Environmentally benign synthesis and anti-mycobacterial evaluation of 9,10-dihydro-4-methyl-chromeno[8,7-e][1,3]oxazin-2(8H)-one derivatives

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In the present study, a series of 9,10-dihydro-4-methyl-chromeno[8,7-e][1,3]oxazin-2(8H)-one derivatives (4a-j) have been synthesized by using one-pot Mannich type condensation cyclization reaction of 7-hydroxy-4-methylcoumarin, formaldehyde and primary amines in water at 80-90°C. These products have been characterized using various spectroscopic techniques (1H and 13C NMR, IR, and MS) followed by evaluation of their in-vitro anti-tubercular activity against Mycobacterium tuberculosis H37Rv. The compounds 4g and 4h display the lowest MIC of 5 µg/mL and offer a remarkable viability of HEK-293 cells at the highest test concentration (100 µg/mL) in toxicity assay.

Keywords: Anti-mycobacterial activity, chromeno[8,7-e][1,3]oxazin-2(8H)-ones, eco-friendly synthesis, Mycobacterium tuberculosis, toxicity assay

Tuberculosis (TB) is one of the oldest, persistent and irrepressible scourges of humanity affecting people across the globe. Even though synthetic drugs have been available for over half a century, TB continues to affect about one-third of the world’s total population. Leaving no country free, the deadly disease has become a global pandemic with African and Asian countries being the most affected. According to World Health Organization (WHO) estimates, approximately 9 million people were reported to have fallen ill with TB in 2013, including around 0.55 million children. Also, there were around 1.5 million deaths that occurred in the same year. Thus, TB has become a cause of global concern requiring effective control and management. The emergence of multi-drug resistant (MDR) and extensively drug resistant (XDR) strains along with an increase in number of HIV cases and that of latent TB have all increased the gravity of the problem necessitating discovery of new potent molecules that could help to curb the menace.

Certain benzoxazine derivatives such as 6-chloro-3-(4-chlorophenyl)-4-thioxo-2H-1,3-benzoxazine-2(3H)-one and 6-chloro-3-(4-methylphenyl)-2H-1,3-benzoxazine-2,4-(3H)-dithione (Figure 1), have been reported to be more effective against Mycobacterium than isoniazid (INH). They have been shown to inhibit the growth of Mycobacterium tuberculosis, Mycobacterium avium, Mycobacterium kansasii 235/80 and M. kansasii 6509/96 at a concentration of 0.5 µmol/L, 16 µmol/L, 2 µmol/L and 0.5-1 µmol/L, respectively. The corresponding minimum inhibitory concentration (MIC) values for INH have been found to be 4, 500, 8 and 500 µmol/L, respectively. In addition, various dihydro[1,3]oxazines are known to possess potent anti-mycobacterial activity. Similarly, some synthetic and naturally occurring coumarins such as Calanolide A and Calanolide B (Figure 1) have also been shown to be effective in inhibiting the mycobacterial growth. All these facts motivated us to design chemical prototypes bearing both dihydro-1,3-oxazine and coumarin moieties, so that the resultant compounds may demonstrate enhanced efficacy against the Mycobacterium species. Therefore, the present study was planned to synthesize a series of 9-substituted-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-ones 4a-j to assess their antimycobacterial potential. All the prepared compounds have been screened for their anti-tubercular efficacy against M. tuberculosis H37Rv and the results are presented in this paper.
Results and Discussion

The synthetic route to construct the target compounds 4a-j is schematically represented in Scheme I. The starting material, 7-hydroxy-4-methylcoumarin 3 was initially prepared via Pechmann condensation of ethyl acetoacetate 1 and resorcinol 2 in the presence of concentrated sulfuric acid at 25°C\(^\text{15,16}\). As a part of our ongoing research to develop efficient synthetic procedures for various dihydro-1,3-oxazines\(^\text{17-21}\), a series of 9-substituted-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one derivatives 4a-j was synthesized in good yields (70-78%) via Mannich type condensation reaction of 7-hydroxy-4-methylcoumarin 3 with formaldehyde and primary amines in molar ratio 1:2:1 under aqueous conditions at 80-90°C. After chromatographic purification, the structures of all the prepared compounds were established on the basis of their analytical and spectral data.

