H, C_3-H), 7.03–7.70 (m, 6H, Ar-H), 8.45 (s, 1H, Tri-H). MS m/z 429. Anal. Calcd for $C_{24}H_{19}N_3O_5$ (%): Calcd. C, 67.13; H, 4.46; N, 9.79; Found: C, 67.12; H, 4.42; N, 9.82.

4-Methyl-7-((1-((7-methyl-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5b)

White solid; yield, 96%; mp 118–120 °C. IR (KBr) per cm 1719 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.42 (s, 6H, 2CH_3), 5.32 (s, 2H, –CH_2O–), 5.80 (s, 1H, C_3-H), 5.98 (s, 2H, –CH_2N), 6.23 (s, 1H, C_3-H), 7.05–7.71 (m, 6H, Ar-H), 8.44 (s, 1H, Tri-H). MS m/z 429. Anal. Calcd for $C_{24}H_{19}N_3O_5$ (%): Calcd. C, 67.13; H, 4.46; N, 9.79; Found: C, 67.15; H, 4.44; N, 9.80.

4-Methyl-7-((1-((6-methoxy-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5c)

White solid; yield, 96%; mp 218–220 °C. IR (KBr) per cm 1711 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s, 3H, CH_3), 3.79 (s, 3H, OCH_3), 5.33 (s, 2H, –CH_2O–), 5.89 (s, 1H, C_3-H), 6.01 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 7.02–7.70 (m, 6H, Ar-H), 8.45 (s, 1H, Tri-H). MS m/z 445. Anal. Calcd for $C_{24}H_{19}N_3O_6$ (%): Calcd. C, 64.72; H, 4.30; N, 9.43; Found: C, 64.74; H, 4.29; N, 9.40.

4-Methyl-7-((1-((7-methoxy-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5d)

White solid; yield, 96%; mp 220–222 °C. IR (KBr) per cm 1711(C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.39 (s, 3H, CH_3), 3.86 (s, 3H, -OCH_3), 5.32 (s, 2H, –CH_2O–), 5.68 (s, 1H, C_3-H), 5.97 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 6.98–7.95 (m, 6H, Ar-H), 8.44 (s, 1H, Tri-H). MS m/z 445. Anal. Calcd for $C_{24}H_{19}N_3O_6$ (%): Calcd. C, 64.72; H, 4.30; N, 9.43; Found: C, 64.75; H, 4.32; N, 9.42.

4-Methyl-7-((1-((5,7-dimethyl-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5e)

White solid; yield, 94%; mp 248–250 °C. IR (KBr) per cm 1712 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.36 (s, 3H, CH_3), 2.40 (s, 3H, CH_3), 2.70 (s, 3H, CH_3), 5.10 (s, 1H, C_3-H), 5.37 (s, 2H, –CH_2O–), 6.19 (s, 2H, –CH_2N), 6.23 (s, 1H, C_3-H), 7.07–7.71 (m, 5H, Ar-H), 8.37 (s, 1H, Tri-H). MS m/z 443. Anal. Calcd for $C_{25}H_{21}N_3O_5$ (%): Calcd. C, 67.71; H, 4.77; N, 9.48; Found: C, 67.75; H, 4.73; N, 9.52.

4-Methyl-7-((1-((7,8-dimethyl-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5f)

White solid; yield, 96%; mp 210–212 °C. IR (KBr) per cm 1714 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.28 (s, 3H, CH_3), 2.36 (s, 3H, CH_3), 2.39 (s, 3H, CH_3), 5.31 (s, 2H, –CH_2O–), 5.80 (s, 1H, C_3-H), 5.97 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 7.02–7.69 (m, 5H, Ar-H), 8.45 (s, 1H, Tri-H). MS m/z 443. Anal. Calcd for $C_{25}H_{21}N_3O_5$ (%): Calcd. C, 67.71; H, 4.77; N, 9.48; Found: C, 67.68; H, 4.76; N, 9.50.

