CHAPTER-I, SECTION-3

Design and synthesis of novel isoxazoline derivatives of 7-hydroxy coumarin as potential immunopotentiators
1.3.1. Introduction

Encouraging results from immunopharmacological studies based on the concept of immunomodulation have generated a considerable interest among researchers. It is in this regard that currently many groups are actively pursuing the studies aimed at designing of appropriate, less toxic and effective immunopotentiators. As an outcome of this intense research activity going on world over, a huge library of immunopotentiators, is known today. Among the currently known immunopotentiators like; muramyl dipeptide derivatives, levamisole, niridazole, oxysuran, flagyl and those from the groups of interferons, interleukins, leukotrienes, corticosteroids and cyclosporine, many have been found to express undesirable side effects and/or high toxicity during the clinical trials. In view of these safety and affectivity concerns about currently known immunopotentiators, studies related to design of novel and effective immunopotentiators associated with fewer side effects are currently in great demand. The development of novel low molecular weight immunopotentiators is considered as one of the attractive approaches in this direction. Preliminary immunostimulatory studies about coumarins and isoxazolines in this regard have projected them as promising lead compounds for the design of immunostimulators with desired characteristics.

1.3.2. Present work

From the literature documented advantages about coumarins and isoxazolines, it appears that conjugation of bioactive pharmacophoric heterocycles with 7-hydroxycoumarin is a promising synthetic strategy for the design of effective and target selective immunostimulators. In this section, we present results from our studies about design and synthesis of novel immunopotentiators through conjugation of bioactive pharmacophoric heterocycles (isoxazolines) with 7-hydroxycoumarin (7-OHC). A focused library of novel isoxazoline conjugates of 7-OHC (Table 1) was synthesized and investigated to probe the relevant structure activity relationship (SAR) with regard to their immunostimulatory activity. To the best of our knowledge, such attempts are not reported in literature so far.
1.3.3. Experimental

1.3.3.1 Synthesis

Synthesis of coumarin-isoxazoline conjugates

For the synthesis of aimed conjugates, the strategy of 1,3-dipolar cyclization between an allylated coumarin and various substituted nitrile oxides (Scheme 1) was followed. Dipolarophile was obtained by alkylating 7-OHC with allylbromide using $K_2CO_3$. Nitrile oxides used as 1,3-dipoles, were synthesized according to the literature procedures. The novel isoxazoline conjugates of 7-OHC synthesized in the ensuing work are presented in Table 1.

Typical procedure for the synthesis of 7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl) methoxy} -2H-chromen-2-one (1):

\[
\text{Scheme 1: Synthesis of bis-heterocycles encompassing umbelliferone and isoxazolines.}
\]

7-(Allyloxy)-2H-chromen-2-one (1c):

In a typical procedure, 1b (0.50 g, 3.08 mmol) was dissolved in dry acetone (20 mL). $K_2CO_3$ (4.26 g, 30.85 mmol) was added and the reaction mixture stirred for 5 min at ambient temperature. 1a (0.44 g, 3.70 mmol) was charged to the above reaction mixture and the reaction mixture allowed to reflux for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to attain room temperature, filtered and the filtrate concentrated under vacuum to afford 1c in pure form (100%).
Phenylhydroxymoyl chloride (1d):

Phenylhydroxymoyl chloride was synthesized as per the literature procedure. In a typical procedure, NH₂OH.HCl (0.50 g, 7.22 mmol) was dissolved in water and neutralized with NaOH. To the neutralized solution of hydroxylamine hydrochloride, benzaldehyde (1.00 g, 9.43 mmol) was added and the reaction mixture stirred for 1 h at ambient temperature. After completion of the reaction (monitored by TLC), excess of water was added to the reaction mixture and the organic compound extracted with EtOAc (2 × 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum to afford pure oxime in 99% yield. Benzaldoxime (1.00 g, 6.42 mmol) was dissolved in DMF (20 mL). N-chlorosuccinimide (0.95 g, 7.18 mmol) was added to the above solution and the reaction mixture stirred for 8-10 h. After completion of the reaction (monitored by TLC), excess of water was added to the reaction mixture and organic compound extracted with Et₂O (3 × 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum to afford pure 1d (74%).

1H NMR (200 MHz, CDCl₃):  δ 7.42 (m, 3H), 7.85 (m, 2H).

Mass (ESI-MS):  155.58 (M⁺).

C, H, N analysis for C₇H₆ClNO:
Calculated C, 54.04; H, 3.89; N, 9.00. Found C, 53.98; H, 4.00; N, 9.11.

7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (1):

In a typical procedure, 1d (0.10 g, 0.04 mmol) was dissolved in THF (4 mL) and to it was added Et₃N (0.02 g, 0.02 mmol). A solution of 1c (0.05 g, 0.02 mmol) in THF (4 mL) was added to the above solution. The reaction mixture was stirred at
ambient temperature for 4 h. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 80 mL of water and extracted with EtOAc (2 × 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase] to yield the pure 7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (1) as a white solid (90%).

<table>
<thead>
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<th>Compound</th>
<th>Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Compound</th>
<th>Yield (%)&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>(6)</td>
<td>90</td>
<td>(13)</td>
<td>87</td>
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</tbody>
</table>

Table 1: Synthesis of various isoxazoline derivatives of umbelliferone

<sup>a</sup>Isolated yields after chromatographic purification.

Structural determination

Formation of 3,5-disubstituted isoxazolines possessing O-allyl derivative of 7-OHC at 5<sup>th</sup> position, was established through the characteristic chemical shift values with
multiplets at 4.00-4.26 ppm for the C5 protons and 3.00-3.65 ppm for C4 protons. Similarly, characteristic chemical shift values for CH₂ group of O-allyl derivative was established with double-doublets/multiplets in the range of 4.06-4.30 ppm and 5.05-5.29 ppm for the two sets of protons.

1.3.3.2. Spectral data

7-{(3-Phenyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (1):

![Chemical Structure]

White solid; mp: 132-133 °C.

\(^1\)H NMR (500 MHz, CDCl₃): \(\delta\) 3.39-3.42 (dd, 1H, \(J_1 = 15.54\) Hz, \(J_2 = 6.93\) Hz), 3.54-3.59 (dd, 1H, \(J_1 = 15.55\) Hz, \(J_2 = 10.82\) Hz), 4.16-4.22 (m, 2H), 5.04-5.09 (m, 1H), 6.15 (d, 1H, \(J = 8.78\) Hz), 6.27 (d, 1H, \(J = 8.46\) Hz), 6.83 (m, 3H), 7.38 (d, 1H, \(J = 8.56\) Hz), 7.63 (d, 1H, \(J = 9.47\) Hz), 7.71 (d, 2H, \(J = 9.61\) Hz).

\(^{13}\)C NMR (500 MHz, CDCl₃): \(\delta\) 37.50, 69.03, 78.35, 101.75, 112.80, 113.05, 113.56, 126.79, 128.81, 128.89, 129.11, 129.57, 130.39, 143.29, 155.74, 156.42, 161.06, 161.50, 176.86.

IR (KBr, cm⁻¹): 693, 761, 853, 913, 1124, 1230, 1280, 1355, 1402, 1448, 1507, 1558, 1614, 1723, 2358, 2853, 2924, 3386.

Mass (ESI-MS): 321.8 (M⁺).

C, H, N analysis for C₁₉H₁₅NO₄: Calculated C, 71.02; H, 4.71; N, 4.36. Found C, 71.04; H, 4.74; N, 4.30.
7-{(3-(4-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (2):

![Chemical Structure](image)

White solid; mp: 140-141 °C.