The IR spectrum of a representative compound 4a showed the characteristic absorption peaks at 1217 and 1048 cm\(^{-1}\) corresponding to the C-O-C asymmetric and C-O-C symmetric stretching, respectively due to the formation of oxazine ring. In the \(^1\)H NMR spectrum of 4a, two peaks appeared at \(\delta\) 4.66 and 5.28 corresponding to the two methylene groups N-CH\(_2\)-Ar and O-CH\(_2\)-N, respectively. Similarly, two characteristic peaks at \(\delta\) 46.3 and 80.9 in the \(^13\)C NMR spectrum of 4a were assigned to the methylene carbons of the oxazine ring. Further, the formation of 9-(2-chlorophenyl)-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one 4a was confirmed by mass spectral analysis which showed \([M+H]^+\) ion peak at \(m/z\) 328.1 corresponding to the molecular formula C\(_{18}\)H\(_{15}\)ClNO\(_3\).

The preliminary in vitro anti-mycobacterial efficacy of compounds 4a-j was assessed against \(M.\) \(\text{tuberculosis}\) H37Rv strain by using microplate alamar blue assay (MABA)\(^\text{13,24}\). INH, rifampicin, streptomycin and ethambutol were used as the reference drugs. The anti-tubercular activity results are shown in Table I. The compounds 4c, 4g and 4h exhibited considerable anti-tubercular activity with the MIC values <10 \(\mu\)g/mL. The oxazines (4g and 4h) containing \(p\)-tolyl and \(p\)-anisyl substituents, respectively were found to be the most active compounds of the series with the MIC value 5 \(\mu\)g/mL. In contrast, the presence of 4-halo substituent in the aryl ring as in the case of compounds 4c-e led to the decrease in anti-mycobacterial activity in the order of 4-Br > 4-Cl > 4-F. Other compounds having 2-chlorophenyl, 3-chlorophenyl and phenyl groups at position 9 as in case of compounds 4a, 4f and 4j did not show any significant activity even at higher concentrations. Further, the replacement of an aryl ring by a benzyl moiety as in the compound 4i also led to the decrease in anti-mycobacterial efficacy. In order to understand the effect of multi-substitution in the aromatic ring present at position 9 on biological
activity, the compound 4b was prepared. On biological evaluation, it displayed higher MIC value (25 μg/mL) as compared to the most active compounds (4g and 4h). Thus, the structure-activity relationship as observed revealed that the substituent at position 9 in structure 4 may be responsible for the anti-mycobacterial action of this class of molecules.

Furthermore, the cytotoxic effect of the lead compounds (4g and 4h) was determined against human embryonic kidney cells 293 (HEK-293) by using MTT assay. Both the compounds are found to be devoid of any significant cytotoxicity at the highest test concentration (100 μg/mL).

**Experimental Section**

The chemicals required for the present study were purchased from Sigma Aldrich and used without further purification. The progress of reactions was monitored by thin layer chromatography using silica gel 60 F254 (pre-coated aluminium sheets) from Merck. $^1$H and $^{13}$C NMR spectra were obtained in CDCl$_3$ by using TMS as an internal standard on Bruker 300 MHz NMR spectrometer and Jeol ECX 400 MHz NMR spectrometer. The chemical shifts and coupling constants (J) are reported in parts per million (δ, ppm) and Hertz (Hz), respectively. Infrared spectra were recorded on Perkin-Elmer IR spectrometer and absorption maxima (ν$_{max}$) are given in cm$^{-1}$. The mass spectra were recorded on Micromass LCT (Waters) mass spectrometer and a JEOLE-ACCUSOF JMS-Y100LC Mass spectrometer having a DART (Direct Analysis in Real Time) source. Elemental analyses were performed on Elementar AnalyseSysteme GmbH VarioEL elemental analyzer. The melting points were recorded on Perkin Elmer Differential Scanning Calorimeter.

**General procedure for the synthesis of 9-substituted-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-ones, 4a-j**

To a mixture of 7-hydroxy-4-methylcoumarin 3 (0.568 mmol) and primary amine (0.568 mmol) in water (1 mL), formalin (37%, w/v, 1.136 mmol) was added. The reaction mixture was stirred at 80-90°C for 1 h. After completion of the reaction, the product was extracted with ethyl acetate (2×10 mL). The organic layers were combined and washed with brine solution (2×10 mL) followed by water (25 mL). The organic layer was dried over anhyd. Na$_2$SO$_4$ and concentrated under reduced pressure. The crude product was purified by column chromatography over silica gel using 20-30% ethyl acetate in heptane as eluent to afford the desired products in 70-78% isolated yields.