4-Methyl-7-((1-((7-hydroxy-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl) methoxy)-2H-chromen-2-one (5g)

Yellow solid; yield, 93%; mp 224–226 °C. IR (KBr) per cm 1709 (C=O), 3437 (OH); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s, 3H, CH_3), 3.43 (s, 1H, OH), 5.33 (s, 2H, –CH_2O–), 5.57 (s, 1H, C_3-H), 5.95 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 6.80–7.70 (m, 6H, Ar-H), 8.45 (s, 1H, Tri-H). MS m/z 431. Anal. Calcd for $C_{23}H_{17}N_3O_6$ (%): Calcd. C, 64.04; H, 3.97; N, 9.74; Found: C, 64.06; H, 3.97; N, 9.79.

4-Methyl-7-((1-((6-chloro-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5h)

Yellow solid; yield, 95%; mp 190–192 °C. IR (KBr) per cm 1709 (C=O), 1724 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.39 (s, 3H, CH_3), 5.33 (s, 2H, –CH_2O–), 5.90 (s, 1H, C_3-H), 6.02 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 7.03–7.94 (m, 6H, Ar-H), 8.43 (s, 1H, Tri-H). ^{13}C-NMR (DMSO-d_6, 400 MHz, TMS): δ ppm 18.08, 48.99, 61.53, 101.67, 111.33, 112.63, 113.42, 115.05, 118.49, 118.78, 124.31, 125.96, 126.47, 128.59, 132.19, 142.72, 149.08, 151.76, 153.32, 154.61, 158.84, 160.06, 160.86. MS m/z 449. Anal. Calcd for $C_{23}H_{16}ClN_3O_5$ (%): Calcd. C, 61.41; H, 3.59; N, 9.34; Found: C, 61.42; H, 3.57; N, 9.36.

4-Methyl-7-((1-((7-chloro-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5i)

Yellow solid; yield, 94%; mp 232–234 °C IR (KBr) per cm 1713 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s, 3H, CH_3), 5.32 (s, 2H, –CH_2O–), 5.92 (s, 1H, C_3-H), 6.00 (s, 2H, –CH_2N), 6.22 (s, 1H, C_3-H), 7.02–7.87 (m, 6H, Ar-H), 8.43 (s, 1H, Tri-H). MS m/z 449. Anal. Calcd for $C_{23}H_{16}ClN_3O_5$ (%): Calcd. C, 61.41; H, 3.59; N, 9.34; Found: C, 61.43; H, 4.02; N, 9.29.

1-((4-((4-methyl-2-oxo-2H-chromen-7-yloxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)-3H-benzo[f]chromen-3-one (5j)

Yellow solid; yield, 96%; mp 256–258 °C. IR (KBr) per cm 1719.7(C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s,

3H, CH_3), 5.34 (s, 2H, $-CH_2O-$), 5.50 (s, 1H, C_3-H), 6.22 (s, 1H, C_3-H), 6.49 (s, 2H, $-CH_2N$), 7.61–8.46 (m, 10H, Ar-H, Tri-H). MS m/z 465. Anal. Calcd for $C_{27}H_{19}N_3O_5$ (%): Calcd. C, 69.67; H, 4.11; N, 9.03; Found: C, 69.69; H, 4.13; N, 9.02.

4-((4-((4-Methyl-2-oxo-2H-chromen-7-yloxy)methyl)-1H-1,2,3-triazol-1-yl)methyl)-2H-benzo[h]chromen-2-one (5k)

Yellow solid; yield, 96%; mp 230–232 °C. IR (KBr) per cm 1712 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.38 (s, 3H, CH_3), 5.33 (s, 2H, $-CH_2O-$), 5.97 (s, 1H, C_3-H), 6.11 (s, 1H, C_3-H), 6.21 (s, 2H, $-CH_2N$), 7.04–8.48 (m, 10H, Ar-H, Tri-H). MS m/z 465. Anal. Calcd for $C_{27}H_{19}N_3O_5$ (%): Calcd. C, 69.67; H, 4.11; N, 9.03; Found: C, 69.65; H, 4.14; N, 9.01.