$^1$H NMR (500 MHz, CDCl$_3$):  
δ 3.25-3.29 (dd, 1H, $J_1 = 16.45$ Hz, $J_2 = 6.82$ Hz), 3.42-3.47 (dd, 1H, $J_1 = 16.65$ Hz, $J_2 = 10.75$ Hz), 3.88 (s, 3H), 4.06-4.09 (m, 1H), 4.12-4.15 (dd, 1H, $J_1 = 9.75$ Hz, $J_2 = 4.98$ Hz), 5.05-5.08 (dd, 1H, $J_1 = 10.93$ Hz, $J_2 = 5.44$ Hz), 6.20 (d, 1H, $J = 9.47$ Hz), 6.75 (s, 1H), 6.79 (d, 1H, $J = 8.56$ Hz), 6.90 (d, 2H, $J = 8.64$ Hz), 7.31 (d, 1H, $J = 8.52$ Hz), 7.52 (d, 1H, $J = 8.46$ Hz), 7.56 (d, 2H, $J = 9.52$ Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$):  
δ 39.69, 55.72, 69.10, 79.15, 101.77, 112.83, 113.07, 113.57, 114.98, 117.69, 124.20, 128.90, 129.22, 131.41, 143.29, 155.78, 156.56, 158.29, 161.04, 161.56.

IR (KBr, cm$^{-1}$):  
669, 810, 837, 897, 1022, 1063, 1095, 1126, 1232, 1275, 1356, 1402, 1507, 1558, 1615, 1650, 1714, 2337, 2361, 2852, 3389.

MALDI-mass: 352 (M$^+$ + H).

C, H, N analysis for C$_{20}$H$_{17}$NO$_5$:  
Calculated C, 68.37; H, 4.88; N, 3.99. Found C, 68.32; H, 4.85; N, 3.94.

7-{(3-(3-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (3):

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1H NMR (500 MHz, CDCl3): $\delta$ 3.48-3.51 (dd, 1H, $J_1 = 16.50$ Hz, $J_2 = 6.93$ Hz), 3.65-3.69 (dd, 1H, $J_1 = 14.64$ Hz, $J_2 = 10.54$ Hz), 3.82 (s, 3H), 4.10-4.14 (m, 2H), 5.13-5.16 (dd, 1H, $J_1 = 11.68$ Hz, $J_2 = 6.72$ Hz), 6.27 (d, 1H, $J = 9.47$ Hz), 6.85-6.95 (m, 3H), 7.20 (d, 1H, $J = 8.56$ Hz), 7.30-7.41 (m, 3H), 7.64 (d, 1H, $J = 9.50$ Hz).

13C NMR (500 MHz, CDCl3): $\delta$ 37.81, 55.39, 69.18, 78.11, 101.85, 112.79, 113.35, 114.25, 114.83, 124.53, 128.35, 128.89, 129.40, 143.26, 155.10, 155.97, 160.34, 161.31, 161.61.

IR (KBr, cm$^{-1}$): 759, 835, 908, 1027, 1124, 1231, 1291, 1377, 1402, 1462, 1507, 1557, 1613, 1728, 2360, 2850, 2921, 2955, 3416.

Mass (ESI-MS): 374 (M$^+$ + Na).

C, H, N analysis for C$_{20}$H$_{17}$NO$_5$: Calculated C, 68.37; H, 4.88; N, 3.99. Found 68.39; H, 4.84; N, 3.91.

7-{(3-(2-Methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (4):

White solid; mp: 96-97 °C.

1H NMR (500 MHz, CDCl$_3$): $\delta$ 3.41-3.44 (dd, 1H, $J_1 = 16.56$ Hz, $J_2 = 7.05$ Hz), 3.62-3.67 (dd, 1H, $J_1 = 16.00$ Hz, $J_2 = 10.80$ Hz), 3.86 (s, 3H), 4.12-4.18 (m, 2H), 5.08 (m, 1H), 6.27 (d, 1H, $J = 9.45$ Hz), 6.83 (s, 1H), 6.86-6.89 (m, 2H), 7.26-7.39 (m, 3H), 7.64 (d, 1H, $J = 9.50$ Hz), 7.74 (d, 1H, $J = 9.40$ Hz)
$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 39.80, 55.95, 69.26, 78.67, 101.75, 111.82, 112.76, 112.89, 113.53, 117.88, 121.11, 128.93, 129.09, 131.12, 143.39, 155.10, 155.97, 160.34, 161.31, 161.61.

IR (KBr, cm$^{-1}$): 755, 808, 835, 891, 1023, 1096, 1124, 1231, 1277, 1340, 1401, 1460, 1487, 1556, 1614, 1727, 2284, 2853, 2924, 3405.

Mass (ESI-MS): 374 (M$^+$ + Na).

Anal Calcd. for C$_{20}$H$_{17}$NO$_5$: Calculated C: 68.37, H: 4.88, N: 3.99; found C: 68.33, H: 4.82, N: 3.93.

7-{(3-p-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (5):

White solid; mp: 158-159 °C.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta$ 2.39 (s, 3H), 3.38-3.39 (m, 2H), 3.49-3.54 (dd, 1H, $J_1 = 15.15$ Hz, $J_2 = 10.86$ Hz), 4.14-4.17 (dd, 1H, $J_1 = 12.39$ Hz, $J_2 = 4.94$ Hz), 5.13-5.14 (m, 1H), 6.27 (d, 1H, $J = 9.50$ Hz), 6.83 (s, 1H), 6.87 (d, 1H, $J = 8.60$ Hz), 7.23 (d, 2H, $J = 7.62$ Hz), 7.38 (d, 1H, $J = 8.59$ Hz), 7.59 (d, 2H, $J = 7.08$ Hz), 7.69 (d, 1H, $J = 8.78$ Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$): $\delta$ 20.44, 36.63, 68.11, 77.17, 100.78, 111.77, 112.03, 112.52, 125.28, 125.77, 125.79, 127.85, 128.22, 128.48, 139.65, 142.35, 154.74, 155.34, 160.02, 160.54.

IR (KBr, cm$^{-1}$): 634, 818, 837, 887, 920, 986, 1027, 1123, 1184, 1231, 1280, 1532, 1394, 1448, 1506, 1616, 1710, 2049, 2854, 2922, 3444.

Mass (ESI-MS): 357.9 (M$^+$ + Na).
C, H, N analysis for
\(C_{20}H_{17}NO_4\):  Calculated C, 71.63; H, 5.11; N, 4.18.  Found C, 71.66; H, 5.15; N, 4.14.

7-{(3-\textit{m}-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (6):

White solid; mp: 133-134 °C.

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 2.34 (s, 3H), 3.48-3.51 (dd, 1H, \(J_1 = 11.56\) Hz, \(J_2 = 6.63\) Hz), 3.65-3.69 (dd, 1H, \(J_1 = 14.49\) Hz, \(J_2 = 10.07\) Hz), 4.20 (m, 2H), 5.13-5.18 (dd, 1H, \(J_1 = 12.01\) Hz, \(J_2 = 5.27\) Hz), 6.27 (d, 1H, \(J = 9.41\) Hz), 6.84 (s, 1H), 6.90-6.95 (m, 3H), 7.19-7.41 (m, 3H), 7.64 (d, 1H, \(J = 9.36\) Hz).

\(^{13}\)C NMR (500 MHz, CDCl\(_3\)): \(\delta\) 23.96, 37.36, 69.10, 78.74, 101.85, 112.85, 113.03, 113.65, 127.80, 128.06, 128.33, 129.05, 129.15, 134.65, 141.33, 143.30, 160.77, 161.38.