<table>
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<tr>
<th>Entry</th>
<th>Compd</th>
<th>R</th>
<th>MIC (µg/mL)/(µmol/L)</th>
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<tbody>
<tr>
<td>1</td>
<td>4a</td>
<td>2-ClC$_6$H$_4$</td>
<td>&gt;80 (&gt; 244.08)</td>
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<tr>
<td>2</td>
<td>4b</td>
<td>2,4-Cl$_2$C$_6$H$_3$</td>
<td>&gt;80 (&gt; 244.08)</td>
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<tr>
<td>3</td>
<td>4c</td>
<td>4-BrC$_6$H$_4$</td>
<td>7 (18.81)</td>
</tr>
<tr>
<td>4</td>
<td>4d</td>
<td>4-ClC$_6$H$_4$</td>
<td>10 (30.51)</td>
</tr>
<tr>
<td>5</td>
<td>4e</td>
<td>4-FC$_6$H$_4$</td>
<td>25 (80.31)</td>
</tr>
<tr>
<td>6</td>
<td>4f</td>
<td>3-ClC$_6$H$_4$</td>
<td>&gt; 40 (&gt; 122.04)</td>
</tr>
<tr>
<td>7</td>
<td>4g</td>
<td>4-(CH$_3$)C$_6$H$_4$</td>
<td>5 (16.27)</td>
</tr>
<tr>
<td>8</td>
<td>4h</td>
<td>4-(CH$_3$O)C$_6$H$_4$</td>
<td>5 (15.46)</td>
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<tr>
<td>9</td>
<td>4i</td>
<td>CH$_2$C$_6$H$_5$</td>
<td>&gt; 40 (&gt; 130.15)</td>
</tr>
<tr>
<td>10</td>
<td>4j</td>
<td>C$_6$H$_5$</td>
<td>&gt;80 (&gt; 272.74)</td>
</tr>
<tr>
<td>11</td>
<td>INH</td>
<td>–</td>
<td>0.03 (0.2188)</td>
</tr>
<tr>
<td>12</td>
<td>RIF</td>
<td>–</td>
<td>0.015 (0.0182)</td>
</tr>
<tr>
<td>13</td>
<td>STR</td>
<td>–</td>
<td>0.25 (0.4299)</td>
</tr>
<tr>
<td>14</td>
<td>EMB</td>
<td>–</td>
<td>2 (9.7890)</td>
</tr>
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INH: isoniazid; RIF: rifampicin; STR: streptomycin; EMB: ethambutol.
J H, 1H, ArH), 7.19-7.22 (m, 1H, ArH), 7.06 (t, J = 7.80 Hz, 1H, ArH), 6.96 (t, J = 7.80 Hz, 1H, ArH), 6.76 (d, J = 8.70 Hz, 1H, ArH), 6.06 (s, 1H, =CH), 5.28 (s, 2H, N-CH2O), 4.66 (s, 2H, N-CH2Ar), 2.32 (s, 3H, CH3); 13C NMR (100 MHz, CDCl3): δ 160.8, 157.1, 152.9, 151.0, 145.6, 130.8, 130.5, 128.7, 127.3, 125.5, 125.1, 123.3, 122.7, 122.3, 113.6, 113.3, 111.8, 111.6, 108.6, 80.9 (N-CH2-O), 46.3 (N-CH2Ar), 18.7 (CH3); DART-MS: m/z 328.1 [M + H]+. Anal. Calcd for C19H15FNO3: m/z 334.0900 [M + Na]+. Found: 334.0806. Anal. Calcd for C18H15FNO3Na: m/z 327.1 [M + Na]+. Found: 328.0700 [M + H]+. Found: C, 69.45; H, 4.53; N, 4.50. Found: C, 69.61; H, 4.41; N, 4.27%.

9-(4-Bromophenyl)-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one, 4f: Yellow solid. Yield 77%. m.p.172.4°C. IR (film): 2922, 2854, 1722 (C=O), 1603, 1514, 1463, 1377, 1262, 1226, 1196, 1166, 1112, 1057, 994, 930, 856, 824, 803, 760, 721 cm−1; 1H NMR (300 MHz, CDCl3): δ 7.37 (d, J = 8.70 Hz, 1H, ArH), 7.07-7.10 (m, 2H, ArH), 6.92-6.96 (m, 2H, ArH), 6.77 (d, J = 8.70 Hz, 1H, ArH), 6.11 (s, 1H, =CH), 5.36 (s, 2H, N-CH2-O), 4.75 (s, 2H, N-CH2Ar), 2.36 (s, 3H, CH3); 13C NMR (100 MHz, CDCl3): δ 160.8, 157.1, 153.0, 151.0, 146.9, 134.9, 126.3, 122.6, 119.2, 118.4, 118.1, 116.3, 113.6, 111.8, 111.6, 108.6, 80.9 (N-CH2-O), 46.3 (N-CH2Ar), 18.7 (CH3); DART-MS: m/z 328.1 [M + H]+. Found: 328.0700 [M + H]+. Found: C, 69.45; H, 4.53; N, 4.50. Found: C, 69.61; H, 4.41; N, 4.27%.