4-Methyl-7-((1-((6-bromo-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5l)

Yellow solid; yield, 93%; mp 188–190 °C. IR (KBr) per cm 1723 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s, 3H, CH_3), 5.34 (s, 2H, $-CH_2O-$), 5.89 (s,1H, C_3-H), 6.02 (s, 2H, $-CH_2N$), 6.22 (s, 1H, C_3-H), 7.05–8.06 (m, 6H, Ar-H), 8.43 (s, 1H, Tri-H). ^{13}C-NMR (DMSO-d_6, 400 MHz, TMS): δ ppm 16.75, 47.67, 60.24, 100.38, 110.02, 111.32, 112.12, 113.68, 115.13, 117.64, 117.73, 124.62, 125.15, 125.90, 133.69, 141.40, 147.71, 150.87, 151.99, 153.31, 157.47, 158.73, 159.56. MS m/z 493. Anal. Calcd for $C_{23}H_{16}BrN_3O_5$(%): Calcd. C, 55.89; H, 3.26; N, 8.50; Found: C, 55.91; H, 3.24; N, 8.53.

4-Methyl-7-((1-((7-methyl-6,8-dinitro-2-oxo-2H-chromen-4-yl)methyl)-1H-1,2,3-triazol-4-yl)methoxy)-2H-chromen-2-one (5m)

Yellow solid; yield, 93%; mp 206–208 °C. IR (KBr) per cm 1757 (C=O), 1714 (C=O); ^{1}H-NMR (DMSO-d_6, 300 MHz, TMS): δ ppm 2.40 (s, 3H, CH_3), 2.51 (s, 3H, CH_3), 5.32 (s, 2H, $-CH_2O-$), 6.07 (s, 2H, $-CH_2N$), 6.22 (s, 2H, $2C_3$-H), 7.04–8.41 (m, 4H, Ar-H), 8.72 (s, 1H, Tri-H). MS m/z 519. Anal. Calcd for $C_{24}H_{17}N_5O_9$ (%): Calcd. C, 61.47; H, 4.13; N, 11.47; Found: C, 61.48; H, 4.12; N,11.43.

Biological evaluation

Anti-TB activity using Alamar Blue Dye

Antimycobacterial activity of compounds was assessed against *M. tuberculosis* using microplate Alamar Blue assay (MABA) (24). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with proportional and BACTEC radiometric method. Briefly, 200 μL of sterile deionized water was added to all outer perimeter wells of sterile 96-well plate to minimize evaporation of medium in the test wells during incubation. The 96-well plate received 100 μL of the Middlebrook 7H9 broth, and serial dilution of compounds was made directly on plate. The final drug concentrations tested were 100–0.2 μg/mL. Plates were covered and sealed with parafilm and incubated at 37 °C for 5 days. After this time, 25 μL of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 were added to the plate and incubated for 24 h. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration that prevented the colour change from blue to pink.

Antimicrobial screening

Nine dilutions of each drug have to be performed with BHI for MIC (25). In the initial tube, 20 μL of drug was added into the 380 μL of BHI broth. For dilutions, 200 μL of BHI broth was added into the next nine tubes separately. Then from the initial tube, 200 μL was transferred to the first tube containing 200 μL of BHI broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube, 200 μL was transferred to second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-9} dilution for each drug. From the maintained stock cultures of required organisms, 5 μL was taken and added into 2 mL of BHI (brain heart infusion) broth. In each serially diluted tube, 200 μL of above culture suspension was added. The tubes were incubated for 24 h and observed for turbidity.