IR (KBr, cm\(^{-1}\)): 694, 757, 787, 835, 912, 1025, 1123, 1158, 1231, 1280, 1349, 1401, 1507, 1615, 1711, 2109, 2850, 2918, 3441.

Mass (ESI-MS): 358 (M\(^{+}\) + Na).

C, H, N analysis for
\(C_{20}H_{17}NO_4\):  Calculated C, 71.63; H, 5.11; N, 4.18.  Found C, 71.64; H, 5.19; N, 4.20.

7-{(3-\textit{o}-Tolyl-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (7):

White solid; mp: 92-93 °C.

\(^1\)H NMR (500 MHz, CDCl\(_3\)): \(\delta\) 2.51 (s, 3H), 3.00-3.04 (dd, 1H, \(J_1 = 15.32\) Hz, \(J_2 = 9.71\) Hz), 3.32-3.36 (dd, 1H, \(J_1 = 16.42\) Hz, \(J_2 = 7.41\)
7-\{3-(2,4-Dimethoxyphenyl)-4,5-dihydroisoxazol-5-yl\}methoxy-2H-chromen-2-one (8):

White solid; mp: 141-142 °C.

$^1$H NMR (500 MHz, CDCl$_3$):
$\delta$ 3.55-3.59 (dd, 1H, $J_1 = 13.34$ Hz, $J_2 = 6.76$ Hz), 3.72-3.76 (dd, 1H, $J_1 = 14.65$ Hz, $J_2 = 10.74$ Hz), 3.90 (s, 3H), 3.91 (s, 3H), 4.17 (m, 1H), 4.18-4.21 (dd, 1H, $J_1 = 12.46$ Hz, $J_2 = 5.20$ Hz), 5.14-5.16 (m, 1H), 6.27 (d, 1H, $J = 9.46$ Hz), 6.84 (s, 1H), 6.85-6.90 (m, 3H), 7.27 (d, 1H, $J = 8.22$ Hz), 7.39 (d, 1H, $J = 8.58$ Hz), 7.64 (d, 1H, $J = 9.48$ Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$):
$\delta$ 39.84, 56.27, 69.16, 79.15, 101.74, 112.19, 112.89, 113.16, 113.54, 113.90, 124.20, 128.93, 129.30, 143.38, 155.78, 156.39, 156.56, 158.29, 161.04, 161.56.
IR (KBr, cm$^{-1}$):  664, 792, 841, 868, 934, 990, 1052, 1129, 1161, 1172, 1210, 1232, 1255, 1281, 1430, 1457, 1518, 1548, 1618, 1735, 2049, 2927, 3452.

Mass (ESI-MS):  381 (M$^+$).


7-{(3-(4-Fluorophenyl)-4,5-dihydroisoazol-5-yl)methoxy}-2H-chromen-2-one (9):

White solid; mp:  114-115 °C.

$^1$H NMR (500 MHz, CDCl$_3$):  $\delta$ 3.34-3.39 (dd, 1H, $J_1 = 17.21$ Hz, $J_2 = 7.04$ Hz), 3.51-3.56 (m, 1H), 4.15-4.18 (dd, 1H, $J_1 = 9.86$ Hz, $J_2 = 4.84$ Hz), 4.21-4.23 (dd, 1H, $J_1 = 9.68$ Hz, $J_2 = 4.75$ Hz), 5.14-5.17 (m, 1H), 6.26 (d, 1H, $J = 9.44$ Hz), 6.82 (s, 1H), 6.85 (d, 1H, $J = 8.54$ Hz), 7.12-7.15 (m, 1H), 7.37-7.46 (m, 4H), 7.63 (d, 1H, $J = 9.44$ Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$):  $\delta$ 38.71, 70.38, 80.18, 103.16, 114.26, 114.55, 115.00, 115.05, 118.71, 124.02, 130.38, 131.88, 131.94, 144.76, 157.07, 157.18, 162.51, 162.87, 165.22.

IR (KBr, cm$^{-1}$):  615, 718, 759, 835, 892, 989, 1031, 1123, 1230, 1279, 1347, 1377, 1400, 1456, 1493, 1507, 1556, 1613, 1726, 2110, 2853, 2869, 2924, 2956, 3384.

Mass (ESI-MS):  361.8 (M$^+$ + Na).

C, H, N analysis for C$_{19}$H$_{14}$FNO$_4$:  Calculated C, 67.25; H, 4.16; N, 4.13.  Found C, 67.22; H, 4.19; N, 4.18.
7-{(3-(4-Chlorophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (10):

![Chemical Structure](image)

White solid; mp: 111-112 °C.

$^1$H NMR (500 MHz, CDCl$_3$):
\[ \delta 3.27-3.32 \text{ (dd, 1H, } J_1 = 17.93 \text{ Hz, } J_2 = 7.08 \text{ Hz)}, \]
\[ 3.44-3.50 \text{ (dd, 1H, } J_1 = 19.04 \text{ Hz, } J_2 = 10.87 \text{ Hz),} \]
\[ 3.53 \text{ (m, 1H), 4.08-4.11 \text{ (dd, 1H, } J_1 = 10.12 \text{ Hz, } J_2 = 4.93 \text{ Hz)}, 5.13-5.15 \text{ (m, 1H), 6.12 \text{ (d, 1H, } J = 8.95 \text{ Hz),} 6.75 \text{ (s, 1H), 6.81 \text{ (d, 1H, } J = 9.48 \text{ Hz), 7.30-7.34 \text{ (m, 3H), 7.56-7.58 \text{ (m, 3H).} \]

$^{13}$C NMR (500 MHz, CDCl$_3$):
\[ \delta 37.02, 68.74, 78.40, 101.85, 111.56, 112.85, 113.65, 127.37, 128.43, 128.63, 128.81, 128.90, 129.22, 131.41, 143.00, 155.10, 155.97, 160.34, 161.31. \]

IR (KBr, cm$^{-1}$): 666, 760, 919, 1049, 1093, 1159, 1230, 1351, 1402, 1558, 1615, 1650, 1714, 2360, 2853, 2924, 3416, 3672.

Mass (ESI-MS): 355.3 (M$^+$).

C, H, N analysis for C$_{19}$H$_{14}$ClNO$_4$:
Calculated C, 64.14; H, 3.97; N, 3.94. Found C, 64.12; H, 3.93; N, 3.91.

7-{(3-(4-Bromophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (11):

Brown solid; mp: 134-135 °C.

$^1$H NMR (500 MHz, CDCl$_3$):
\[ \delta 3.28-3.32 \text{ (dd, 1H, } J_1 = 13.95 \text{ Hz, } J_2 = 5.93 \text{ Hz),} \]
\[ 3.50-3.56 \text{ (dd, 1H, } J_1 = 18.45 \text{ Hz, } J_2 = 11.04 \text{ Hz),} \]
\[ 3.64 \text{ (m, 1H), 4.26 \text{ (m, 1H), 5.26 \text{ (m, 1H), 6.29 \text{ (d,} \]
1H, J = 9.17 Hz), 6.90 (s, 1H), 6.95 (d, 1H, J = 6.79 Hz), 7.17 (m, 2H), 7.46 (d, 2H, J = 8.43 Hz), 7.74 (d, 2H, J = 9.15 Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$): \( \delta \) 36.23, 67.89, 77.69, 100.67, 111.78, 112.51, 112.56, 116.22, 116.39, 121.55, 128.89, 129.39, 129.45, 142.27, 158.43, 159.64, 163.70, 164.24.