9-(3-Chlorophenyl)-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one, 4f: Viscous liquid. Yield 75%. IR (film): 2962, 2924, 2854, 1725 (C=O), 1627, 1605, 1593, 1484, 1438, 1382, 1368, 1263, 1222, 1164, 1110, 1057, 992, 963, 935, 912, 850, 812, 761, 687 cm−1; 1H NMR (300 MHz, CDCl3): δ 7.30 (d, J = 8.79 Hz, 1H, ArH), 7.10 (t, J = 8.08 Hz, 1H, ArH), 7.04 (s, 1H, ArH), 6.92 (dd, J = 8.21 Hz, 1J = 1.60 Hz, 1H, ArH), 6.83 (d, J = 7.82 Hz, 1H, ArH), 6.71 (d, J = 8.79 Hz, 1H, ArH), 6.05 (s, 1H, =CH), 5.31 (s, 2H, N-CH2-O), 4.70 (s, 2H, N-CH2Ar), 2.30 (s, 3H, CH3); 13C NMR (100 MHz, CDCl3): δ 160.7, 157.1, 153.0, 150.9, 143.3, 126.3, 121.7, 118.1, 116.3, 113.6, 113.3, 108.4, 79.0 (O-CH2-N), 46.2 (N-CH2Ar), 18.6 (CH3); HRMS (ESI): Calcd for C19H15FNO3Na: m/z 334.0900 [M + Na]+. Found: 334.0806. Anal. Calcd for C18H15FNO3: C, 69.45; H, 4.53; N, 4.50. Found: C, 69.61; H, 4.41; N, 4.27%.
72.14; H, 5.74; N, 4.43. Found: C, 71.87; H, 5.67; N, 4.74%.

9-(4-Methoxyphenyl)-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one, 4h: White solid. Yield 72%. m.p.160.6°C [162-64°C]22. IR (film): 2921, 2851, 1723 (C=O), 1602, 1510, 1438, 1394, 1382, 1269, 1298, 1265, 1244, 1226, 1184, 1186, 1162, 1103, 1059, 1033, 984, 962, 931, 897, 830, 817, 765, 753, 731, 690, 669, 635 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.37 (d, $J = 8.05$ Hz, 1H, ArH), 7.09 (d, $J = 8.79$ Hz, 2H, ArH), 6.81 (d, $J = 8.79$ Hz, 2H, ArH), 6.78 (d, $J = 8.79$ Hz, 1H, ArH), 6.12 (s, 1H, =CH), 5.36 (s, 2H, N-CH$_2$-O), 4.75 (s, 2H, N-CH$_2$-Ar), 3.74 (s, 3H, OCH$_3$), 2.37 (s, 3H, CH$_3$); DART-MS: m/z 324 [M + H]$^+$. Anal. Caled for C$_{18}$H$_{15}$N$\text{O}_{3}$: C, 73.71; H, 5.15; N, 4.78.

9-Benzyl-4-methyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one, 4i: Yellow solid. Yield 77%. m.p.128.1°C [134-36°C]$^{23}$. IR (film): 3063, 3029, 2924, 2853, 1728 (C=O), 1602, 1497, 1454, 1393, 1368, 1352, 1310, 1264, 1223, 1189, 1156, 1137, 1069, 1025, 1004, 929, 898, 872, 850, 814, 754, 735, 699 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.41 (d, $J = 8.73$ Hz, 1H, ArH), 7.28-7.35 (m, 4H, ArH), 6.81 (d, $J = 8.73$ Hz, 1H, ArH), 6.12 (s, 1H, =CH), 4.92 (s, 2H, N-CH$_2$-O), 4.19 (s, 2H, N-CH$_2$-Ar), 3.90 (s, 2H, CH$_2$Ar), 2.40 (s, 3H, OCH$_3$); DART-MS: m/z 308.2 [M + H]$^+$. Anal. Caled for C$_{19}$H$_{17}$N$\text{O}_{3}$: C, 74.25; H, 5.58; N, 4.56. Found: C, 73.91; H, 5.91; N, 4.19%.