Cytotoxic study

The cytotoxic effect of drugs on normal cells (V79 and HBL100) was assessed by MTT assay (26). In brief, exponentially growing cells (1×10^4 cells/well) were plated in 96-well plates and allowed to adhere for 24 h prior to extract addition. The drugs were dissolved in 0.1% DMSO and then diluted with the medium. The cells were then exposed to different concentrations of drug (5–200 μg/mL) for 24 h. The cells in the control wells received medium containing the same volume of DMSO (0.1%). After the incubation, 100 μL of MTT reagent (1 mg/mL in PBS) was added, and cells were incubated for an additional 4 h. The formazan produced by the viable cells was solubilized by addition of 100 μL DMSO. The suspension was placed on a microvibrator for 5 min, and absorbance was recorded at 540 nm by the plate reader (ELx800; BioTek, Winooski, VT, USA). The experiment was performed in triplicate. Doxorubicin was used as positive control. The percentage of growth inhibition was calculated with respect to vehicle control using the formula:

% Inhibition = [{(Control absorbance − Blank absorbance) − (Test absorbance − Blank absorbance)}/(Control absorbance − Blank absorbance)] × 100.

DNA cleavage experiment

Preparation of culture media

Potato dextrose broth (PDB): 250 g of peeled potato was boiled for 20 min and squeezed and filtered (27). To this filtrate, 20 g of dextrose was added, and the volume was made up to 1000 mL by distilled water. The spores of the culture were inoculated in to the autoclaved media and grown at ~27 °C for 48 h.

Isolation of DNA

Two gram of sample was grounded using 25 mL of prechilled mortar and pestle. 1 mL of lysozyme solution was added to the above sus-

pension and incubated at 37 °C for 30 min, shaking occasionally. After the incubation, the lysis was completed by adding 2 mL of sodium dodecyl sulphate (SDS) solution, this preparation was heated for 10 min at a 60 °C water bath, and finally, the solution was cooled to room temperature. 5 M sodium chloride solution was added to the lysed preparation. An equal volume of chloroform–ethanol mixture (24:1) was added to the lysed preparation suspended in 1 M sodium chloride and slowly shaken (30–60 oscillations/min) in a tightly stoppered flask for 30 min at room temperature. The resulting emulsion was separated by centrifugation for 5 min at 10 000 × *g* at room temperature. After centrifuging, the top aqueous solution was carefully pipetted out from the coagulated protein emulsion at the interphase. The pipetted aqueous phase was kept in a beaker. The nucleic acid solution was gently stirred with a sterilized glass rod while slowly adding 95% ethanol down the side of a beaker, so that ethanol is layered over the viscous aqueous phase. The preparation was stirred to mix ethanol throughout the entire aqueous phase, and all the gelatinous thread-like DNA-rich precipitate was spooled off by the glass rod. Excess fluid from the spooled crude DNA was drained off by pressing the rod against the wall of the beaker until no further fluid can be squeezed from the spooled preparation. The crude DNA was dissolved on stirring it in 9 mL of dilute (1/10 fold) saline citrate in a test tube or small beaker. 3 M sodium acetate and 1 mM EDTA were added to the even suspension (pH 7.0). The preparation was transferred to a 100-mL beaker, and the sample was gently swirled by dripping in 5.4 mL isopropanol. The pellet was centrifuged at 8 500 g for 10 min and dried at room temperature. The pellet was dissolved in minimum volume of Tris–HCl buffer (50 mM, pH 8.2).