IR (KBr, cm$^{-1}$): 683, 835, 896, 1032, 1125, 1158, 1231, 1281, 1351, 1403, 1512, 1556, 1614, 1726, 2361, 2852, 2923, 3411.

Mass (ESI-MS): 401 (M$^+$ + H).

C, H, N analysis for C$_{19}$H$_{14}$BrNO$_4$: Calculated C, 57.02; H, 3.53; N, 3.50. Found C, 57.10; H, 3.58; N, 3.52.

7-{(3-(4-Nitrophenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (12):

Yellow solid; mp: 130-131 °C.

$^1$H NMR (500 MHz, CDCl$_3$): \( \delta \) 3.40-3.45 (dd, 1H, $J_1 = 17.01$ Hz, $J_2 = 7.25$ Hz), 3.55-3.61 (dd, 1H, $J_1 = 19.21$ Hz, $J_2 = 10.71$ Hz), 4.23 (m, 2H), 5.20-5.29 (m, 1H), 6.28 (d, 1H, $J = 9.48$ Hz), 6.83 (s, 1H), 7.39 (d, 1H, $J = 8.20$ Hz), 7.60-7.67 (m, 2H), 8.13 (d, 2H, $J = 7.85$ Hz), 8.30 (d, 2H, $J = 7.40$ Hz).

$^{13}$C NMR (500 MHz, CDCl$_3$): \( \delta \) 37.68, 73.35, 76.92, 101.44, 102.20, 109.68, 109.75, 115.05, 122.73, 126.83, 127.17, 132.58, 136.99, 137.01, 151.39, 151.44, 157.30.

IR (KBr, cm$^{-1}$): 679, 738, 854, 983, 1097, 1191, 1236, 1298, 1345, 1404, 1490, 1523, 1533, 1557, 1623, 1730, 2058, 2927, 3450.
Mass (ESI-MS): 389 (M⁺ + Na).

C, H, N analysis for C₁₉H₁₄N₂O₆:
Calculated C, 62.30; H, 3.85; N, 7.65. Found C, 62.38; H, 3.82; N, 7.69.

7-[(3-(Naphthalen-1-yl)-4,5-dihydroisoxazol-5-yl)methoxy]-2H-chromen-2-one (13):

White solid; mp: 119-120 °C.

¹H NMR (500 MHz, CDCl₃):
δ 3.56-3.60 (dd, 1H, J₁ = 13.86 Hz, J₂ = 6.13 Hz), 3.75-3.80 (dd, 1H, J₁ = 15.32 Hz, J₂ = 10.92 Hz), 4.11-4.24 (m, 2H), 5.16 (m, 1H), 6.26 (d, 1H, J = 9.15 Hz), 6.84 (s, 1H), 7.36 (d, 1H, J = 8.04 Hz), 7.50 (d, 1H, J = 7.46 Hz), 7.56-7.63 (m, 4H), 7.89-7.93 (m, 3H), 8.99 (d, 1H, J = 8.01 Hz).

¹³C NMR (500 MHz, CDCl₃):
δ 37.45, 69.30, 77.31, 104.44, 115.47, 115.72, 116.19, 126.49, 126.80, 127.45, 128.90, 129.15, 129.60, 130.30, 130.42, 133.31, 133.72, 136.68, 145.95, 158.43, 159.64, 163.70, 164.24.

IR (KBr, cm⁻¹):
615, 751, 775, 800, 834, 1026, 1122, 1230, 1279, 1348, 1401, 1508, 1612, 1724, 2341, 2361, 2852, 2923, 2955, 3423.

Mass (ESI-MS): 394 (M⁺ + Na).

C, H, N analysis for C₂₃H₁₇NO₄:
Calculated C, 74.38; H, 4.61; N, 3.77. Found C, 74.32; H, 4.67; N, 3.74.
1.3.3. Biological experiments

To probe the structure activity relationship (SAR) of synthesized isoxazoline conjugates, we investigated their immunopotentiating activity both in vitro and in vivo. Initially all the synthesized compounds were subjected to in vitro lymphocyte proliferation assay followed by in vivo studies of active compounds to determine their influence on various aspects of immune system like splenocyte proliferation ex vivo (T cell and B cell proliferation), antibody production (IgM and IgG), DTH reaction, T-Cell subtypes (CD4 and CD8), cytokine production (IL-2, IFN-γ, IL-4), NO (macrophage) production and toxic effects. Levamisole, a known immunostimulator reported to augment the antibody response, was given orally as positive control, at a dose of 2.5 mg/kg body weight.

**In vitro lymphocyte proliferation by MTT assay**

Briefly, single cell suspensions were prepared by teasing the tissue between two glass slides and cells were centrifuged at 400 x g for 10 min at 4 °C. Erythrocytes present were lysed with red cell lysis buffer (0.5 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM disodium ethylene diamine tetraacetic acid, pH 7.2) for 5 min. Lymphocytes obtained were then washed thrice with PBS. Cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%. To evaluate the effect of test compounds (0.1-10 μg) on the proliferation of splenic lymphocytes, spleen cell suspension (2 x 10⁶ cell/mL) was pipetted into 96 well plates (200 μL/well) in the presence of Con A (5 μg/mL) and LPS (10 μg/mL) cultured at 37 °C for 48 h. The plates were centrifuged at 1400 x g, 5 min and supernatant collected for the estimation of cytokines in cell culture supernatant. 100 μL fresh complete media was added in 96 well plates and again incubated for 24 h. 20 μL of MTT solution (5 mg/mL) were added to each well and incubated for 4 h. The plates were centrifuged (1400 x g, 5 min) and the untransformed MTT removed carefully by pipetting. To each well, 100 μL of a DMSO working solution (192 μL DMSO with 8 μL 1 M HCl) was added and the absorbance evaluated in an ELISA reader at 570 nm after 15 min. Absorbance was recorded at 570 nm. Values are expressed as mean ± S.E. of three observations. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).
Animals

The study was conducted on male BALB/c mice (18-22 g). The Ethical Committee of the Indian Institute of Integrative Medicine (IIIM, CSIR) instituted for animal handling approved all the protocols. The animals were bred and maintained under standard laboratory conditions: temperature (25 ± 2 °C) and a photoperiod of 12 h. Commercial pellet diet and water were given ad libitum.

Treatment

SRBC collected in Alsever’s solution, were washed three times in large volumes of pyrogen-free 0.9% normal saline and adjusted to a concentration of $5 \times 10^9$ cells/mL for immunization and challenge. The animals were divided into five groups of six animals each. Group I (control) received 1% gum acacia; group II received levamisole (2.5 mg/kg b.wt); group III received test sample (compound 2) (0.001 mg/kg b.wt); group IV received test sample (compound 2) (0.01 mg/kg b.wt) and group V received test sample (compound 2) (0.1 mg/kg b.wt). Test sample (compound 2) dissolved in 1% gum acacia was administered orally for 14 days. The dose volume was 0.2 mL. Similar studies were carried out with compounds 4 and 8.

Antibody Titre

The animals were immunized by injecting 0.2 mL of 10% of fresh SRBC suspension intraperitonially on day 0. Blood samples were collected in microcentrifuge tubes from individual animals by retro-orbital plexus on day 7 for primary antibody titre and day 14 for secondary antibody titre. Serum was separated and antibody levels were determined through haemagglutination technique described by Nelson and Mildenhall. Briefly, equal volumes of individual serum samples of each group were pooled. Two fold dilutions of pooled serum samples were made in 25 μL volumes of normal saline in a microtitration plate to which was added 25 μL of 1% suspension of SRBC in saline. After mixing, the plates were incubated at room temperature for 1 h and examined for haemagglutination under the microscope. The reciprocal of the highest dilution of the test serum giving agglutination was taken as the antibody titre.