4-Methyl-9-phenyl-9,10-dihydrochromeno[8,7-e][1,3]oxazin-2(8H)-one, 4j: Yellow solid. Yield 76%. m.p.142.5°C [144-46°C]$^{22,23}$. IR (film): 2923, 2853, 1726 (C=O), 1600, 1497, 1382, 1265, 1218, 1164, 1106, 1058, 1030, 985, 961, 932, 947, 849, 842, 755 695 cm$^{-1}$; $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 7.37 (d, $J = 9.52$ Hz, 1H, ArH), 7.25-7.29 (m, 2H, ArH), 7.13-7.15 (m, 2H, ArH), 6.96 (t, $J = 7.69$ Hz, 1H, ArH), 6.78 (d, $J = 8.79$ Hz, 1H, ArH), 6.13 (s, 1H, =CH), 5.43 (s, 2H, N-CH$_2$-O), 4.83 (s, 2H, N-CH$_2$-Ar), 2.37 (s, 3H, CH$_3$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$ 160.9, 157.4, 153.0, 151.0, 147.7, 129.2, 123.4, 121.8, 118.3, 113.5, 113.1, 111.5, 108.7, 79.7 (N-CH$_2$-O), 46.2 (N-CH$_2$-Ar), 18.6 (CH$_3$); DART-MS: m/z 294 [M + H]$^+$. Anal. Caled for C$_{19}$H$_{17}$N$\text{O}_{3}$: C, 74.25; H, 5.52; N, 4.12%.

Anti-mycobacterial assay

The in vitro anti-tubercular efficacy of the synthesized compounds 4a-j was assessed against M. tuberculosis H37Rv strain using microplate alamar blue assay (MABA) as reported earlier$^{13,24}$. The 96-well U-bottom plates were used for the antimycobacterial assay. To minimize the evaporation, sterile water was added to all the peripheral wells of a U-bottom plate. A single cell suspension was prepared from a log phase culture of M. tuberculosis H37Rv by passing it through a 8 µm filter. The absorbance was measured at a wavelength of 600 nm using a Cary UV-Vis spectrophotometer and the cell density was then calculated using McFarland standards. The calculated amount of drug and 7H9 medium along with $10^6$ cells were added to each well of the plate so as to make the total volume of each well to 200 µL. Positive and negative controls were also included to check the sterility of the medium as well as the viability of the inoculum. The plates were then sealed with paraffilm and incubated at 37°C. After 10 days of incubation, 0.02% resazurin solution was added to each well and the plates were incubated overnight at 37°C prior to assessing a colour development. A colour change from blue to pink was considered as growth and a blue colour indicated the growth inhibition of M. tuberculosis H37Rv. The MIC value defined as the lowest drug concentration required for the complete inhibition of bacterial growth was determined by visual inspection. Isoniazid, rifampicin, streptomycin and ethambutol were used as standard drugs for the present study.

Cytotoxicity assay

The human embryonic kidney cells (HEK-293) were seeded at $5 \times 10^3$ cells per well into 96-well microtiter plates containing 100 µL of DMEM medium supplemented with 1% penicillin-streptomycin-glutamine solution and 10% fetal bovine serum. The serial drug dilutions of eight 2-fold dilution steps covering a range from 100 to 0.78 µg/mL were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to assure the growth of controls and sterile conditions. 10 µL of MTT reagent (5 mg MTT dissolved in 1 mL PBS) was then added to each well and the plates were incubated for 2-4 h in the cell culture incubator. Then, 100 µL of detergent reagent (90% isopropanol, 9.999% Triton X-100 and 0.001% conc. HCl) was added to each well and the plates were incubated for another 2 h in the dark at RT. The absorbance of each well was then read at 570 nm by using a Biotek Synergy HT microplate reader and the data was analyzed with the help of microplate reader software.
Each CC50 value obtained is the mean of at least two separate experiments performed in duplicate.

Conclusions

In conclusion, a series of chromeno[1,3]oxazines were prepared in good yields by using an eco-friendly one-pot methodology. After spectroscopic characterization, all the compounds were evaluated for their efficacy against *M. tuberculosis* H37Rv strain by using microplate alamar blue assay. Six of the tested compounds (4g and 4h) are found to be non-toxic to the HEK-293 cells even at the highest test concentration (100 μg/mL).

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References