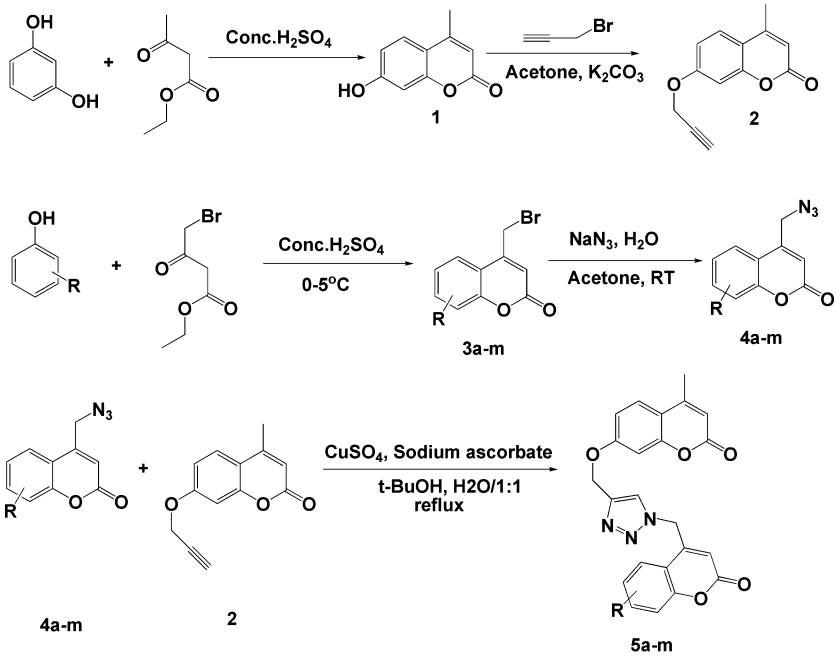
Agarose gel electrophoresis

A total of 200 mg of agarose was weighed and dissolved in 25 mL of TAE buffer (4.84 g Tris base, pH 8.0, 0.5 M EDTA/L) by boiling. When the gel attains ~55 °C, it was poured into the gel cassette fitted with comb. After the gel was solidified, the comb was carefully removed, and the gel was placed in the electrophoresis chamber flooded with TAE buffer. 20 μL of DNA sample (mixed with bromophenol blue dye at 1:1 ratio) was loaded carefully into the wells, along with standard DNA marker and pass the constant 50 V of electricity for around 30 min. The gel was removed and carefully stained with ethidium bromide solution (2 μg/mL) for 10–15 min, and the bands were observed under UV transilluminator (UVP, Germany).

Result and Discussion

Chemistry

Acetylenic dipolarophile used in this investigation, 4-methyl-7-(prop-2-ynyloxy)-2*H*-chromen-2-one **2**, was synthesized by the reaction of 7-hydroxy 4-methyl 2*H*-chromen-2-one **1** with 3-bromoprop-1-yne using potassium carbonate as base in acetone medium. Ethyl 4-bromoacetoacetate obtained from the bromination of ethylacetoacetate was treated with substituted phenols under Pechmann cyclization conditions using neat sulphuric acid as the condensing agent. The reaction resulted in the formation of 4-(bromomethyl)-2*H*-chromen-2-ones **3a**–**m**. The reactivity of 4-(bromomethyl)-2*H*-chromen-2-ones has been explored in a cascaded manner via an allylic displacement. The required dipolar azide intermediates **4a**–**m** were synthesized by the reaction of sodium azide with various 4-(bromomethyl)-2*H*-chromen-2-ones **3a**–**m** in aqueous acetone at room temperature and were quite stable even above 100 °C. Copper catalysed the reaction of various dipolar 2*H*-chromen-2-one azides with acetylenic dipolarophile in the presence of sodium ascorbate in aqueous medium resulting in the exclusive formation of 1,4-substituted bis-chromenyl triazole hybrids, which is in accordance with the earlier observations on click reactions (14) (Scheme1).



Scheme 1: Synthesis of 4-methyl-7-((1-((2-oxo-2*H*-chromen-4-yl)methyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-2*H*-chromen-2-ones **5a**–**m**.