DTH reaction

The method of Doherty was followed to assess SRBC induced DTH response in mice. Mice were immunized by injecting 20 μL of $5 \times 10^9$ SRBC/mL subcutaneously into the right hind foot pad. Compounds 2, 4 and 8 were administered 2 h after SRBC injection and once daily on consecutive days. Seven days later, the thickness of the
left hind footpad was measured with spheromicrometer (pitch, 0.01 mm) and considered as the control. The mice were challenged by injecting same amount of SRBC intradermally into the left hind footpad. The foot thickness was measured again after 24 and 48 h after challenge.\textsuperscript{10}

**Spleenocyte proliferation assay (ex vivo)**

Spleen collected under aseptic conditions in HBSS, was minced using a pair of scissors and passed through a fine steel mesh to obtain a homogeneous cell suspension and the erythrocytes were lysed with NH\textsubscript{4}Cl (0.8%, w/v). After centrifugation (380 x g at 4 °C for 10 min), the pelleted cells were washed three times with PBS and resuspended in complete medium [RPMI 1640 supplemented with 12 mM HEPES (pH 7.1), 0.05 mM 2-mercaptoethanol, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% FCS]. The cell number was counted with a haemocytometer by the trypan blue dye exclusion technique. Cell viability exceeded 95%.\textsuperscript{7} To evaluate the effect of compound 2 on the proliferation of splenic lymphocytes, the spleen cell suspension (1 x 10\textsuperscript{7} cell/mL) was pipetted into 96-well plates (200 μL/well) and cultured at 37 °C for 72 h in a humid saturated atmosphere containing 5% CO\textsubscript{2} in the presence of Con-A (5 μg/mL) and LPS (10 μg/mL). After 72 h, 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The plates were centrifuged (1400 x g, 5 min) and the untransformed MTT removed carefully by pipetting. To each well, 100 μL of a DMSO working solution (192 μL DMSO with 8 μL 1 M HCl) was added and the absorbance evaluated in an ELISA absorbance reader at 570 nm after 15 min. Similar studies were carried out for compounds 4 and 8.

**Spleen T-cell subtyping**

T-Cell sub-typing was performed as described in literature.\textsuperscript{11} Briefly, spleenocyte single cell suspension in RPMI-1640 (10\textsuperscript{6} cell/mL) was prepared and after counting viable cells by tryphan-blue dye exclusion method, spleen cellularity was obtained. The CD\textsuperscript{4\textsuperscript{+}}/CD8\textsuperscript{−} and CD\textsuperscript{4\textsuperscript{−}}/CD8\textsuperscript{+} T-Cell subtypes were measured using flowcytometer and mouse anti-CD4 and CD8 monoclonal antibody conjugated with flourescein-isothiocyanate (FITC) and phycoerythrin (PE). By multiplying differential ratios of each CD4 and CD8 subtypes to the total spleen cell contents, their total amounts in spleen were calculated.
Cytokine production from spleenocytes
Cytokines from mouse spleenocytes were assayed using the cytokine kits (Quantikine R & D SYSTEMS).\textsuperscript{12}

Nitric oxide assay
The amount of stable nitrite, the end product of NO generation by the activated macrophages, was determined by a colorimetric assay. Briefly, 50 μL of culture supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% H\textsubscript{3}PO\textsubscript{4}). This mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was read on a spectrophotometer. The nitrite concentration was determined by extrapolation from a sodium nitrite standard curve.

Statistical analysis
The data were expressed as mean ± S.E. and analysed statistically with ANOVA (Bonferroni multiple comparison test).

Toxicity studies
The test compounds were tested for possible cellular toxic effects on spleen cells by MTT assay.\textsuperscript{13}

1.3.4. Results
Effect of test compounds on \textit{in vitro} lymphocyte proliferation by MTT assay
All the investigated compounds showed dose-related increase or decrease of titre (\textit{Figure 1}). Among the tested compounds, compounds 2, 4 and 8 were found to be potentially immunostimulating even at lower doses (0.1 μg/mL) in comparison to levamisole (Lev) and umbelliferone (UM). Compounds 3, 5 and 7 also enhanced immunostimulation to a significant extent. Immunostimulatory effect was found to decrease with increase in dosage from 0.1 μg/mL to 10 μg/mL in compounds 9-13. These compounds did not enhance the lymphocyte proliferation significantly.
Figure 1: Effect of test compounds on lymphocyte proliferation in vitro. The proliferation was calculated based on MTT assay. Absorbance was recorded at 570 nm. Values are expressed as mean ± S.E. of three observations. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

Effect on antibody titre

The compounds were tested for their possible role in B-cell activation by determining IgM (primary antibody synthesis) and IgG (secondary antibody synthesis) titre. All the compounds showed a substantial increase at all doses (0.001 mg/kg, 0.01 mg/kg and 0.1 mg/kg) in primary and secondary antibody synthesis in comparison to that of standard (Figure 2). Comparatively, compound 2 showed maximum stimulation at a dose of 0.1 mg/kg. From the data it is evident that the increase in IgG titre is more than that in IgM titre.
Figure 2: Effect of compounds 2, 4 and 8 on antibody titres in mice. Data are mean ± S.E. of six animals. *P < 0.05; **P < 0.01; ***P < 0.001 when compared with control group determined by one-way ANOVA (Bonferroni correction multiple comparison test).

Delayed type hypersensitivity (DTH) response

The effect of compounds 2, 4 and 8 on SRBC induced DTH reaction was assessed in mice following various doses (Figure 3). The DTH response values were higher than that observed with Lev even at lower doses of 0.001 mg/kg. Out of the three compounds evaluated, compound 2 induced significantly higher DTH response at a dose of 0.1 mg/kg. The other two compounds also induced DTH response to a significant extent. All the tested compounds could induce better DTH response at 24 h study followed by 48 h and 72 h respectively.
**Figure 3**: Effect of compounds 2, 4 and 8 on DTH response. Data are expressed as mean ± S.E. of five observations of left hind foot pad thickness measured at 24, 48 and 72 h. *P < 0.05; **P < 0.01; ***P < 0.001 as compared to control determined by one-way Anova (Bonferroni correction multiple comparison test).

**Effect on spleenocyte proliferation ex vivo (T and B cell proliferation)**

The effect of compounds 2, 4 and 8 on T and B cell proliferation was assessed in mice following various doses under ex vivo conditions (Figure 4). Cell proliferation showed a regular increase with increase in the dose for all these compounds. As is evident, compound 2 resulted in a significant increase in T and B cell proliferation at all doses in comparison to Lev and the other two compounds. Compound 4 showed proliferation equivalent to Lev at a dose of 0.01 mg/kg. Compound 8 also increased cell proliferation to a significant extent.
Effect on spleen T-cell subtyping

Spleen single cell suspension (10⁶ cell/mL) was studied for CD4⁺/CD8⁺ T-cell subtypes by anti-CD4 and CD8 monoclonal antibodies conjugated with fluoresceine-isothio-cyanate (FITC) and phycoerythrin (PE) using flowcytometer. By multiplying differential ratios of each CD4 and CD8 subtypes to the total spleen cell contents, their total amounts in spleen were calculated and the results enlisted in Table 2. The control values were 20.70% of CD4⁺ and 13.3% of CD8⁺ T cells. Maximum effect of 2 was obtained at 0.1 mg/kg dose, 40.2% CD4⁺ and 21.6% CD8⁺ T cells. This shows a significant increase in CD4⁺ T cell count. Lev at 2.5 mg/kg oral dose stimulated both CD4⁺ and CD8⁺ T cells, showing 30.8% of CD4⁺ and 18.3% of CD8⁺ T cells.
Table 2: Effect of different doses of compounds 2, 4 and 8 on spleen T cell subtypes

Number of observation = 6

(a) \( P < 0.01 \); (b) \( P < 0.05 \)

Effect on cytokine release (IL-2, IFN-\( \gamma \) & IL-4)

In order to understand the specific effects of compounds 2, 4 and 8 on cytokine profiles, characteristic IL-2, IL-4 and IFN-\( \gamma \) were analyzed. All these compounds stimulated IL-2, IL-4 and IFN-\( \gamma \) release in a dose related manner (Figure 5). Among these three compounds, compound 2 was found to be most effective at a dose of 0.1 mg/kg. Compound 8 substantially increased the release of cytokines at a dose of 0.1 mg/kg. Compound 4 showed effect comparable to that of Lev at a dose of 0.01 mg/kg but was found to be more active than the standard at a dose of 0.1 mg/kg. Altogether, release of IL-4 was least among the three types of cytokines.
Figure 5: Effect of compounds 2, 4 and 8 on IL-2, IFN-γ and IL-4 cytokine production. Each bar represents the mean value of triplicate readings ± SE. Mouse spleen cells (2 × 10^6 cells/mL) were stimulated with and without (control) 2.5 µg/well Con-A in the presence of each of the compounds for 48 h. Cell supernatant was collected to see the effect of these compounds on the production of IL-2, IFN-γ and IL-4, measured by commercial kits (Quantikine, R&D Systems).

Effect on NO production (macrophage production)

Compounds 2, 4 and 8 were tested for their possible role in macrophage production. All these compounds expressed dose related increase of macrophage production (Figure 6). In comparison to the standard, these compounds showed a substantial increase at all doses (0.001 mg/kg, 0.01 mg/kg and 0.1 mg/kg). Comparatively, compound 2 showed maximum stimulation at a dose of 0.1 mg/kg followed by compounds 4 and 8 respectively.
Toxicity studies
The results indicate that the test compounds (as high as 100 μg/mL) did not exhibit any toxic effect after 72 h incubation.

1.3.5. Enantioselective synthesis
The isoxazoline derivatives as presented in table 1, possess one chiral centre in the isoxazoline ring thereby raising the possibility that derivatives synthesised as per Scheme 1, would be a mixture of two possible enantiomers. In order to investigate the impact of stereochemistry of bis-heterocyclic conjugates on their immune potentiating potential, enantioselective synthesis of the possible enantiomers of most active compound (2) was carried out following the steps depicted in Scheme 2.
The lipase B from *Candida antarctica* (CALB) was employed for the kinetic resolution of the racemic ester (2c). The lipase showed excellent differential enantioselectivity in catalysing the hydrolysis of racemic ester. It was observed that the enzyme in presence of S-isomer selectively hydrolysed the R-counterpart without affecting S-isomer, producing the corresponding enantiopure alcohol with high enantiomeric excesses (>99% ee) in accordance with the Kazlauskas rule. However in
the absence of \(R\)-counterpart, the ester with \(S\)-configuration at chiral carbon in the ring is slowly hydrolysed to the corresponding enantiopure alcohol with high enantiomeric excesses (>99% ee). The enantiomeric excesses of acetate \((S)-2c\), alcohols \((S)-2d, (R)-2d\) and bromo compounds \((S)-2e, (R)-2e\) were determined by chiral column chromatography. The absolute configurations of compounds \((S)-2c, (S)-2d, (R)-2d, (S)-2e\) and \((R)-2e\) were determined comparing the specific rotation signs measured for the products with that reported in the literature [14-17]. The spectroscopic data (\(^1\)H-NMR, MS and IR) and melting point of compounds \(2c, 2d\) and \(2e\) matches well with the data reported in the literature [15, 17-18]. The known absolute configuration of enantiomers \((S)-2d, (R)-2d, (S)-2e\) and \((R)-2e\) provided the way to determine the absolute configurations of the bis-heterocycles \([(R)-2\) and \((S)-2\]. Detailed procedure for the synthetic steps in scheme 2 is as follows:

\(\{3-(4\text{-methoxyphenyl})\text{-4,5-dihydroisoxazol-5-yl}\}\text{methanol (2b):}\)

In a typical procedure, \(2a\) (0.20 g, 1.07 mmol) was dissolved in THF (5 mL) and to it was added \(\text{Et}_3\text{N}\) (0.054 g, 0.534 mmol) at 0 °C. A solution of allyl alcohol (0.062 g, 1.07 mmol) in THF (4 mL) was added to the above solution. The reaction mixture was stirred for 8 h between 0-5 °C. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 60 mL of water and extracted with \(\text{EtOAc} (2 \times 30 \text{ mL})\). The combined organic layers were dried over anhydrous \(\text{Na}_2\text{SO}_4\) and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: \(\text{EtOAc}\); (7:3) as mobile phase] to yield the pure racemic \(3-(4\text{-methoxyphenyl})\text{-4,5-dihydroisoxazol-5-yl}\text{methanol (90%).}\)

White solid; mp: 166-167 °C.

\(^1\)H NMR (200 MHz, \(\text{CDCl}_3\)): \(\delta\) 3.18-3.31 (m, 2H), 3.57-3.63 (dd, 1H, \(J_1 = 6.00\) Hz, \(J_2 = 4.00\) Hz), 3.74-3.80 (m, 1H, 3.87 (s, 3H), 4.76-4.82 (dd, 1H, \(J_1 = 10.00\) Hz, \(J_2 = 8.00\) Hz), 6.85 (d, 2H, \(J = 8.00\) Hz), 7.47 (d, 2H, \(J = 8.00\) Hz).

IR (KBr, cm\(^{-1}\)): 626, 707, 814, 906, 955, 1018, 1063, 1178, 1258, 1274, 1300, 1343, 1415, 1459, 1508, 1560, 1604, 2849, 2925, 3391.

Mass (LC-MS): 208 (\(\text{M}^+ + \text{H}\)).
C, H, N analysis for
\( \text{C}_{11}\text{H}_{13}\text{NO}_3 \):
Calculated C, 63.76; H, 6.32; N, 6.76. Found C, 63.62; H, 6.41; N, 6.69.

\{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl\}methyl acetate (2c):

2b (0.19 g, 0.917 mmol) was dissolved in DCM (5 mL). Acetic anhydride (0.093 g, 0.917 mmol) was charged to the above solution followed by the addition of DMAP (catalytic amount). The reaction mixture was then allowed to stir at ambient temperature for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was quenched with NaHCO₃ solution and extracted with DCM (2 × 50 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum. The semisolid left behind was recrystallized from hexane-ethyl acetate to yield racemic compound 2c in pure form (100%).

White solid; mp: 82-83 °C.

\(^1\)H NMR (200 MHz, CDCl₃):
\( \delta \) 2.02 (s, 3H), 2.98-3.09 (dd, 1H, \( J_1 = 14 \) Hz, \( J_2 = 8.00 \) Hz), 3.39-3.38 (dd, 1H, \( J_1 = 8.00 \) Hz, \( J_2 = 6.00 \) Hz), 3.74-3.78 (m, 1H), 3.88 (s, 3H), 4.16-4.19 (m, 1H), 4.83-4.94 (dd, 1H, \( J_1 = 14 \) Hz, \( J_2 = 10.00 \) Hz), 6.87 (d, 2H, \( J = 8.00 \) Hz), 7.49 (d, 2H, \( J = 10 \) Hz).