Formation of acetylenic dipolarophile **2** was supported by the appearance of two singlets in the ^{1}H-NMR at 2.59 and 4.77 ppm because of C≡C–**H** and C≡C–C**H**$_2$ protons, respectively. In the ^{1}H-NMR spectrum of the azides (**4e, 4f, 4l, 4m**), the C_4-CH_2 protons linked to the azide group were observed between 4.54 and 4.65 ppm, and the C_3-H of 2*H*-chromen-2-one appeared as a singlet between 6.45 and 6.70 ppm. In IR spectrum of azide, band at 2117–2132 per cm was observed because of the presence of azide group. The IR spectrum of triazole adduct **5a**–**m** showed absence of azide band at 2117–2132 per cm, and the ^{1}H-NMR spectrum showed two interesting features viz., (i) C_4-CH_2 protons showed a downfield shift and were observed at 6.00 ppm when compared with 4.56 ppm observed in the case of 4-arylamino methyl 2*H*-chromen-2-one. (ii) The C_3-H of 2*H*-chromen-2-one in the triazole adduct experienced an upfield shift and was observed as a singlet at 5.78 ppm, as against 6.60–6.70 ppm in the case of both 4-arylaminomehtyl and 4-phenoxymethyl 2*H*-chromen-2-one (20,28). The observed results are in agreement with our earlier report (18). This rare type of shielding effect of 2*H*-chromen-2-one C_3-H and simultaneous pronounced deshielding of the C_4-CH_2 protons were because of the triazole on the C_4-CH_2 group, which has been consistently observed in spectral data of all the synthesized bis-chromenyl triazole hybrids.

Biology

Anti-TB activity using Alamar Blue Dye

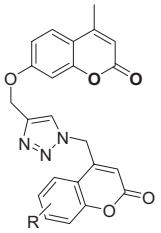
All the synthesized titled compounds were evaluated for their antimycobacterial activity against *M. tuberculosis* using MABA. The data (Table 1) revealed that compounds **5b** (C_7-Me), **5c** (C_6-OMe), **5d** (C_7-OMe), **5e** (C_5,C_7-diMe) and **5f** (C_7,C_8-diMe) showed weak activity with MIC > 100 μg/mL. Compounds **5a** (C_6-Me), **5g** (C_7-OH), **5k** (benzo[h] fused chromen), **5l** (C_6-Br), **5m** (C_7-Me-C_6,C_8-diNO_2) showed good activity with MIC of 12.50 μg/mL. Compounds **5h** (C_6-Cl), **5i** (C_7-Cl) and **5j** (benzo[f] fused chromen derivative) were as highly active as streptomycin with MIC of 6.25 μg/mL. Above observations clearly indicate that chloro and benzo substituents on the coumarin ring have remarkable impact on the antitubercular activity of the title compounds. Thus, chloro and benzo substituents reinforce the antitubercular activity of coumarin triazole hybrids.

2*H*-chromen-2-one derivatives possessing hydrazone moiety (8) and benzyl triazoles (1) exhibited antimycobacterial activity with MIC of 50–100 μg/mL and 16 μg/mL, respectively. Compared with the above compounds, bis-chromenyl triazole hybrids exhibited higher antimycobacterial (6.25 μg/mL) activity. Thus, the attachment of triazole moiety to the 2*H*-chromen-2-one dramatically increases the antimycobacterial activity of the parental 2*H*-chromen-2-one compared with chromenes possessing the hydrazone unit. This comparison clearly supports the existence of a better effect in 2*H*-chromen-2-one triazole hybrids.