IR (KBr, cm\(^{-1}\)):
626, 706, 816, 904, 1045, 1064, 1103, 1175, 1249, 1274, 1301, 1344, 1362, 1441, 1462, 1510, 1605, 1742, 2855, 2930, 3458.

Mass (LC-MS):
288 (M\(^+\) + K).

C, H, N analysis for
\( \text{C}_{13}\text{H}_{15}\text{NO}_4 \):

Typical lipase-catalyzed kinetic resolution of (±)-2c:
The racemic acetate (.100 g, .401 mmol), aqueous phosphate buffer (4 mL, 0.1 M, pH 7.0), toluene (300 µL) and immobilized lipase B from *Candida antarctica* (80 mg) were shaken (150 rpm) continuously at 25 ± 1 °C for 7 min. After a certain degree of conversion (~50%) as indicated by high performance liquid chromatography (HPLC), the reaction was terminated by adding ethyl acetate and centrifuging the mixture at 10,000–15,000 g to remove the enzyme and the suspended particles. The clear
solution was decanted and the centrifuged mass extracted separately with ethyl acetate (3 × 30 mL). The organic layer was combined and washed with water. The combined organic layers were then dried and evaporated under reduced pressure to furnish a mixture of hydrolyzed alcohol \((R)-2d\) and unhydrolyzed ester \((S)-2c\), which were separated by column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase]. The unhydrolyzed ester \((S)-2c\) (0.040 g, 0.16 mmol), aqueous phosphate buffer (2.5 mL, 0.1 M, pH 7.0), toluene (200 µL) and immobilized lipase B from \textit{C. antarctica} (30 mg) were shaken (150 rpm) continuously at 25 ± 1 °C for 12 h. After complete hydrolysis (as indicated by TLC), the mixture was filtered with suction, and the filtrate concentrated in vacuo to afford pure \((S)-2d\).

Optical rotations were measured on Perkin-Elmer 241 polarimeter at 25 °C using sodium D light. Enantiomeric purities of \((S)-2c\), \((R)-2d\) and \((S)-2d\) were determined by chiral HPLC (Therme Finnigan-UV-VIS Detector):

\((S)-\{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl\}methyl acetate 2c:

HPLC purity >99%; HPLC ee >99%; \([ \alpha ]_D^{25} = +41.90 \) (c 0.5, CHCl3), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 62.767 min (Figure 7).

**Figure 7:** Chromatogram of \((S)-2c\) recorded during analysis with Chiral HPLC.
(R)-{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol 2d:
HPLC purity >99%; HPLC ee >99%; \([\alpha]_D^{25} = -94.50\) (c 0.5, CHCl₃), HPLC condition
(OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min),
retention time 31.53 min (Figure 8).

Figure 8: Chromatogram of (R)-2d recorded during analysis with Chiral HPLC.

(S)-{3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl}methanol 2d:
HPLC purity >99%; HPLC ee >99%; \([\alpha]_D^{25} = +96.00\) (c 0.5, CHCl₃), HPLC condition
(OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min),
retention time 52.00 min (Figure 9).
**Figure 9:** Chromatogram of (S)-2d recorded during analysis with Chiral HPLC.

**(R)-5-({bromomethyl})-3-(4-methoxyphenyl)-4,5-dihydroisoxazole 2e:**

**(R)-2d** (.04 g, 0.193 mmol) was dissolved in DCM (5 mL) and CBr₄ (.096 g, 0.289 mmol) was charged to the above solution. TPP (.075 g, 0.289 mmol) was added to the reaction mixture slowly for 10 min at 0 ºC. The reaction mixture was then allowed to stir at ambient temperature for 2 h. After completion of the reaction (monitored by TLC), the reaction mixture was diluted with 60 mL of water and extracted with DCM (2 × 30 mL). The combined organic layers were dried over anhydrous Na₂SO₄ and concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (9:1) as mobile phase] to yield the pure **(R)-2e** (90%).

HPLC purity >99%; HPLC ee >99%; [α]ᵦ²⁵ = -21.37 (c 0.5, CHCl₃), HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 49.995 min (**Figure 10**).
Figure 10: Chromatogram of (R)-2e recorded during analysis with Chiral HPLC.

White solid; mp: 73-75 °C.

1H NMR (200 MHz, CDCl3): \( \delta \) 3.19-3.34 (m, 2H), 3.37-3.50 (dd, 1H, \( J_1 = 12.00 \) Hz, \( J_2 = 6.00 \) Hz), 3.55-3.60 (m, 1H), 3.95 (s, 3H), 4.94-5.04 (dd, 1H, \( J_1 = 14.00 \) Hz, \( J_2 = 8.00 \) Hz), 6.93 (d, 2H, \( J = 8.00 \) Hz), 7.55 (d, 2H, \( J = 6.00 \) Hz).

IR (KBr, cm\(^{-1}\)): 666, 706, 755, 813, 902, 1020, 1064, 1215, 1259, 1274, 1361, 1416, 1462, 1507, 1602, 2848, 2916, 3018, 3400.

Mass (LC-MS): 269 (M\(^+\)).

C, H, N analysis for C\(_{11}\)H\(_{12}\)BrNO\(_2\): Calculated C, 48.91; H, 4.48; N, 5.19. Found C, 48.83; H, 4.41; N, 5.27.

(S)-5-{bromomethyl)-3-(4-methoxyphenyl)-4,5-dihydroisoxazole 2e:

Same procedure as described for (R)-2e was used. Yield of the compound was found to be 90%. HPLC purity >99%; HPLC ee >99%; \([\alpha]_D^{25} = +20.99 \) (c 0.5, CHCl\(_3\)).
HPLC condition (OJH chiral column, eluent 2-propanol–hexane (1:9), flow rate: 0.8 mL/min), retention time 52.248 min (Figure 11).

Figure 11: Chromatogram of (S)-2e recorded during analysis with Chiral HPLC.

(R)-7-{(3-(4-methoxyphenyl)-4,5-dihydroisoxazol-5-yl)methoxy}-2H-chromen-2-one (2):
In a typical procedure, umbelliferone (0.269 g, 1.66 mmol) was dissolved in dry acetone (5 mL). K$_2$CO$_3$ (4.26 g, 30.85 mmol) was added and the reaction mixture stirred for 5 min at ambient temperature. (R)-2e (0.30 g, 1.11 mmol) was charged to the above reaction mixture and the reaction mixture allowed to reflux for 3 h. After completion of the reaction (monitored by TLC), the reaction mixture was allowed to attain room temperature, filtered and the filtrate concentrated under vacuum to afford crude product which was subjected to column chromatography [silica gel 230-400 mesh as stationary phase, hexane: EtOAc; (7:3) as mobile phase] to yield the pure (R)-2 (92%).

White solid; mp: 140-141.5 °C.
Specific rotation $[\alpha]_D^{25}$ - 32.51 (c 0.5, CHCl$_3$).
\( \text{\textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3})}: \delta 3.25-3.29 \ (dd, 1H, J_1 = 16.45 \ Hz, J_2 = 6.82 \ Hz), \ 3.41-3.48 \ (dd, 1H, J_1 = 16.65 \ Hz, J_2 = 10.75 \ Hz), \ 3.88 \ (s, 3H), 4.05-4.10 \ (m, 1H), 4.13-4.18 \ (dd, 1H, J_1 = 10 \ Hz, J_2 = 4.98 \ Hz), 5.04-5.10 \ (dd, 1H, J_1 = 10.00 \ Hz, J_2 = 5.00 \ Hz), 6.19 \ (d, 1H, J = 9.47 \ Hz), 6.76 \ (s, 1H), 6.80 \ (d, 1H, J = 8.56 \ Hz), 6.92 \ (d, 2H, J = 8.64 \ Hz), 7.33 \ (d, 1H, J = 8.52 \ Hz), 7.45 \ (d, 1H, J = 8.46 \ Hz), 7.56 \ (d, 2H, J = 8.00 \ Hz). \)

\( \text{IR (KBr, cm}^{-1})\): 669, 810, 837, 897, 1022, 1063, 1095, 1126, 1232, 1275, 1356, 1402, 1507, 1558, 1615, 1650, 1714, 2337, 2361, 2852, 3389.

Mass (LC-MS): 351 (M\(^+\)).

C, H, N analysis for \( \text{C}_{20}\text{H}_{17}\text{NO}_5 \):
Calculated C, 68.37; H, 4.88; N, 3.99. Found C, 68.32; H, 4.85; N, 3.94.

\((S)-7-\{(3-(4\text{-methoxyphenyl})-4,5\text{-dihydroisoazol-5-yl)methoxy}\}-2\text{H-chromen-2-one (2)}:\)
Same procedure as described for compound \((R)-2\) was used. Yield of the compound was found to be 92%.
White solid; mp: 140-141.5 °C.
Specific rotation \([\alpha]_D^{25}\) = +31.91 (c 0.5, CHCl\textsubscript{3}).

\textbf{1.3.6. Comparative Activity Studies}
Both the compounds [(\textit{R})-2 and \textit{(S)}-2] as well as their racemate were explored for their \textit{in vitro} lymphocyte proliferation potential. It was observed that both the tested enantiomers as well as their racemate show excellent dose-related increase of titre (\textbf{Figure 12}) even at lower doses (0.1 µg/mL) in comparison to levamisole (Lev). It was observed that the activity of the enantiomers in their pure form as well as their racemate form is almost similar at all doses. We attribute this stereo-independent biological activity of the tested bis-hetrocycles to their flexible molecular structure. The enantiomers therefore are expected to exhibit similar stereo-independent activity under \textit{in vivo} conditions.
1.3.7. Discussion

For exploration of SAR in isoxazoline conjugates of 7-OHC as immunopotentiators, correlation of their activity with the substitution pattern in the basic skeleton seems quite informative. To decipher such information, we attempted varied type of substitutions on the aryl ring of 7-OHC from simple H, electron donating (OCH₃ and CH₃) and electron withdrawing groups (F, Cl, Br and NO₂) to bulky aryl (naphthyl) groups for the synthesis of coumarin-isoxazoline conjugates. The results obtained from immunomodulatory experiments of synthesized conjugates showed a higher immunostimulating potential of isoxazoline derivatives in comparison to control and 7-OHC. In the biological screening experiments, all the screened compounds displayed a characteristic immunopotentiating activity that was dependent on the dosage selection in relation to antigen. The results of preliminary assays and
structures of synthesized conjugates indicate that isoxazolines possessing electron donating groups on the aryl ring (2-8) are stronger immunopotentiators (4-OCH₃ > 2,4-OCH₃ > 2-OCH₃ > 3-OCH₃ > 4-CH₃ > 2-CH₃ > 3-CH₃) in comparison to those possessing electron withdrawing groups (9-12) (4-Cl > 4-Br > 4-F ≈ 4-NO₂). The results further indicate that the immunostimulatory activity is enhanced by increasing dosage of electron rich isoxazoline derivatives and reverse is the case with electron deficient aryl rings of isoxazoline moieties. Introducing a bulky aryl (naphthyl) group (13) decreases the immunostimulating potential with increase in dose. This may be attributed to high molecular mass and overcrowding of aryl rings, which inhibits their interaction with the binding receptors. The attachment of umbelliferone at 5th position via an O-allyl intermediate formation on the isoxazoline ring together with appropriately functionalized aromatic ring at the 3rd position of isoxazoline ring seems to impart immune-enhancing activity to the molecule.

Coumarins are reported to interact with ubiquitous intracellular receptor proteins through binding of their aromatic hydrocarbons. Hence it can be inferred that electron donating groups at ortho or para positions with respect to isoxazoline ring increase the overall electron density and hence lead to their stronger interaction with the receptor binders. Moreover, single bond rotations between an aryl ring and isoxazoline ring in conjugates possessing electron donating groups may lead to orientation that ensures their proper binding with intracellular receptor proteins. This is well in agreement with the results we obtained in the current study. Compounds with OCH₃ and CH₃ groups on the phenyl rings ortho/para to isoxazoline ring (compounds 2, 4, 5 and 7) displayed significantly higher activity than OCH₃ and CH₃ groups meta to isoxazoline ring (compounds 3 and 6). Conjugate occupied with OCH₃ group at both ortho and para positions with respect to isoxazoline ring (compound 8) also resulted in better activity.

Compounds 2, 4 and 8 showed significant activity under in vitro experiments. Results from in vivo studies of these compounds, where many assays were used to explore their impact on spleenocyte proliferation ex vivo (T cell and B cell proliferation), antibody production (IgM and IgG), DTH reaction, T-Cell subtypes (CD4 and CD8), cytokine production (IL-2, IFN-γ, IL-4) and NO (macrophage) activation, were quite encouraging. Since a well-known synergism exists between these cytokines in iNOS
expression, the induction of cytokines after treatment with the test compounds in these experiments could be attributed to the release of significant amounts of NO. In all \textit{in vivo} experiments, compound 2 exhibited highest activity at all doses in comparison to control, levamisole and compounds 4 and 8. At lower doses compound 4 showed activities almost similar to that of standard; however at higher doses the activity was found to increase with increase in dosage per kg body weight. The difference observed in activity of 2 and 4 may be attributed to the fact that compound 2 being \textit{para} substituted is more symmetrical and stable, that leads to its better interaction with the receptor binders in comparison to compound 4. In contrast, compound 8 being slightly bulkier and less symmetrical than 2 may show lesser interaction with the receptors. Synthesis of secondary antibodies (IgG) was more than primary antibody synthesis (IgM), hence resulting in generation of a good number of memory cells. The observed enhancement in CD4\textsuperscript{+} values for these compounds clearly implies the immunogenic response through MHC-class II pathway. Given the short half-life of the coumarins, it is conceivable that multiple administrations through oral or intravenous routes will be required for threshold saturation of the receptors involved in the subsequent activation of lymphocytes and macrophages. Compounds 2 and 8 clearly exert a significant immunostimulatory activity on the immune system and hence can be advocated as a suitable immunostimulatory lead compounds for future research.

\textbf{1.3.6. Conclusion}

The results presented in this chapter signify that conjugates of appropriately substituted isoxazoline derivatives with 7-OH coumarins can act as potential immunostimulators.
1.3.8. References


$^1$H NMR spectrum of compound 2
Maldi-mass of compound 2
$^{13}$C NMR spectrum of compound 2
DEPT NMR spectrum of compound 2
IR spectrum of compound 2
$^1$H NMR spectrum of compound 9
ESI-MS spectrum of compound 9
$^{13}$C NMR spectrum of compound 9
DEPT NMR spectrum of compound 9
IR spectrum of compound 9
$^1$HNMR spectrum of (S)-2c
LC-MS spectrum of (S)-2c
IR spectrum of (S)-2c
$^1$HNMR spectrum of (R)-2d
LC-MS of (R)-2d
IR spectrum of (R)-2d
$1^1$HNMR spectrum of (R)-2e
LC-MS spectrum of (R)-2e
IR-spectrum of (R)-2e
\textbf{1HNMR spectrum of (R)-2}
LC-MS of (R)-2