Antibacterial screening

All the synthesized titled compounds were evaluated for their antibacterial activity against (i) Gram-positive bacteria: *Streptococcus faecalis* (MTCC 3382) and *Staphylococcus aureus* (MTCC 3160) and (ii) Gram-negative bacteria: *Pseudomonas aeruginosa* (MTCC 1034) and *Escherichia coli* (MTCC 1089). The antibacterial data (Table 1) revealed that some of the bis-chromenyl triazole hybrids exhibited

Table 1: Results of biological evaluation of compounds **5a–m** MICs (μg/mL)



Compounds	R	Antibacterial activity: Gram-positive *Streptococcus faecalis*	Gram-positive v*Streptococcus aureus*	Gram-negative *Pseudomonas aeruginosa*	Gram-negative *Escherichia coli*	Antifungal activity: *Candida albicans*	Antifungal activity: *Aspergillus niger*	Anti tubercular activity
5a	C_6-Me	>100	50	>100	>100	25	50	12.5
5b	C_7-Me	50	25	>100	>100	50	25	>100
5c	C_6-OMe	50	50	25	25	50	6.25	>100
5d	C_7-OMe	50	50	>100	25	50	25	>100
5e	C_5,C_7-diMe	100	50	>100	25	50	25	>100
5f	C_7,C_8-diMe	50	25	>100	50	25	50	>100
5g	C_7-OH	>100	25	>100	50	>100	50	12.5
5h	C_6-Cl	50	25	25	50	50	25	6.25
5i	C_7-Cl	50	25	50	50	50	25	6.25
5j	benzo[f]	50	25	50	50	50	50	6.25
5k	benzo[h]	50	50	>100	50	50	25	12.5
5l	C_6-Br	50	50	>100	25	25	25	12.5
5m	C_7-Me-C_6,C_8-diNO_2	>100	25	>100	50	50	50	12.5
Ciprofloxacin	–	1	1	1	1	–	–	–
Streptomycin	–	–	–	–	–	–	–	6.25
Fluconazole	–	–	–	–	–	16	8	–

Table 2: IC_{50} value of after 24 h drug incubation with V79 and HBL100 cell lines by MTT assay

	IC_{50} (μg/mL)	
Compounds	V79	HBL100
C_6-Cl	44.24 ± 2.15	36.91 ± 1.63
C_7-Cl	61.88 ± 3.74	54.39 ± 2.07
Ben[f]	48.25 ± 5.36	52.03 ± 2.55
Doxorubicin	0.19 ± 0.02	0.30 ± 0.03 μM

moderate activity. Compounds **5b** (C_7-Me), **5f** (C_7,C_8-diMe), **5g** (C_7-OH), **5h** (C_6-Cl), **5i** (C_7-Cl), **5j** (benzo[f] fused chromen) and **5m** (C_7-Me-C_6,C_8-diNO_2) showed moderate activity with MIC of 25 μg/mL against *S. aureus*. Compounds **5c** (C_6-OMe) and **5h** (C_6-Cl) groups showed moderate activity with MIC of 25 μg/mL against *P. aeruginosa*. Compounds **5c** (C_6-OMe), **5d** (C_7-OMe), **5e** (C_5,C_7-diMe) and **5l** (C_6-Br) showed moderate activity with MIC of 25 μg/mL against *E. coli*. All compounds showed weak activity against *S. faecalis*. The compounds screened had no significant specificity for Gram-positive or Gram-negative species. In all the cases, -OCH_3 and -Cl groups had the best effect on the antimicrobial activity of the tested compounds.

Antifungal screening

All the synthesized title compounds were screened for their antifungal activity against *Candida albicans* and *Aspergillus niger*. The antifungal data (Table 1) revealed that some of the bis-chromenyl triazole hybrids exhibited moderate activity. Compounds **5a** (C_6-Me), **5f** (C_7,C_8-diMe) and **5l** (C_6-Br) showed moderate activity with MIC of 25 μg/mL against *C. albicans*. Compounds **5b** (C_7-Me), **5d** (C_7-OMe), **5e** (C_5, C_7-diMe), **5h** (C_6-Cl), **5i** (C_7-Cl), **5k** (benzo[h] fused chromen) and **5l** (C_6-Br) showed moderate activity with MIC of 25 μg/mL against *A. niger*. Compound **5c** (C_6-OMe) is highly active, which was higher than the activity of fluconazole, with MIC of 6.25 μg/mL against *A. niger*. The tested compounds possessing electron-donating groups at C-6 and C-7 in the coumarin moiety enhanced the antifungal activity, which was more significant against A.niger than C. albicans. A fourfold reduction in MIC was observed in the 6-methoxy-substituted compound **5c** against A. niger, which confirms the effect of electron-donating groups on the antifungal activity.

Cytotoxic study

The potent compounds **5h, 5i** and **5j** (C_6-Cl, C_7-Cl and benzo[f]) were tested in normal cells (V79 and HBL100) for toxicity and showed IC_{50} ranging between 40 and 60 μg/mL (Table 2). As observed from the MIC values (6.25 μg/mL), the compounds were microbicidal at significantly lower doses, four to six times lesser than IC_{50} values. Therefore, it may be inferred that the compounds do not inhibit the growth of normal cell lines (V79 and HBL100), at concentrations active for microbicidal action. These classes of compounds may be taken further for *in vivo* testing to confirm their microbicidal activity.

DNA cleavage analysis

The DNA cleavage studies of **5c** (C_6-OMe), 4-methyl-7-((1-((6-methoxy-2-oxo-2*H*-chromen-4-yl)methyl)-1*H*-1,2,3-triazol-4-yl)methoxy)-2*H*-chromen-2-one, have been carried out against *A. niger* by agarose gel electrophoresis method. Gel electrophoresis technique works on the migration of DNA under the influence of electric potential. The photograph (Figure 2) shows the molecular weight differences compared with control and is the differentiating criterion for the DNA cleaving ability of the tested compounds with *A. niger*. Control experiments using DNA alone does not indicate any significant cleavage of DNA even after long exposure time. After marker M and control C, the first six lanes correspond to six different concentrations viz., 50, 25, 12.5, 6.25, 3.125, 1.562 μg/mL. Compound **5c** with concentration 50 μg/mL shows the absence of the control band, which could be attributed to the DNA cleavage, but later the extent of cleavage has decreased with the decrease in the concentration of compound **5c**.

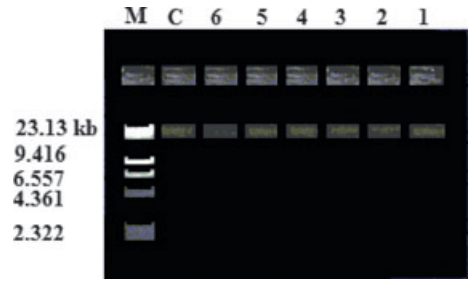


Figure 2: Gel electrophoresis picture of compound **5c**. Photograph showing the effect of representative compound **5c** on DNA of A. niger. Lane M: DNA marker, Lane C: untreated DNA, Lanes 1, 2, 3, 4, 5 and 6 correspond to 1.562, 3.125, 6.25, 12.5, 25, 50 μg/mL.

Conclusions

We have synthesized a series of bis-chromenyl triazole hybrids under click reaction conditions. Antimycobacterial screening data reveal that the synthesized compounds showed significant antimycobacterial activity against *M. tuberculosis*. Compounds **5h** (C_6-Cl), **5i** (C_7-Cl) and **5j** (benzo[f] fused chromen) showed activity equivalent to streptomycin. All the compounds showed moderate activity against microbials. On the other hand, **5c** (C_6-OMe) showed excellent fungicidal activity, which was higher than fluconazole, and DNA cleavage against *A. niger*. These compounds act as potent antitubercular agent rather than antimicrobial agent and are active against Gram-positive bacteria compared with Gram-negative bacteria. Cytotoxicity study reveals that compounds do not inhibit the growth of normal cell lines (V79 and HBL100) even at higher concentrations. Higher activity of these compounds in antitubercular studies indicates that these compounds may act as good structural leads in developing new antimycobacterial drugs.

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Conflicts of Interest

Authors have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. ^{1}H and ^{13}C NMR spectra.

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